Principles of Bone Biology

SECOND EDITION

Volume 1

EDITED BY

John P. Bilezikian

Lawrence G. Raisz

Gideon A. Rodan

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Contents

VOLUME 1

Contributors xiii
Preface to the Second Edition xxi
Preface to the First Edition xxiii

PART I
BASIC PRINCIPLES

A. Cell Biology

CHAPTER 1
Structure and Development of the Skeleton 3
Sandy C. Marks, Jr., and Paul R. Odgren

CHAPTER 2
Biomechanics of Bone 17
Dennis M. Cullinane and Thomas A. Einhorn

CHAPTER 3
Embryonic Development of Bone and the Molecular Regulation of Intramembranous and Endochondral Bone Formation 33
Andrew C. Karaplis

CHAPTER 4
Mesenchymal Stem Cells and Osteoblast Differentiation 59
Jane E. Aubin and James T. Triffitt

CHAPTER 5
Transcriptional Control of Osteoblast Differentiation and Function 83
Thorsten Schinke and Gerard Karsenty

CHAPTER 6
The Osteocyte 93
P. J. Nijweide, E. H. Burger, and J. Klein-Nulend

CHAPTER 7
Cells of Bone: Osteoclast Generation 109
Naoyuki Takahashi, Nobuyuki Udagawa, Masamichi Takami, and Tatsuo Suda

CHAPTER 8
Osteoclast Function: Biology and Mechanisms 127
Kalervo Väänänen and Haibo Zhao

CHAPTER 9
Integrin and Calcitonin Receptor Signaling in the Regulation of the Cytoskeleton and Function of Osteoclasts 141
Le T. Duong, Archana Sanjay, William Horne, Roland Baron, and Gideon A. Rodan

CHAPTER 10
Apoptosis in Bone Cells 151
Brendan F. Boyce, Lianping Xing, Robert J. Jilka, Teresita Bellido, Robert S. Weinstein, A. Michael Parfitt, and Stavros C. Manolagas

CHAPTER 11
Involvement of Nuclear Architecture in Regulating Gene Expression in Bone Cells 169
Gary S. Stein, Jane B. Lian, Martin Montecino, André J. van Wijnen, Janet L. Stein, Amjad Javed, and Kaleem Zaidi

B. Biochemistry

CHAPTER 12
Type I Collagen: Structure, Synthesis, and Regulation 189
Jerome Rossert and Benoit de Crombrugghe

CHAPTER 13
Collagen Cross-Linking and Metabolism 211
Simon P. Robins and Jeffrey D. Brady

CHAPTER 14
Bone Matrix Proteoglycans and Glycoproteins 225
Pamela Gehron Robey

CHAPTER 15
Osteopontin 239
Masaki Noda and David T. Denhardt

CHAPTER 16
Bone Proteinases 251
Richard C. D'Alonzo, Nagarajan Selvamurugan, Stephen M. Krane, and Nicola C. Partridge

CHAPTER 17
Integrins and Other Cell Surface Attachment Molecules of Bone Cells 265
Michael A. Horton, Stephen A. Nesbitt, Jon H. Bennett, and Gudrun Stenbeck

CHAPTER 18
Intercellular Junctions and Cell–Cell Communication in Bone 287
Roberto Civitelli, Fernando Lecanda, Niklas R. Jørgensen, and Thomas H. Steinberg

C. Bone Remodeling and Mineral Homeostasis

CHAPTER 19
Histomorphometric Analysis of Bone Remodeling 303
Susan M. Ott

CHAPTER 20
Phosphorus Homeostasis and Related Disorders 321
Marc K. Drezner

CHAPTER 21
Magnesium Homeostasis 339
Robert K. Rude

CHAPTER 22
Metals in Bone: Aluminum, Boron, Cadmium, Chromium, Lead, Silicon, and Strontium 359
Felix Bronner

CHAPTER 23
Biology of the Extracellular Ca^{2+}-Sensing Receptor (CaR) 371
Edward M. Brown

D. The Hormones of Bone

CHAPTER 24
Receptors for Parathyroid Hormone (PTH) and PTH-Related Peptide 389
Thomas J. Gardella, Harald Jüppner, F. Richard Bringhurst, and John T. Potts, Jr.

CHAPTER 25
Parathyroid Hormone: Molecular Biology 407
Justin Silver, Tally Naveh-Many, and Henry M. Kronenberg

CHAPTER 26
Parathyroid Hormone–Receptor Interactions 423
Michael Chorev and Michael Rosenblatt

CHAPTER 27
Actions of Parathyroid Hormone 463
Janet M. Hock, Lorraine A. Fitzpatrick, and John Bilezikian

CHAPTER 28
Renal and Skeletal Actions of Parathyroid Hormone (PTH) and PTH-Related Protein 483
F. Richard Bringhurst and Gordon J. Strewler

CHAPTER 29
Physiological Actions of Parathyroid Hormone (PTH) and PTH-Related Protein: Epidermal, Mammary, Reproductive, and Pancreatic Tissues 515
John J. Wysolmerski, Andrew F. Stewart, and T. John Martin

CHAPTER 30
Vascular, Cardiovascular, and Neurological Actions of Parathyroid-Related Protein 531
Thomas L. Clemens and Arthur E. Broadus

CHAPTER 31
1α,25(OH)$_2$Vitamin D$_3$: Nuclear Receptor Structure (VDR) and Ligand Specificities for Genomic and Rapid Biological Responses 545
Anthony W. Norman

CHAPTER 32
Vitamin D Gene Regulation 573
Sylvia Christakos

CHAPTER 33
Photobiology and Noncalcemic Actions of Vitamin D 587
Michael F. Holick

CHAPTER 34
Structure and Molecular Biology of the Calcitonin Receptor 603
Deborah L. Galson and Steven R. Goldring

CHAPTER 35
Calcitonin Gene Family of Peptides: Structure, Molecular Biology, and Effects 619
Kenneth L. Becker, Beat Müller, Eric S. Nylén, Régis Cohen, Jon C. White, and Richard H. Snider, Jr.

CHAPTER 36
Amylin and Calcitonin Gene-Related Peptide 641
Ian R. Reid and Jill Cornish

E. Other Systemic Hormones That Influence Bone Metabolism

CHAPTER 37
Estrogens and Progestins 655
David Rickard, Steven A. Harris, Russell Turner, Sundeep Khosla, and Thomas C. Spelsburg

CHAPTER 38
Selective Estrogen Receptor Modulators 677
Douglas B. Muchmore and Geoffrey Greene

CHAPTER 39
Mechanisms of Estrogen Action in Bone 693
Roberto Pacifici

CHAPTER 40
Thyroid Hormone and Bone 707
Paula H. Stern

CHAPTER 41
Clinical and Basic Aspects of Glucocorticoid Action in Bone 723
Barbara E. Kream and Barbara P. Lukert

CHAPTER 42
Effects of Diabetes and Insulin on Bone Physiology 741
Johan Verhaeghe and Roger Bouillon

CHAPTER 43
Androgens: Receptor Expression and Steroid Action in Bone 757
Kristine M. Wiren and Eric S. Orwoll

CHAPTER 44
Kinins and Neuro-osteogenic Factors **773**
Ulf H. Lerner and Pernilla Lundberg

F. Local Regulators

CHAPTER 45
The Role of Insulin-like Growth Factors and Binding Proteins in Bone Cell Biology **801**
Cheryl A. Conover and Clifford Rosen

CHAPTER 46
Platelet-Derived Growth Factor and the Skeleton **817**
Ernesto Canalis and Sheila Rydziel

CHAPTER 47
Fibroblast Growth Factor (FGF) and FGF Receptor Families in Bone **825**
Marja M. Hurley, Pierre J. Marie, and Robert Z. Florkiewicz

Index for Volumes 1 and 2 853

VOLUME 2

Contributors xi
Preface to the Second Edition xix
Preface to the First Edition xxi

CHAPTER 48
Vascular Endothelial Growth Factors **883**
Shun-ichi Harada and Kenneth A. Thomas

CHAPTER 49
Transforming Growth Factor-β **903**
L. F. Boneward

CHAPTER 50
Bone Morphogenetic Proteins **919**
Vicki Rosen and John M. Wozney

CHAPTER 51
Bone Morphogenetic Protein Receptors and Actions **929**
Kohei Miyazono

CHAPTER 52
Colony-Stimulating Factors **943**
Willy Hofstetter and Matthew T. Gillespie

CHAPTER 53
Local Regulators of Bone: IL-1, TNF, Lymphotoxin, Interferon-γ, IL-8, IL-10, IL-4, the LIF/IL-6 Family, and Additional Cytokines **961**
Mark C. Horowitz and Joseph A. Lorenzo

CHAPTER 54
Prostaglandins and Bone Metabolism **979**
Carol C. Pilbeam, John R. Harrison, and Lawrence G. Raisz

CHAPTER 55
Nitric Oxide and Other Vasoactive Agents **995**
Lee D. K. Buttery, Lucia Mancini, Niloufar Moradi-Bidhendi, Meg C. O'Shaughnessy, Julia M. Polak, and Iain MacIntyre

PART II
MOLECULAR MECHANISIMS OF METABOLIC BONE DISEASES

CHAPTER 56
Molecular Basis of PTH Overexpression **1017**
Geoffrey N. Hendy and Andrew Arnold

CHAPTER 57
Familial Benign Hypocalciuric Hypercalcemia and Neonatal Primary Hyperparathyroidism **1031**
Ghada El-Hajj Fuleihan, Edward M. Brown, and Hunter Heath III

CHAPTER 58
Multiple Endocrine Neoplasia Type 1 **1047**
Maria Luisa Brandi, Cesare Bordi, Francesco Tonelli, Alberto Falchetti, and Stephen J. Marx

CHAPTER 59
The Role of the *RET* Protooncogene in Multiple Endocrine Neoplasia Type 2 **1067**
Robert F. Gagel and Gilbert J. Cote

CHAPTER 60
Systemic Factors in Skeletal Manifestations of Malignancy **1079**
Janet E. Henderson, Richard Kremer, and David Goltzman

CHAPTER 61
Local Factors in Skeletal Malignancy **1093**
Gregory R. Mundy, Toshiyuki Yoneda, Theresa A. Guise, and Babatunde Oyajobi

CHAPTER 62
Molecular Basis of PTH Underexpression **1105**
R. V. Thakker

CHAPTER 63
Jansen's Metaphysical Chondrodysplasia and Blomstrand's Lethal Chondrodysplasia: Two Genetic Disorders Caused by PTH/PTHrP Receptor Mutations **1117**
Harald Jüppner, Ernestina Schipani, and Caroline Silve

CHAPTER 64
Pseudohypoparathyroidism **1137**
Michael A. Levine

CHAPTER 65
Other Skeletal Diseases Resulting from G Protein Defects: Fibrous Dysplasia of Bone and McCune–Albright Syndrome **1165**
Lee S. Weinstein

CHAPTER 66
Osteogenesis Imperfecta **1177**
David W. Rowe

CHAPTER 67
Hereditary Deficiencies in Vitamin D Action **1195**
Uri A. Liberman

CHAPTER 68
Oncogenic Osteomalacia **1209**
Kenneth W. Lyles

CHAPTER 69
Osteopetrosis **1217**
L. Lyndon Key, Jr., and William L. Ries

CHAPTER 70
Hypophosphatasia: Nature's Window on Alkaline Phosphatase Function in Man **1229**
Michael P. Whyte

CHAPTER 71
Paget's Disease of Bone **1249**
Frederick R. Singer and G. David Roodman

CHAPTER 72
Genetic Determinants of Bone Mass and Osteoporotic Fracture **1259**
Stuart H. Ralston

CHAPTER 73
Pathophysiology of Osteoporosis **1275**
Gideon A. Rodan, Lawrence G. Raisz, and John P. Bilezikian

CHAPTER 74
Evaluation of Risk for Osteoporosis Fractures **1291**
Patrick Garnero and Pierre D. Delmas

PART III
PHARMACOLOGICAL MECHANISMS OF THERAPEUTICS

CHAPTER 75
Parathyroid Hormone **1305**
A. B. Hodsman, D. A. Hanley, P. H. Watson, and L. J. Fraher

CHAPTER 76
Calcium **1325**
Robert P. Heaney

CHAPTER 77
Calcium Receptors as Novel Drug Targets 1339
Edward F. Nemeth

CHAPTER 78
Bisphosphonates: Mechanisms of Action 1361
Herbert Fleisch, Alfred Reszka, Gideon A. Rodan, and Michael Rogers

CHAPTER 79
Fluoride in Osteoporosis 1387
Johann D. Ringe

CHAPTER 80
The Pharmacology of Estrogens in Osteoporosis 1401
Robert Lindsay and Felicia Cosman

CHAPTER 81
Vitamin D and Analogs 1407
Glenville Jones

CHAPTER 82
Molecular and Clinical Pharmacology of Calcitonin 1423
Mone Zaidi, Angela M. Inzerillo, Bruce Troen, Baljit S. Moonga, Etsuko Abe, and Peter Burckhardt

CHAPTER 83
Growth Hormone and Insulin-like Growth Factor-I Treatment for Metabolic Bone Diseases 1441
Clifford J. Rosen

CHAPTER 84
Anabolic Steroid Effects on Bone in Women 1455
Azriel Schmidt, Shun-ichi Harada, and Gideon A. Rodan

CHAPTER 85
Estrogen Effects on Bone in the Male Skeleton 1467
John P. Bilezikian, Sundeep Khosla, and B. Lawrence Riggs

CHAPTER 86
Mechanisms of Exercise Effects on Bone 1477
Robert Marcus

PART IV
METHODS IN BONE RESEARCH

CHAPTER 87
Application of Transgenic Mice to Problems of Skeletal Biology 1491
Stephen Clark and David Rowe

CHAPTER 88
Use of Cultured Osteoblastic Cells to Identify and Characterize Transcriptional Regulatory Complexes 1503
Dwight A. Towler and Rene St. Arnaud

CHAPTER 89
Current Methodologic Issues in Cell and Tissue Culture 1529
Robert J. Majeska and Gloria A. Gronowicz

CHAPTER 90
Biochemical Markers of Bone Metabolism 1543
Markus J. Seibel, Richard Eastell, Caren M. Gundberg, Rosemary Hannon, and Huibert A. P. Pols

CHAPTER 91
Methods and Clinical Issues in Bone Densitometry and Quantitative Ultrasonometry 1573
Glen M. Blake and Ignac Fogelman

CHAPTER 92
Controversial Issues in Bone Densitometry 1587
Paul D. Miller

CHAPTER 93
Macro- and Microimaging of Bone Architecture 1599
Yebin Jiang, Jenny Zhao, and Harry K. Genant

CHAPTER 94
Transilial Bone Biopsy **1625**
Robert R. Recker and
M. Janet Barger-Lux

CHAPTER 95
Animal Models in Osteoporosis Research **1635**
Donald B. Kimmel

CHAPTER 96
Defining the Genetics of Osteoporosis: Using the Mouse to Understand Man **1657**
C. J. Rosen, L. R. Donahue, and W. G. Beamer

Index for Volumes 1 and 2 1667

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Preface to the Second Edition

The success of the first edition of *Principles of Bone Biology* clearly indicated that this text met an important need in our field. Well-worn copies (often with a cracked spine!) can be found on the shelves of bone biology research laboratories and offices throughout the world. We knew from the outset that undertaking the first edition would include a commitment to producing a second one. Advances in bone biology over the past five years have moved forward at a dizzying pace, clearly justifying the need for a second edition at this time. The elucidation of the molecular interactions between osteoblasts and osteoclasts is one of many examples documenting this point. Studies of animals in which critical genes have been deleted or over-expressed have produced some surprises and added still further complexity to what we have already recognized as an extremely complex regulatory system controlling the development and maintenance of skeletal structures. These and many other advances have provided the background for further development of effective therapeutic approaches to metabolic bone diseases.

In preparing the second edition, we have asked all authors to provide extensive revisions of their chapters. Additionally, the second edition features new authors who have written 10 new chapters. Some chapters from the first edition have been consolidated or otherwise reconfigured to keep the total number of chapters essentially the same as in the first edition. Although the number of chapters and their organizational structure has been retained, the extraordinary amount of new information has led to an increase in size of many of the chapters along with more extensive referencing. As a result, the substantially larger second edition is being published in two volumes. Each volume contains a full table of contents and full indexing to help the reader find specific information. The somewhat smaller individual volumes should be easier to handle and hold up better to the extensive use we expect from readers.

As was the case in the first edition, we asked our authors to meet a tight schedule so that the text would be as up-to-date as possible. We are indebted to our many authors who successfully met this challenge. The updated chapters as well as the new ones have, therefore, been written in such a way that the newest and most exciting breakthroughs in our field are still fresh. This task could not have been completed without the help of the staff at Academic Press. We acknowledge, in particular, Jasna Markovac and Mica Haley. They have been enormously helpful in all phases of this effort.

We have enjoyed very much the task of bringing this second edition to you. We trust that this second edition will be even more useful to you than the first. Enjoy the book!

John P. Bilezikian
Lawrence G. Raisz
Gideon A. Rodan

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Preface to the First Edition

The world of modern science is undergoing a number of spectacular events that are redefining our understanding of ourselves. As with any revolution, we should take stock of where we have been, where we are, and where we are going. Our special world of bone biology is participating in and taking advantage of the larger global revolution in modern science. Often with shocking but delightful suddenness, we are gaining new insights into difficult issues, discovering new concepts to explain old observations, developing new approaches to perennial mysteries, and applying novel technological advances from other fields to our own. The pace with which the bone world is advancing is impressive not only to the most ardent optimists, who did not expect so much so soon, but also to the more sober minded who, only several years ago, would have brushed off the notion that progress could come with such lightening speed.

The rationale for this book is rooted in the recognition of the revolution in bone biology. We need a new repository of knowledge, bringing us both to the core and to the edge of our universe. Our goal is to provide complete, truly up-to-date, and detailed coverage of this exciting and rapidly developing field. To achieve this, we assembled experts from all over the world and asked them to focus on the current state of knowledge and the prospects for new knowledge in their area of expertise. To this end, *Principles of Bone Biology* was conceived. It is designed to be useful to students who are becoming interested in the field and to young investigators at the graduate or postgraduate level who are beginning their research careers. It is also designed for more established scientists who want to keep up with the changing nature of our field, who want to mine this lode to enrich their own research programs, or who are changing their career direction. Finally, this book is written for anyone who simply strives for greater understanding of bone biology.

This book is intended to be comprehensive but readable. Each chapter is relatively brief. The charge to each author has been to limit size while giving the reader information so complete that it can be appreciated on its own, without necessary recourse to the entire volume. Nevertheless, the book is also designed with a logic that might compel someone to read on, and on, and on!

The framework of organization is fourfold. The first 53 chapters, in a section titled "Basic Principles," cover the cells themselves: the osteoblast, the osteoclast, and the osteocyte; how they are generated; how they act and interact; what turns them on; what turns them off; and how they die. In this section, also, the biochemistry of collagenous and noncollagenous bone proteins is covered. Newer understandings of calcium, phosphorus, and magnesium metabolism and the hormones that help to control them, namely, parathyroid hormone, vitamin D metabolites, calcitonin, and related molecules, are presented. A discussion of other systemic and local regulators of bone metabolism completes this section.

The second section of this book, "Molecular Mechanisms of Metabolic Bone Diseases," is specifically devoted to basic mechanisms of a variety of important bone diseases. The intention of these 17 chapters is not to describe the diseases in clinical, diagnostic, or therapeutic terms but rather to illustrate our current understanding of underlying mechanisms. The application of the new knowledge summarized in Part I to pathophysiological, pathogenetic, and molecular mechanisms of disease has relevance to the major metabolic bone disorders such as osteoporosis, primary hyperparathyroidism, and hypercalcemia of malignancy as well as to the more uncommon disorders such as familial benign hypocalciuric hypercalcemia, pseudohypoparathyroidism, and osteopetrosis.

The third section of this book, "Pharmacological Mechanisms of Therapeutics," addresses the great advances that have been made in elucidating how old and new drugs act to improve abnormalities in bone metabolism. Some of these drugs are indeed endogenous hormones that under

specified circumstances are useful therapies: estrogens, vitamin D, calcitonin, and parathyroid hormone are representative examples. Others agents such as the bisphosphonates, fluoride, and calcium are reviewed. Finally, agents with therapeutic potential but still in development such as calcimimetics, insulin-like growth factors, transforming growth factor, bone morphogenetic protein, and fibroblast growth factor are presented with a view to the future. The intent of this 12-chapter section is not to provide step-by-step "how-to" instructions for the clinical uses of these agents. Such prescribing information for established therapies is readily found in other texts. Rather, the underlying mechanisms by which these agents are currently believed to work is the central point of this section.

The fourth and final section of this book, "Methods in Bone Research," recognizes the revolution in investigative methodologies in our field. Those who want to know about the latest methods to clone genes, to knock genes out, to target genes, and to modify gene function by transfection and by transcriptional control will find relevant information in this section. In addition, the selection and characteristics of growth conditions for osteoblastic, osteoclastic, and stem cells; animal models of bone diseases; assay methodologies for bone formation and bone resorption and surrogate bone markers; and signal transduction pathways are all covered. Finally, the basic principles of bone densitometry and bone biopsies have both investigative and clinical relevance. This 15-chapter section is intended to be a useful reference for those who need access to basic information about these new research technologies.

The task of assembling a large number of international experts who would agree to work together to complete this ambitious project was formidable. Even more daunting was the notion that we would successfully coax, cajole, and otherwise persuade authors of 97 chapters to complete their tasks within a six-month period. For a book to be timely and still fresh, such a short time leash was necessary. We are indebted to all the authors for delivering their chapters on time.

Finally, such a monumental undertaking succeeds only with the aid of others who helped conceive the idea and to implement it. In particular, we are grateful to Jasna Markovac of Academic Press, who worked tirelessly with us to bring this exciting volume to you. We also want to thank Tari Paschall of Academic Press, who, with Jasna, helped to keep us on time and on the right course. We trust our work will be useful to you whoever you are and for whatever reason you have become attracted to this book and our field. Enjoy the book. We enjoyed editing it for you.

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PART I

Basic Principles

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CHAPTER 1

Structure and Development of the Skeleton

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Introduction

This brief overview of the structure and development of the skeleton focuses primarily on bone and its cells (Fig. 1). We review the structure and function of these cells, their divisions of labor within the skeleton, the emerging complexities of their changing regulation with age, and the emerging knowledge of the molecular regulation of the skeleton. We also look briefly at emerging knowledge of molecular regulation of the skeleton. Because neither the complexities of the cellular microenvironment nor the influences of nonosseous tissues on bone cells can be duplicated *in vitro,* these parameters of bone metabolism must be evaluated eventually in the complexities of the *in vivo* environment. To assist both the reader and the investigator in interpreting these and other studies of the structure, development, and regulation of bone, we offer a brief critical analysis review of various methods used to examine bone metabolism.

The interested reader is referred to reviews of this topic from different perspectives (Buckwalter *et al.,* 1996a,b; Hall, 1987; Marks and Popoff, 1988; Schenk, 1992).

Cells of The Skeleton: Development, Structure, and Function

Bone is a highly specialized form of connective tissue that is nature's provision for an internal support system in all higher vertebrates. It is a complex living tissue in which the extracellular matrix is mineralized, conferring marked rigidity and strength to the skeleton while still maintaining some degree of elasticity. In addition to its supportive and protective functions, bone is a major source of inorganic ions, actively participating in calcium homeostasis in the body. There is increasing evidence that the central control of development and renewal of the skeleton is more sophisticated than previously appreciated (Ducy *et al.,* 2000).

Bone is composed of an organic matrix that is strengthened by deposits of calcium salts. Type I collagen constitutes approximately 95% of the organic matrix; the remaining 5% is composed of proteoglycans and numerous noncollagenous proteins (see chapters to follow). Crystalline salts deposited in the organic matrix of bone under cellular control are primarily calcium and phosphate in the form of hydroxyapatite.

Morphologically, there are two forms of bone: cortical (compact) and cancellous (spongy). In cortical bone, densely packed collagen fibrils form concentric lamellae, and the fibrils in adjacent lamellae run in perpendicular planes as in plywood (Fig. 2). Cancellous bone has a loosely organized, porous matrix. Differences between cortical and cancellous bone are both structural and functional. Differences in the structural arrangements of the two bone types are related to their primary functions: cortical bone provides mechanical and protective functions and cancellous bone provides metabolic functions.

Bone Cell Structure and Function

Bone is composed of four different cell types (Fig. 1). Osteoblasts, osteoclasts, and bone lining cells are present on bone surfaces, whereas osteocytes permeate the mineralized interior. Osteoblasts, osteocytes, and bone-lining cells originate from local osteoprogenitor cells (Fig. 3A), whereas osteoclasts arise from the fusion of mononuclear

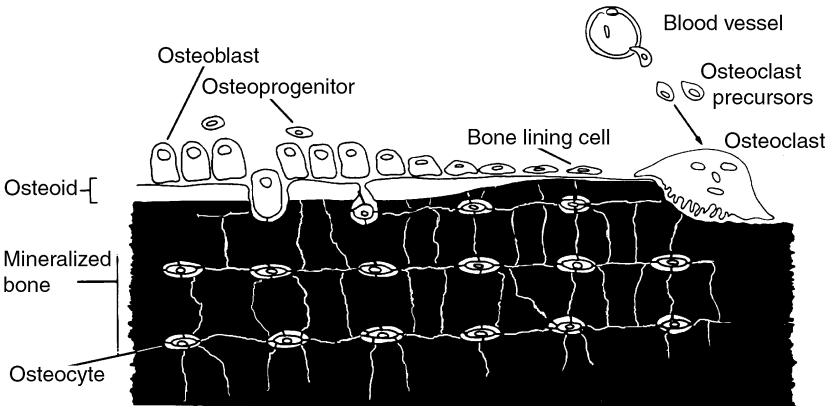

Figure 1 The origins and locations of bone cells. Taken from Marks and Popoff (1988). Reprinted by permission of John Wiley and Sons, Inc.

precursors, which originate in the various hemopoietic tissues. The apical and basal surfaces of bone cells are defined in an opposite sense from those of epithelia. Apical surfaces are those that are attached to the extracellular matrix and basal surfaces are those that are away from the matrix.

Osteoblasts are fully differentiated cells responsible for the production of the bone matrix. Portions of four osteoblasts are shown in Figs. 2 and 3B. An osteoblast is a typical protein-producing cell with a prominent Golgi apparatus and well-developed rough endoplasmic reticulum. It secretes the type I collagen and the noncollagenous proteins of the bone matrix (see Chapters 4 and 5). The staggered overlap of the individual collagen molecules provides the characteristic periodicity of type I collagen in bone matrix. Numerous noncollagenous proteins have been isolated from bone matrix (Sandberg, 1991), but to date there is no consensus for a definitive function of any of them.

Osteoblasts regulate mineralization of bone matrix, although the mechanism(s) is not completely understood. In woven bone, mineralization is initiated away from the cell surface in matrix vesicles that bud from the plasma membrane of osteoblasts. This is similar to the well-documented role of matrix vesicles in cartilage mineralization (Hohling *et al.,* 1978). In lamellar bone, the mechanism of mineralization appears to be different. Mineralization begins in the hole region between overlapped collagen molecules where there are few, if any, matrix vesicles Landis *et al.,* 1993) and appears to be initiated by components of the collagen molecule itself or noncollagenous proteins at this site. Whatever the mechanisms of mineralization, collagen is

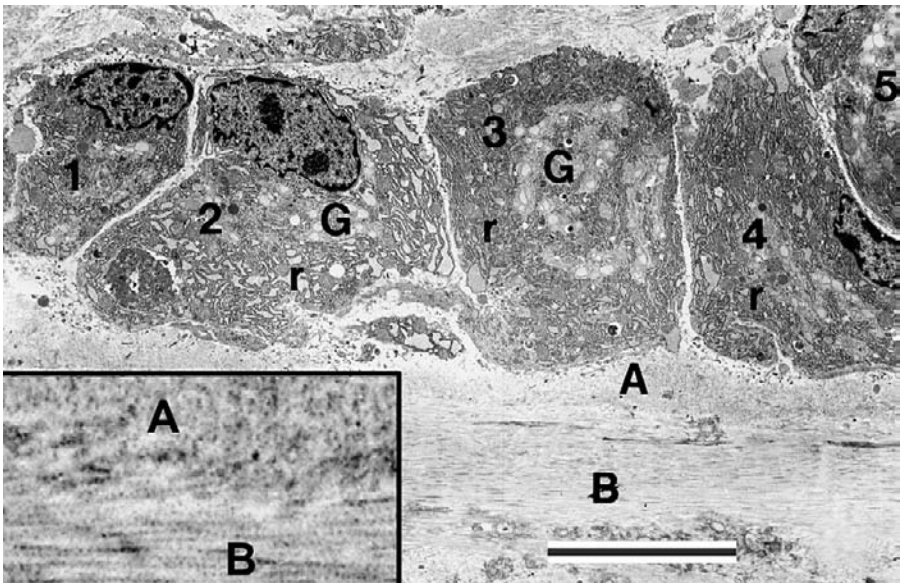

Figure 2 Transmission electron micrograph of osteoblasts (numbered) on a bone surface in which the collagenous matrix has been deposited in two layers (A and B) at right angles to each other. The Golgi apparatus (G) and rough endoplasmic reticulum (r) are prominent cytoplasmic organelles in osteoblasts. (Original magnification: ×2800. Bar: 0.1 μm.)

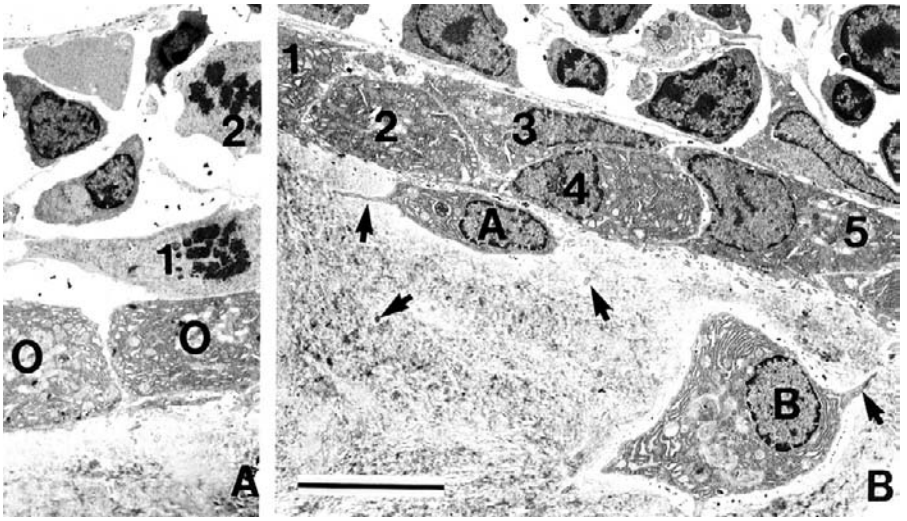

Figure 3 (A) Transmission electron micrograph of an osteoblast (O) and daughter cells (1 and 2) of a dividing osteoprogenitor cell. (Original magnification: ×2100.) (B) Transmission electron micrograph of five osteoblasts (numbered) and two osteocytes (A and B) in the process of being embedded in bone matrix. Arrows identify processes extending from the osteocytes and within the bone matrix that will serve as their metabolic and regulatory lifelines via gap junctions between adjacent cells. (Original magnification: ×2100. Bar: 0.1 μm.)

at least a template for its initiation and propagation and there is always a layer of unmineralized bone matrix (osteoid) on the surface under osteoblasts. Matrix deposition is usually polarized toward the bone surface, but periodically becomes generalized, surrounding the osteoblast and producing the next layer of osteocytes. Deposition of mineral makes the matrix impermeable, and to ensure a metabolic lifeline, osteocytes establish numerous cytoplasmic connections with adjacent cells before mineralization.

The osteocyte (Fig. 3B) is a mature osteoblast within the bone matrix and is responsible for its maintenance (Buckwalter *et al.,* 1996a). These cells have the capacity not only to synthesize, but also to resorb matrix to a limited extent. Each osteocyte occupies a space, or lacunae, within the matrix and extends filopodial processes through canaliculi in the matrix (Figs. 4A and B) to contact processes of adjacent cells (Figs. 5A and B) by means of gap junctions. Because the diffusion of nutrients and metabolites through the mineralized matrix is limited, filopodial connections permit communication between neighboring osteocytes, internal and external surfaces of bone, and with the blood vessels traversing the matrix. The functional capacities of osteocytes can be easily ascertained from their structure. Matrix-producing osteocytes have the cellular organelles characteristic of osteoblasts (Fig. 5A), whereas osteolytic osteocytes contain lysosomal vacuoles and other features typical of phagocytic cells (Fig. 5B). (For a review of osteocyte functions, see Chapter 6.)

Bone lining cells are flat, elongated, inactive cells that cover bone surfaces that are undergoing neither bone formation nor resorption (Fig. 6). Because these cells are inactive, they have few cytoplasmic organelles. Little is known regarding the function of these cells; however, it has been speculated that bone lining cells can be precursors for osteoblasts.

Osteoclasts are large, multinucleated cells that resorb bone (Fig. 7). When active, osteoclasts rest directly on the bone surface and have two plasma membrane specializations: a ruffled border and a clear zone. The ruffled border is the central, highly infolded area of the plasma membrane where bone resorption takes place. The clear zone is a microfilament-rich, organelle-free area of the plasma membrane that surrounds the ruffled border and serves as the point of attachment of the osteoclast to the underlying bone matrix. Active osteoclasts exhibit a characteristic polarity. Nuclei are typically located in the part of the cell most removed from the bone surface and are interconnected by cytoskeletal proteins (Watanabe *et al.,* 1995). Osteoclasts contain multiple circumnuclear Golgi stacks, a high density of mitochondria, and abundant lysosomal vesicles that arise from the Golgi and cluster near the ruffled border. A molecular phenotype for osteoclasts is emerging (Horne, 1995; Sakai *et al.,* 1995) (see Chapters 7, 8, and 9).

Cellular Divisions of Labor within the Skeleton

Cartilage and bone are two tissues that comprise the skeleton. Despite their shared supportive functions, these tissues are dramatically different (i.e., matrix composition and mineralization state). The cellular activities that occur in each of the two tissues, however, are limited to matrix formation, matrix mineralization, and matrix resorption. In each tissue, different cell types perform different, yet sometimes overlapping, functions (Fig. 8). In cartilage, matrix is produced and mineralized by chondrocytes. Mineralization and resorption of cartilage are activities associated with hypertrophied chondrocytes.

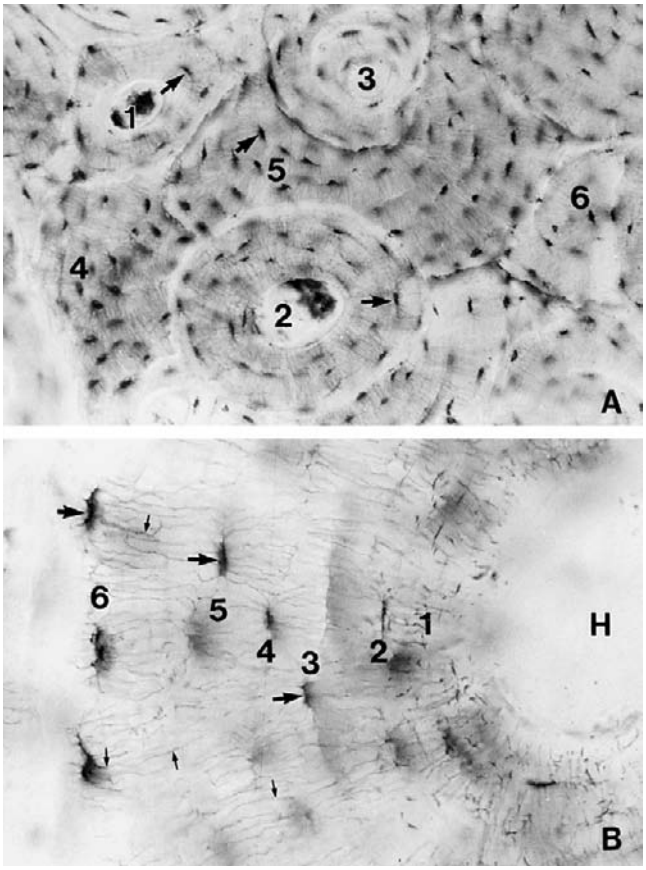

Figure 4 (A) A thin-ground crosssection of human cortical bone in which osteocyte lacunae (arrows) and canaliculi have been stained with India ink. Osteocytes are arranged around a central vascular channel to constitute Haversian systems. Active Haversian systems (1, 2, and 3) have concentric lamellae in this plane. Older Haversian systems (4, 5, and 6) have had parts of their original territories invaded and remodeled. This is seen most clearly where 2 and 3 have invaded the territory originally occupied by 5. (Original magnification: ×185. Bar: 50 μm.) (B) Higher magnification of part of a Haversian system showing the successive layering (numbers) of osteocytes (large arrows) from the central core (H) that contains the vasculature. Small arrows identify the canaliculi that connect osteocyte lacunae in different layers. (Original magnification: ×718. Bar: 50 μm.)

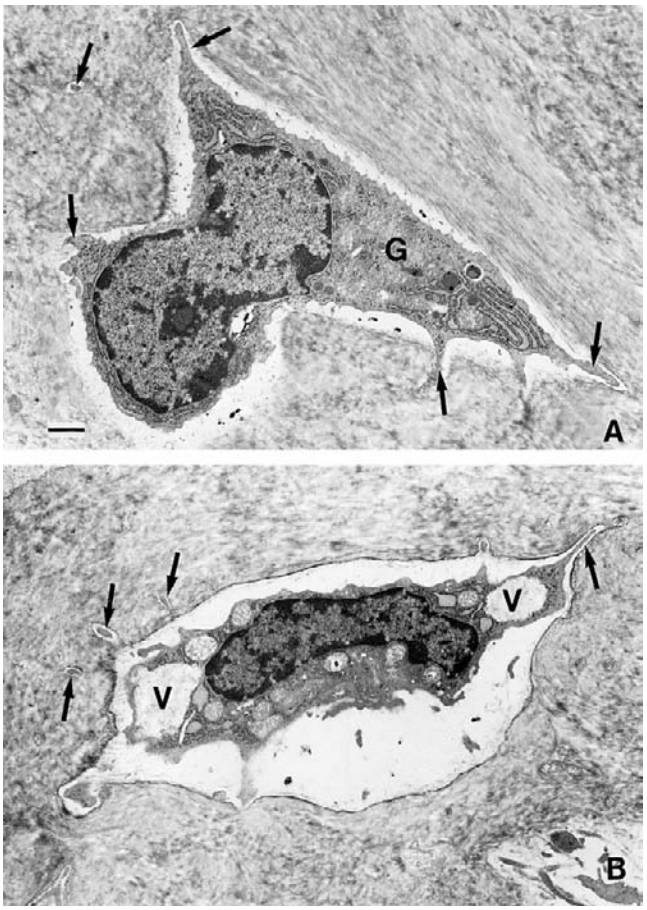

Figure 5 Transmission electron micrographs of two osteocytes of different phenotype and functional states. Young osteocytes (A) have nuclear and cytoplasmic features of osteoblasts: a euchromatic nucleus with a prominent nucleolus, a large Golgi apparatus (G), prominent rough endoplasmic reticulum, and numerous cytoplasmic processes (arrows) projecting into the surrounding matrix. Some older osteocytes (B) can have an osteolytic phenotype with increased lacunar volume, an electron-dense lacunar surface, condensed nuclei, and numerous cytoplasmic vacuoles. (Original magnification: ×7000. Bar: 0.01 μm.)

However, cartilage mineralized in the growth plate is resorbed by osteoclasts (see Figs. 12 and 13). In bone, matrix is produced and mineralized by osteoblasts and osteocytes. Resorption occurs primarily by osteoclasts, but localized perilacunar resorption may occur around osteocytes (Fig. 5B).

Coordination of Cellular Activities during Skeletal Development and Maturation

Variable Activities of Skeletal Cells

The activities of skeletal cells vary considerably over the life span of the organism. This is necessary to build a mineralized tissue where there was none before and to maintain it after reaching maturity. The variable activities of bone formation and resorption in relation to each other during the human life cycle are summarized in Fig. 9. The first two decades are devoted to development of the skeleton, called modeling. During this period, bone formation necessarily precedes and exceeds bone resorption. Thus, although these activities are related temporally and spatially, they are uncoupled in the sense that they are unequal. During the next three decades (and beyond) the adult skeleton is maintained by removing and replacing a fraction each year. This remodeling begins with a localized resorption that is succeeded by a precisely equal formation of bone at the same site (Parfitt, 1994). Thus, bone formation equals bone resorption, a process called coupling (see the section that follows, Fig. 10). In compact bone, resorption by osteoclasts produces a cutting cone through Haversian systems, and the subsequent reformation of these systems produces osteons of unequal age, size, and configurations (Fig. 4A). Sometime after the fifth decade, the formative phase of the remodeling sequence

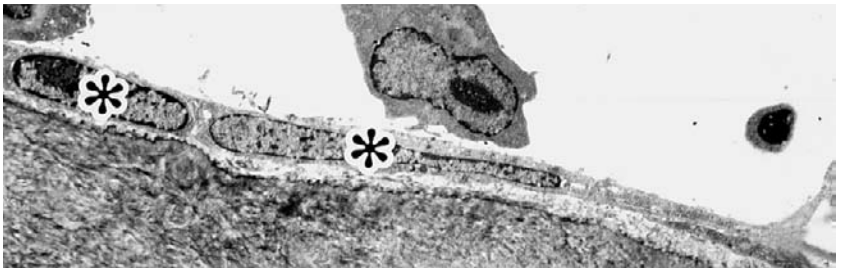

Figure 6 Transmission electron micrograph of bone lining cells (asterisks). These flat cells have few organelles and form a thin cellular layer on inactive bone surface that is often hard to resolve by light microscopy. (Original magnification: ×3000. Bar: 0.1 μm.)

fails to keep pace with resorptive activity and skeletal mass, including the connectivity of trabecular bone, decreases. This reduces skeletal strength and increases the risk of fracture over time, depending on the magnitude by which resorption and formation are uncoupled. Given the apparent inevitability and universality of an osteoporotic trend with age, therapy has focused on increasing skeletal mass during development and/or slowing resorption after the fifth decade. What is needed is a selective, predictable, locally active anabolic agent. This discovery may be more likely if we focus more on skeletal development than its pathology.

It is clear that the coordination of the activities of skeletal cells is a local event. Local factors recruit specific cells and local factors regulate their activity. Furthermore, multiple factors in a precise sequence and concentration are needed for the full expression of a cell's potential, and these factors and their concentrations differ for bone formation and bone resorption. It is also clear that more than one cell type can produce many of these factors and that normal skeletal development is a collaborative effort of cells from diverse lineages (Marks and Popoff, 1988; Yamazaki and Eyden, 1995; Yoder and Williams, 1995). The complexities of skeletal development and maintenance are now being acknowledged along with the poverty of our understanding of these relationships. This book is an attempt to put these factors and cells in some order that has both theoretical (functional) and practical (therapeutic) significance.

General Regulation of Cellular Activities

Most simply put, the challenges of understanding the complexities of skeletal modeling and remodeling, coupling and uncoupling, are illustrated by the influences that osteoblasts have on osteoclasts and vice versa (Marks and Popoff, 1988; Mundy, 1994). These are illustrated schematically in Fig. 10. Osteoblasts, the progeny of local osteoprogenitor cells, produce factors that influence the differentiation and function of osteoclasts (Martin and Ng,

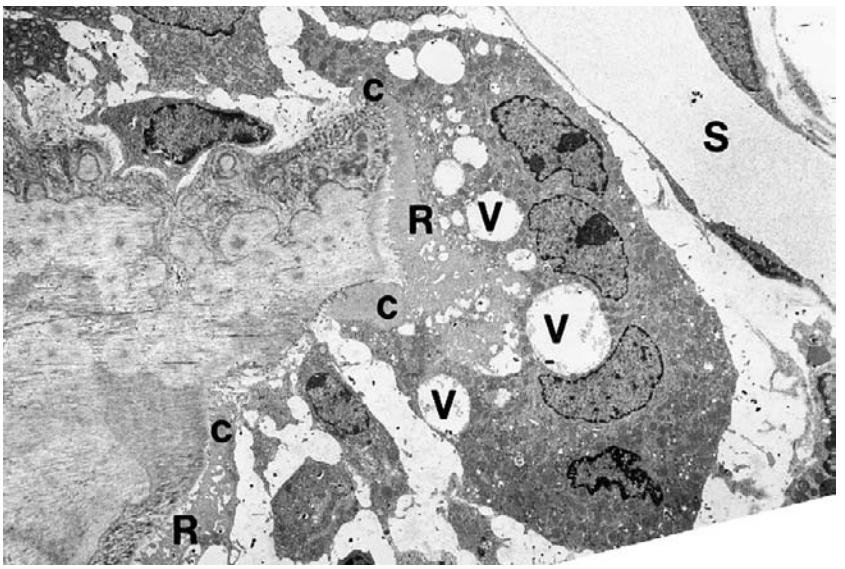

Figure 7 Transmission electron micrograph of parts of two osteoclasts. These multinucleated cells attach to bones at clear zones (C), which create a three-dimensional seal around the ruffled border (R) working area. Active cells have large vacuoles in the cytoplasm next to the ruffled border. S, vascular sinus. (Original magnification: ×2240. Bar: 0.1 μm.)

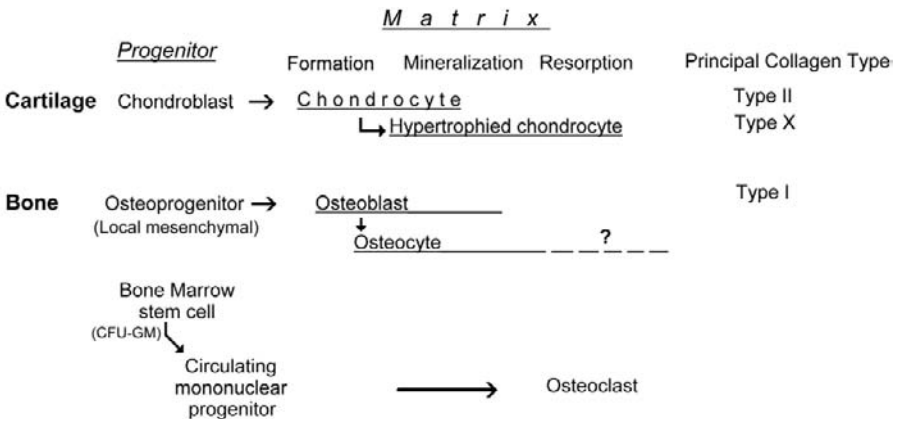

Figure 8 Cellular division of labor in the skeleton. Schematic of the major cells and their functions in cartilage and bone.

1994). Some of these are deposited in bone matrix itself, whereas others appear to be secreted locally in response to hormones or local factors. These conclusions are based on the facts that receptors for most osteolytic factors are found on osteoblasts, not osteoclasts (Rodan and Martin, 1981), that osteoclasts resorb bone in response to factors released into culture media by activated osteoblasts (McSheehy and Chambers, 1986), and that some components of the extracellular matrix of bone can attract and/or activate osteoclasts (Thesingh and Burger, 1983). Osteoclasts, however, are derived from hemopoietic stem cell progeny (monocytes) that use vascular routes to migrate to skeletal sites (Marks, 1983). After exiting the vasculature at specific locations in the skeleton, these mononuclear precursors either fuse with each other or other multinucleated cells to become osteoclasts. Their activation depends in large part on local signals derived from other cells, including but not limited to osteoblasts. However, bone resorption itself produces factors that recruit and activate osteoblasts. Indeed, the ability of supernatants of resorbing bone organ cultures to promote the proliferation and differentiation of osteoblast progenitors began the current interest in identifying the coupling factor(s) (Drivdahl *et al.,* 1981; Farley *et al.,* 1982).

It is clear from the foregoing that the activities of skeletal cells in a particular site change with age, that these changes are controlled by local factors, including weight bearing, and that we have much to learn about the identity and sequence of action of these agents in the changing dynamics of skeletal metabolism (Frost and Jee, 1994; Weryha and Leclere, 1995).

	Development	Maintenance	The Osteoporoses
Age	0-20	20-50	50+
Sequence	BF → BR Modeling	BR→ BF Remodeling	BR → BF
Activity	BF > BR	BF = BR	BF < BR

Figure 9 Development, maintenance, and pathology of the skeleton. Summary of the relative levels of skeletal cell activity during the human life cycle.

Formation of the Skeleton

Formation of the skeleton (ossification) occurs by either a direct (intramembranous) or an indirect (endochondral) process. Both require a solid base and a well-developed vascular supply for the elaboration and mineralization of the extracellular matrix. Mobility or low oxygen tension at the site favors the differentiation of chondrocytes or fibroblasts.

Intramembranous ossification occurs during embryonic development by the direct transformation of mesenchymal cells into osteoblasts. This type of ossification for entire bones is restricted to those of the cranial vault, some facial bones, and parts of the mandible and clavicle. The flat bones of the skull grow toward each other from primary ossification centers in each and meet at sutures. Sutures are fibroelastic cellular domains (Fig. 11) composed of the periostea of adjacent bones. The center of a suture contains a proliferating cell population whose progeny differentiate and move toward adjacent bone surfaces, becoming osteoblasts. During this migration these cells produce type III collagen at low levels, types V and XI transiently, and finally type I, the major bone collagen (Wurtz *et al.,* 1998). This mechanism provides a steady source of osteoblasts and allows bones to expand at their edges. When growth is complete, sutures remain as fibrous connections or disappear, depending on the suture site.

Bones that participate in joints and bear weight form by endochondral ossification, a method by which the unique properties of cartilage and bone are exploited to provide a mechanism the for formation and growth of the skeleton during growth of the individual. In such bones the condensed embryonic mesenchyme transforms into cartilage, which reflects in both position and form the eventual bone to be formed at that site. In the central part of such a bone, endochondral ossification provides for a linear, interstitial proliferation of columns of chondrocytes. Their progressive hypertrophy, mineralization of the intercolumnar cartilage matrix in the long axis of the bone, and the persistence of mineralized cartilage after disappearance of its cells acts as an elongating scaffold for the deposition of subchondral

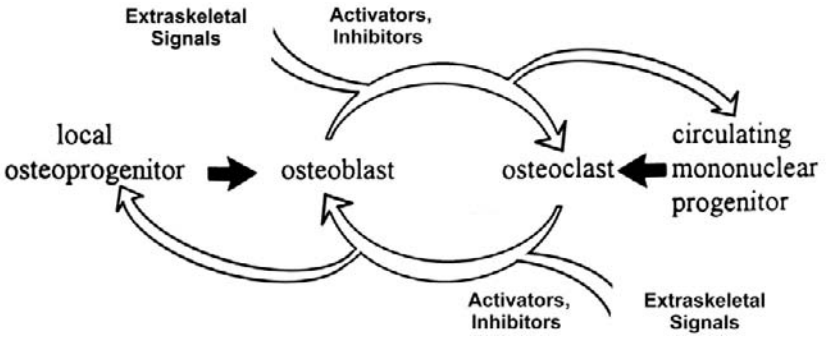

Figure 10 Cellular coordination of skeletal development. Schematic of the divergent origin and interrelated function of the principal bone cells.

(metaphyseal) bone (Hunziker, 1994). In the circumference of such a bone, starting initially at the center and progressing toward the ends, the investing cartilage cells and stroma (perichondrium) transform into osteoblasts that form a periosteal collar after the underlying chondrocytes have hypertrophied and mineralized the matrix. The peripheral osteoblasts (periosteum) arrive with a blood supply whose vessels penetrate the central hypertrophied, mineralized cartilage core and carry to the interior the skeletal cell progenitors for the formation and turnover of bone. Thus, peripherally extension of the periosteum and centrally mineralization of cartilage, hypertrophy, and disappearance of chondrocytes and bone formation on the mineralized cartilagenous scaffold proceed toward the end of each growing long bone.

The cellular events of long bone growth in length by endochondral ossification are illustrated in Figs. 12 and 13. At the top of the figures, chondrocyte proliferation and matrix elaboration in the direction of bone growth and the hypertrophy of these cells are the primary mechanisms for the linear growth of bones (Hunziker, 1994). Chondrocytes mineralize the intercolumnar matrix, producing a rigid scaffold that persists in the metaphysis and becomes the solid base upon which osteoblasts deposit and mineralize bone matrix. The closely packed mineralized cartilage septae at the chondroosseous junction are thinned to about one-third their density (Schenk *et al.,* 1967, 1968) by osteoclasts at this site (Fig. 12), providing space for new bone and a longitudinally oriented vasculature in the metaphysis (Aharinejad *et al.,* 1995). The final component of longitudinal bone growth is resorption of the central (marrow cavity) ends of metaphyseal trabeculae.

The fate of hypertrophied chondrocytes is controversial. Earlier reports of universal cell death conflicted with biochemical data and were perpetuated by poor fixation methods that produced pyknotic cells. Better fixation preserves the morphology of these cells, and it is clear that at least some hypertrophied chondrocytes survive (Farnum *et al.,* 1990; Hunziker and Schenk, 1984; Takechi and Itakura, 1995) after vascular penetration of their lacunae (Figs. 12 and 13) and can differentiate into osteoblasts (Galotto *et al.,* 1994; Roach *et al.,* 1995; Thesingh *et al.,* 1991) at least *in vitro* but that the percentage of such cells may vary among species (Gibson *et al.,* 1995).

Longitudinal bone growth is a precise balance between chondrocyte proliferation, cartilage matrix production and mineralization, and hypertrophy and vascular invasion of the lacuna of the terminal hypertrophied chondrocyte after re-

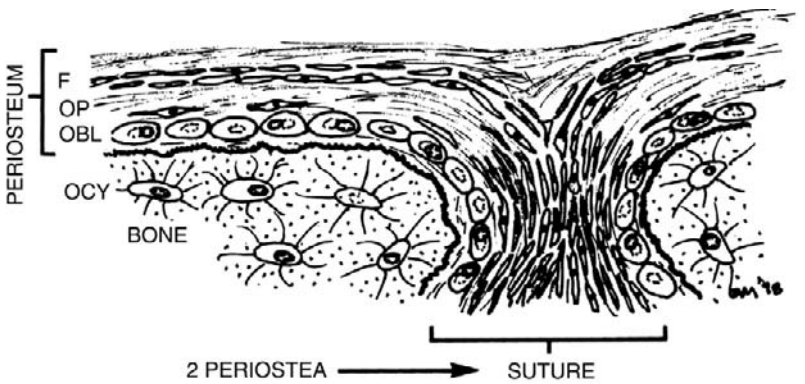

Figure 11 Cellular relationships in a periosteum and a suture. F, fibroblast; OP, osteoprogenitor cell; OBL, osteoblast; OCY, osteocyte. Reprinted from Marks *et al.* (1999), with permission of John Wiley & Sons.

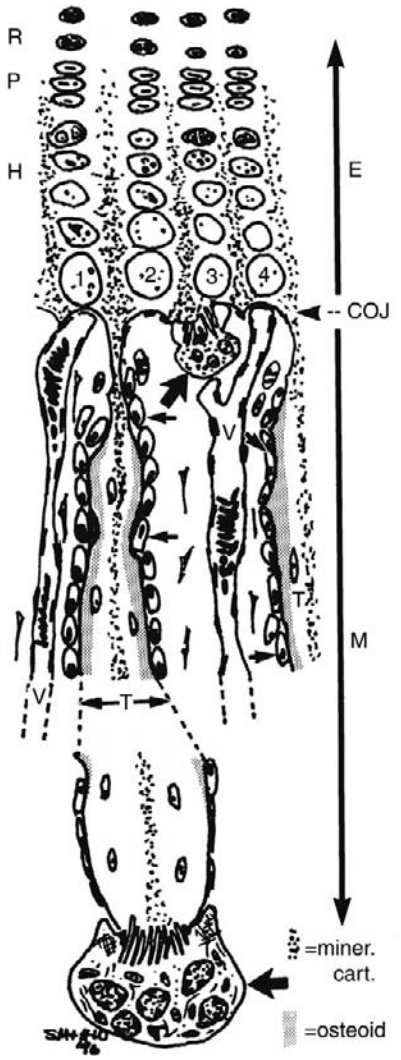

Figure 12 Schematic drawing of cellular locations and activities at the chondroosseous junction (COJ) of growing bone. The physis, or epiphyseal plate (E), consists of resting (R), proliferating (P), and hypertrophied (H) chondrocytes. In the metaphysis (M), trabeculae (T) alternate longitudinally with vascular channels (V). Osteoblasts (small arrows) line trabecular surfaces beginning just below the COJ, and osteoclasts (large arrows) are found in two locations: at the COJ and at the marrow cavity ends of the trabeculae. Chondrocytes are aligned in columns (four are numbered), and their alignments are maintained by mineralization of the longitudinal interterritorial matrix between columns that begins in the zone of proliferating chondrocytes and gets denser in the zone of hypertrophy. These mineralized cartilaginous struts are the surfaces in the metaphysis on which osteoblasts differentiate, produce, and mineralize the extracellular matrix of bone. All trabeculae in the metaphysis have a mineralized cartilage core, which is then resorbed, together with bone, by osteoclasts at the margin of the marrow cavity (bottom). Other osteoclasts at the COJ resorb about two of every three cores of mineralized cartilage that extend from the epiphysis. This provides space in the metaphysis for bone deposition and vascular invasion. The latter is an important regulator of the thickness of the zone of hypertrophied chondrocytes by penetrating the horizontal septum between the oldest such chondrocytes and the metaphysis (illustrated for cells 1 and 4). Reprinted from Marks (1998), with permission of C.V. Mosby.

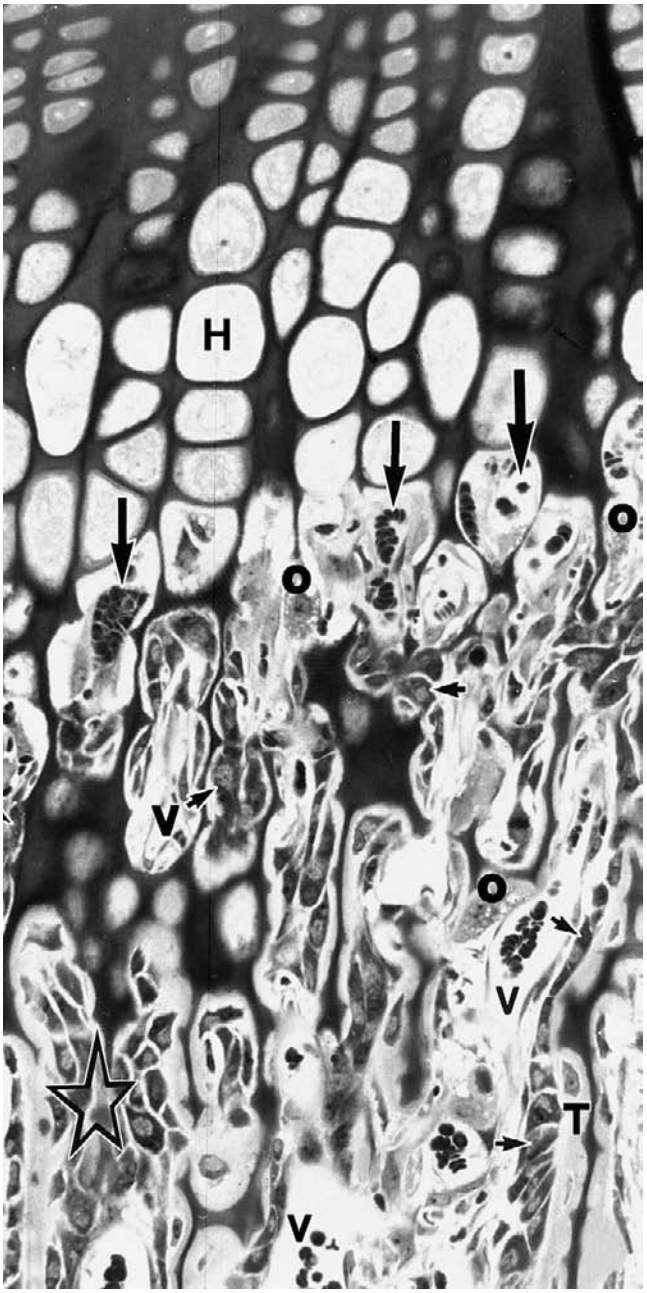

Figure 13 Photomicrograph of the chondroosseous junction in a young rat. The physis is composed primarily of hypertrophied (H) chondrocytes in this field. Vascular invasion of chondrocyte lacunae is occurring at many sites (vertical arrows) along the COJ, and vascular channels (V) are common. Mineralized cartilage in the metaphysis stains darkly. The typical trabecular cross section of a central cartilage core, bone, osteoid, and osteoblasts is clear at the lower right (T) but is obscured in much of the rest of the field due to the obliquity of their planes of section. Osteoblasts (small arrows) can be identified on most of the metaphyseal surfaces, and a large group (star) appears where trabeculae converge just out of the plane of this section. Several osteoclasts (O) can be seen near the COJ. (Toluidine blue stain; ×500.) Reprinted from Marks (1998), with permission of C. V. Mosby.

sorption of the horizontal septum within a column by mononuclear cells (Hunziker, 1994; Price *et al.,* 1994) rich in cathepsin B and with a distinct morphology (Lee *et al.,* 1995). Cartilage proliferation is under the direct influence of a variety of hormones (growth, thyroid, corticosteroids, and parathyroid) and local growth factors (insulin-like growth factors and basic fibroblast growth factor) (Nilsson *et al.,* 1994). Because most studies have been done *in vitro* where three-dimensional relationships of cells and matrices and the complex physiological landscape cannot be duplicated, it is

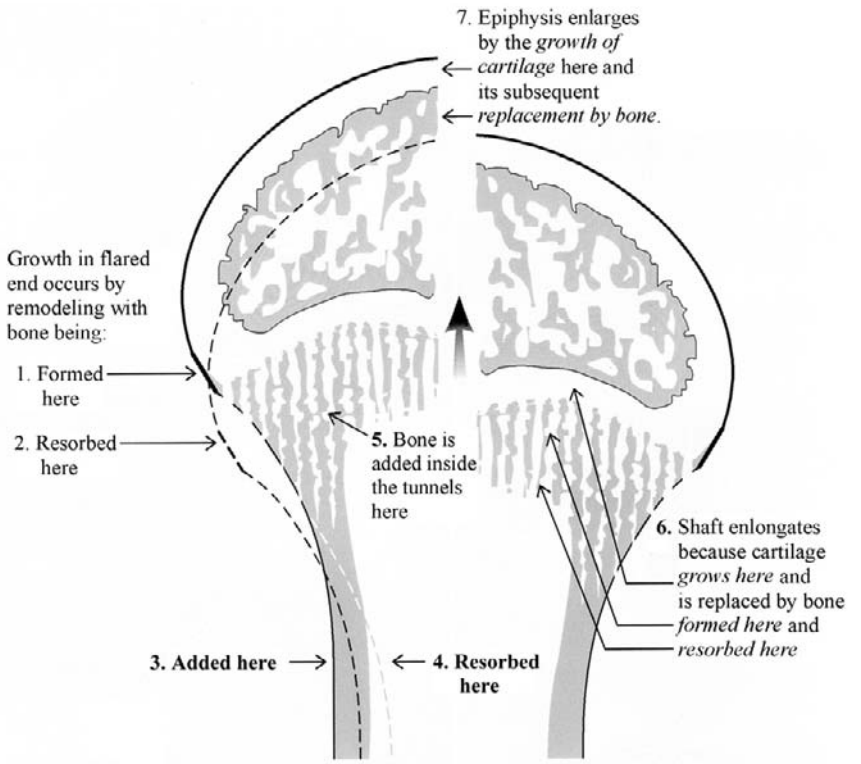

Figure 14 Diagram of regional changes in cartilage and bone that produce growth in the length (large arrow) and width of long bones. Reprinted from Marks (1998), with permission of C. V. Mosby.

not surprising that reports of the effects of individual factors on bone growth conflict and give us incomplete information at best.

Bone growth in diameter (Fig. 14) is accomplished most basically by formation externally (periosteum) and resorption internally (endosteum). This is strictly true only for the central portion of long bones and only if the bone is cylindrical. Because most bones are asymmetrical cylinders centrally and are expanded (flared) unevenly at each end, growth in diameter is more complex than depicted in the process just described and varies by region according to the dynamic changes in bone shape at that site.

At the flared ends of a growing long bone the periosteal collar externally surrounds part of the growth plate cartilage and extends much farther peripherally than the central bone (Fig. 14) of the shaft. Thus, during bone growth, with extension of the new periosteal collar, the old periosteal collar has to be removed and reformed toward the center. This is accomplished by resorption on the periosteal surface and formation on the endosteal surface at this site, a polarization of these activities that is opposite that seen at the center of the shaft. In summary, the succession of metabolic activities on the periosteal surface is (1) formation at the periosteal collar, (2) resorption, and (3) formation toward the center of the shaft. In general, activities in the peripheral endosteum are the opposite. In the metaphysis, bone formation on the mineralized cartilage scaffold takes place after osteoclasts thin the longitudinal mineralized cartilage remnants of the growth plate. This increases the thickness and strength of these trabeculae, which remain until their central ends are resorbed to accommodate longitudinal expansion of the marrow cavity during bone growth.

Bone growth involves the coordination of a variety of cellular activities in specific sites whose onset and rates vary among bones and even within a single bone during its development. These activities are under the influence of a variety of humoral and local factors whose relative concentrations, sites, and sequences of appearance vary during development.

The complexities of skeletal maintenance are unlikely to be substantially less complicated than those of development. Thus, the multiplicity and redundancy of the biological controls of skeletal metabolism need to be appreciated as we seek to interpret all experimental data.

Molecular Regulation of Skeletal Development

The principal physiologic processes of skeletal formation and maintenance might be summarized as pattern formation, transition from cartilage to bone, bone matrix synthesis and secretion, and bone resorption and remodeling. Genes with crucial roles in all these processes have been discovered recently, giving both new depth to our understanding of normal bone biology and hopes for novel clinical strategies and interventions in disease or injury. Some of these discoveries came as surprises in gene knockout or transgenic studies conceived with quite different expected outcomes, demonstrating the critical importance of evaluating gene function in the living organism. Genes essential for bone synthesis,

normal patterning, and bone resorption are discussed in the following brief overview. Subsequent chapters treat these in much greater detail.

Bone Formation

A molecular event crucial for the synthesis and secretion of bone matrix, i.e., for the fully differentiated activity of osteoblasts, is the production by osteoprogenitor cells of the DNA-binding transcription factor cbfa-1. Independent investigations led to its simultaneous discovery by three groups (Ducy *et al.,* 1997; Komori *et al.,* 1997; Mundlos *et al.,* 1997; Otto *et al.,* 1997). People and mice with a haploid insufficiency of the *cbfa-1* gene suffer from skeletal defects that include a ridged skull and lack of clavicles, known clinically as cleidocranial dysplasia. The dramatic, and lethal, phenotypic consequences of diploid defects of *cbfa-1* were seen in knockout mice. Those mice were able to construct a nearly complete cartilage model of the skeleton, but having lost all osteoblastic bone matrix production, failed to mineralize the cartilage model. Clearly, cbfa-1 acts as a master switch in osteoblast differentiation and bone synthesis. In turn, its induction or inhibition by local and systemic factors is central to bone formation. This area has been reviewed by Ducy *et al.,* (2000) and is treated in greater depth in Chapters 3, 4, and 5.

Patterning and Endochondral Ossification: The Changeover from Cartilage to Bone

Growth of the long bones, the spine, and ribs proceeds via the construction of a cartilage model that is then remodeled into bone (see Figs. 12, 13, and 14). This process begins before birth and continues throughout the growth phase. Interestingly, some advances in understanding the complexities of its regulation owe much to basic research done with organisms that have no endoskeleton. The *hedgehog* gene, discovered in *Drosophila melanogaster* as a regulator of body segment polarity, has been conserved through evolution and is present in three versions in mammals, called sonic, desert, and Indian hedgehog. The hedgehog proteins regulate axis polarity and pattern formation in early cartilage modeling. Indian hedgehog, partially through communication with the parathyroid hormone-related protein and its receptor, helps maintain the exquisitely balanced regulation of chondrocyte proliferation and hypertrophy that determines bone growth in the epiphysis (Kronenberg *et al.,* 1997; Philbrick *et al.,* 1996; St-Jacques *et al.,* 1999; van der Eerden *et al.,* 2000; Vortkamp *et al.,* 1998). See Chapter 3 for more information on this process.

Bone Resorption: An Exception to the Redundancy of Critical Functions Rule

The advent of gene knockout technology has necessitated a reevaluation of our thinking about bone resorption. Many genes were knocked out in mice by researchers in various fields who anticipated phenotypic consequences consistent with important gene functions inferred from results of cell culture experiments, only to find that the missing gene's function could be compensated for by other redundant pathway components. While this was not always the case, it did occur with some frequency and produced a general appreciation that evolution has selected for redundancy in many critical functions.

The phenotype of osteopetrosis, however, which results from defective osteoclast development or function, was found unexpectedly in several gene knockout experiments. These include the protooncogenes *c-src* (Soriano *et al.,* 1991) and *c-fos* (Wang *et al.,* 1992); a transcription factor identified in immune system cells, NF-κB (Franzoso *et al.,* 1997; Iotsova *et al.,* 1997); and the hematopoietic transcription factor PU.1 (Tondravi *et al.,* 1997). In addition, genes critical for osteoclast function have been identified in studies of naturally occurring osteopetrotic mutations: the cytokine M-CSF, or CSF-1, in the *op* mouse (Yoshida *et al.,* 1990); and microphthalmia, a transcription factor also active in pigment and mast cells, in the *mi* mouse (Steingrimsson *et al.,* 1994) and the *mib* rat (Weilbaecher *et al.,* 1998). In addition to these, knockouts of osteoclast-specific genes for cathepsin K (Saftig *et al.,* 1998), a cysteine protease, and the vacuolar proton pump Atp6i (Li *et al.,* 1999) also result in osteopetrosis.

The field of immunology contributed another key discovery recently in our understanding of osteoclast formation and activity, the identification of a tumor necrosis factor family member produced by T cells called TRANCE (also known in the literature as RANKL, ODF, and OPGL) (Anderson *et al.,* 1997; Kong *et al.,* 1999; Wong *et al.,* 1997; Yasuda *et al.,* 1998). TRANCE is also produced by osteoblasts, and knockout mice lack both osteoclasts and lymph nodes. The TRANCE receptor (also called RANK) and its intracellular-associated signaling molecule TRAF-6 are both required for osteoclast formation, shown by the severe osteopetrosis in mice in which either of those genes are knocked out (Dougall *et al.,* 1999; Lomaga *et al.,* 1999; Naito *et al.,* 1999). More information about osteoclasts, their formation, and activation is presented in Chapters 7, 8, and 9.

Together, these findings demonstrate that, in contrast to some bodily processes that have redundant means to ensure they take place, bone resorption does not. Bone resorption may in fact be thought of as a highly regulated and specialized form of autoimmunity. It appears that evolution has favored a scenario in which the commitment to resorb bone, which is a unique and potentially debilitating process, requires that many signaling pathways all agree.

Methods for Studying Skeletal Development and Regulation

Mineralization in the skeleton has made cellular access difficult and has impeded progress in understanding bone cell biology. A century ago, studies of the skeleton had to

focus on either the mineral or the cellular components because one had to be destroyed to study the other. Improvements in methods were not sufficient to study both bone cells and their mineralized environment until the advent of electron microscopy, which provided durable embedding media and thin-sectioning procedures. Bone cell cultures were developed later than those for other tissues for similar reasons. As a result, considerable attention was paid to cells outside the skeleton for clues about bone cell function (Kahn *et al.,* 1978). Unfortunately, these data often supported erroneous conclusions because of two facts: cells of a particular family operate differently in different tissues (even the same cells are known to function differently in different site; Cecchini *et al.,* 1994) and no culture conditions *in vitro* can duplicate the complex cell/matrix/humoral interactions that occur in the organism *in vivo*. Detailed discussions of these points have been published (Fox *et al.,* 2000; Marks, 1997; Marks and Hermey, 1996). These principles need to be remembered when trying to reconcile discrepancies between studies of bone cells. The method(s) used will determine or limit the results that are possible.

Fortunately, it is now possible to study the effects of both genes and proteins *in vivo*. Analyses are complex and the results often surprising, but, unlike many *in vitro* studies, the validity of data is unquestioned. The relative ease with which animals can be produced in which a gene has been eliminated, modified, or overexpressed or in which there is cells- or tissue-specific expression has produced a variety of *in vivo* models to study the authentic biological effects of genes and their products. A number of these transgenic and knockout mutations have had surprising skeletal phenotypes. Many of the new developments in bone biology described in this book have been derived from these new discoveries in organismal molecular biology. In short, these targeted gene manipulations, combined with the numerous spontaneous skeletal mutations and an understanding of the predictable, orderly, local events in normal skeletal development, provide a new series of reality checks for bone biologists, replacing the earlier overreliance on *in vitro* methods. Many sites in the body exhibit localized, precisely timed displays of skeletal metabolism, including cell recruitment, activation, function, and senescence and as such are places where the informed investigator can intelligently dissect the crucial elements of these events. Two such sites are the postnatal development of the caudal vertebrae in rodents (unpublished work by Cecchini in Marks and Hermey, 1996) and the skeletal events around erupting teeth (Marks and Schroeder, 1996). We can expect to learn much more about the basic and applied biology of the skeleton using these systems, some of which are reviewed in Chapters 87–96.

Conclusions

The skeleton is a complex association of metabolically active cells attached to, embedded in, or surrounded by a mineralized matrix. The potential activities of each cell type are understood in broad outline, but the complex cellular interactions during development and maturation of the skeleton are under intense scrutiny. These are the new frontiers of skeletal biology and will have required a shift in focus from the isolated *in vitro* cell systems of today to the complex *in vivo* environment. This is accomplished most efficiently by studying the development and progression of reproducible sites of skeletal maturation and the skeletal effects of targeted changes in expression of specific genes. This, in turn, can be facilitated by the application of new molecular and morphologic techniques, such as *in situ* hybridization, polymerase chain reaction, immunocytochemistry, and high-resolution three-dimensional reconstruction.

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CHAPTER 2

Biomechanics of Bone

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Introduction

Bone is a physiologically dynamic tissue whose primary functions are to provide a mechanical support system for muscular activity, provide for the physical protection of organs and soft tissues, and act as a storage facility for systemic mineral homeostasis. The resulting structure of the skeleton then is influenced heavily by mechanical principles, acting both as constraints and as driving forces in its architecture (Christiansen, 1999; Cullinane, 2000; Galileo, 1638; Thompson, 1946). A form–function relationship exists in the architecture of bone, and this relationship guides the evolution, embryogenesis, and continued ontological adaptation of the skeleton.

Since the observations of Galileo it has been recognized that the inherent architecture of bone is not only organized to accommodate normal loading, but also is influenced during ontogeny by the mechanical stresses associated with daily function. Thus, the skeleton is both evolutionarily adapted and has the capacity to adapt as a result of changes in daily activity (Carter, 2000). A formal description of the dynamic structure–function relationship between bone and mechanical load was established in the late 19th century in what has since become known as Wolff's law (Wolff, 1892). Wolff determined that the trabecular elements of the skeleton were not only designed to perform their specific functions, but also responded to load by altering their structural configuration during the lifetime of an individual. Wolff's law has become widely accepted as the general guiding principle of bone regulation, with some more recent modifications (Bertram and Swartz, 1991; Biewener *et al.,* 1996; Fyhrie and Carter, 1986) and recently proposed mechanisms (Carter, 2000; Martin, 2000; Mullender and Huiskes, 1995; Turner and Pavalko, 1998). The skeleton then is not only genetically programmed for a specific configuration, but a degree of morphological plasticity exists that is influenced heavily by an individual's mechanical loading history. These ontological adaptations modify the skeleton in order to optimize its functional capacity during locomotion or other mechanical duties.

To understand how the skeleton moves or how bone responds to impact, it is necessary to appreciate how the mechanical properties of bone determine skeletal responses to both physiological and mechanical load. If details on the structural configuration and the tissue level properties of bone are provided, this information can be used to predict the risk of fractures associated with normal daily activities, athletic activities, advancing age, or metabolic bone diseases. It is imperative then to apprecial that the mechanical behavior of the skeleton is contingent upon how bone functions as a tissue and a whole organ.

Basic Biomechanics

The mechanical behavior of bone may be studied at two levels: material and structural. The material, or tissue, level properties of bone are evaluated by performing standardized mechanical tests on uniform bone tissue samples. Depending on the level of resolution, tissue level testing is relatively independent of bone structure or geometry. Second, by examining the mechanical behavior of bones as whole anatomical units, the contributions of structural properties can be determined. Mechanical properties may also be estimated *in vivo* using densitometric projections, but these are less accurate than actual mechanical testing. Taken together, these two levels of mechanical properties represent the way bones respond to forces *in vivo* and can be observed by means of experiments on sections of bones

(for material properties) or on fully intact bones with normal geometry (for structural properties).

The assessment of bone mechanical properties can be made using techniques ranging from noninvasive imaging to *in vitro* mechanical tests of excised specimens or whole bones. The accuracy of any assessment, however, is contingent upon its degree of dependence on extrapolation from non-mechanical data and its reflection of actual, *in vivo* physiological stresses. Micro-computed tomography (CT), magnetic resonance imaging (MRI), and peripheral qualitative CT (pQCT) methods of imaging, especially in use with finite element models, continue to improve in their accuracy of mechanical property assessment, (Cody *et al.,* 1999; Moisio *et al.,* 2000), as does simple bone mineral density estimation (Toyras *et al.,* 1999), vibration analysis (Weinhold *et al.,* 1999), ultrasonic wave propagation (van der Perre and Lowet, 1996), and duel-energy absorptiometry (Sievanen *et al.,* 1996). Some of these techniques primarily reflect bone tissue level properties (DEXA, wave propagation, vibration, etc.), whereas others incorporate three-dimensional information from architecture with tissue mechanical property estimates to determine structural level properties (MRI, pQCT, and micro-CT with finite element models).

However, when performing mechanical tests on bone, it is important to bear in mind that the differences between tissue level and structural level properties are not always clear. If one considers a small cube of vertebral trabecular bone as a tissue section, then trabecular element preferred orientation may influence what is assumed to be a material property, when in fact it is largely influenced by structural configuration (Keaveny *et al.,* 2000). Likewise, both material and structural properties of bones can change with the level of resolution. As mentioned earlier, vertebral trabecular bone fails by creep as multiple individual elements fail, yet the failure mode of each individual trabecular element is more elastic. These two levels of mechanical properties in bone are also evident during fractures, when what appears to be a structural failure must be accompanied by a tissue level failure. It is important to realize then that a fracture represents a failure of bone tissue at both the material and the whole bone levels (Hayes, 1983).

Stress–Strain Relationships

Like other objects in nature, bone undergoes acceleration, deformation, or both when a force is applied to it. If the bone is constrained over one portion of its structure so that it cannot move when a force is applied or if equal and opposite forces are applied to it, deformation will occur, resulting in the generation of an internal resistance to the applied force. This internal resistance is known as *stress*. Stress is equal in magnitude but opposite in direction to the applied force and is distributed over the cross-sectional area of the bone (in a long bone example). It is expressed in units of force (Newtons = N) per unit area (meters squared = m^2):

$$\text{Stress} = \delta = \text{force/area}.$$

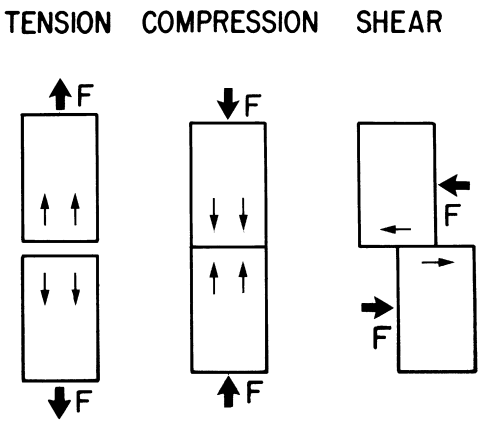

Figure 1 The three basic types of stress into which all complex stress patterns can be resolved: tension, compression, and shear. Reprinted with permission from Craig, R. G. (1989). "Restorative Dental Materials," p. 68. C. V. Mosby, St. Louis.

The standard international unit for stress is the Pascal, which is 1 N of force distributed over 1 m^2, which converts to 1.45×10^{-4} pounds per square inch (psi):

$$1 \text{ Pa} = 1N/m^2 = 1.45 \times 10^{-4} \text{ psi}.$$

Although an externally applied force can be directed at a specimen from any angle, producing complex stress patterns in the material, all stresses can be resolved into three types: tension, compression, and shear (Fig. 1). *Tension* is produced in a material when two forces are directed away from each other along the same line, with resistance to tensile forces coming from the intermolecular attractive forces that resist the material's being torn apart; ultimate tensile strength is a measure of this cohesive force. An example of

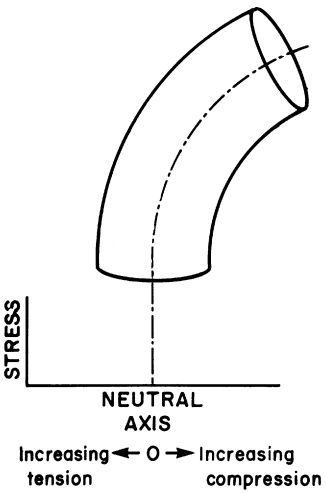

Figure 2 The bending of a simple cylinder results in tensile stresses on the convex side and compressive stresses on the concave side. The magnitude of these stresses increases proportionally to the distance from the neutral axis of bending. Reprinted with permission from Radin, E. L., Simon, S. R., Rose, R. N., and Paul, J. P. (1980). "Practical Biomechanics for the Orthopaedic Surgeon," p. 14. Wiley & Sons, New York.

tension occurs at the tendon–bone interface when a muscle acts via contraction. *Compression* results from two forces, again acting along the same line but directed toward each other; it is resisted by interatomic repulsive forces, which rise sharply at short interatomic distances. An example of compression occurring in the body is when a weight is carried on the head and the compressive load is transduced down the axis of the spine via the vertebral bodies. *Shear* forces occur when two loads act in parallel but in opposite directions from one another and can be linear or rotational. Shear occurs in a vertebral body when the superior end plate surface is loaded anteroposteriorly while the inferior end plate surface experiences a posteroanterior directed load. It must be noted that, *in vivo,* these individual stresses should be looked upon as a predominant rather than a singular stress for they almost always act in concert.

Thus, most stress patterns are complex combinations of these three stress types. Bending, for example, produces a combination of tensile forces on the convex side of a structure or material and compression on the concave side (Fig. 2). *Torsion,* or twisting produces shear stress along the entire length of a structure or material, whereas tensile stresses elongate it and compressive stresses shorten it (Fig. 3). Bending in two directions (X and Y coordinates) simultaneously, even acting on a regularly shaped cantilevered beam, can combine to create more complex

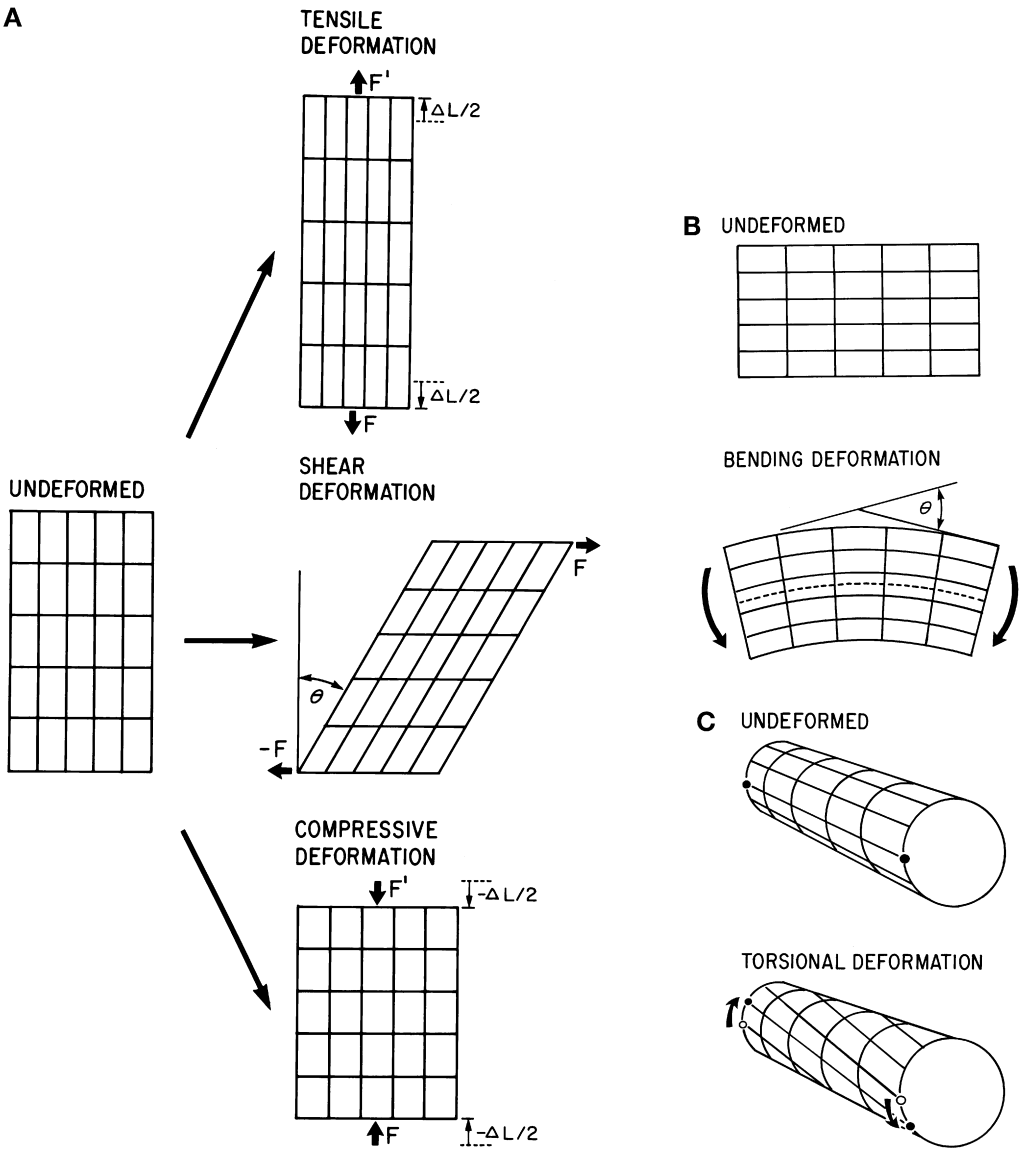

Figure 3 (A) Deformations produced by tensile, shear, and compressive stresses. F' and F are equal and opposite tensile or compressive forces, $-F$ and F are equal and opposite shear forces, θ is the angle of deformation, and Δ is change in length resulting from deformation. (B) Deformation produced by bending stress. θ is the angle of deformation. (C) Deformation produced by torsional stress. Reprinted with permission from Black, J. (1988). "Orthopaedic Biomaterials in Research and Practice." Churchill Livingston, New York.

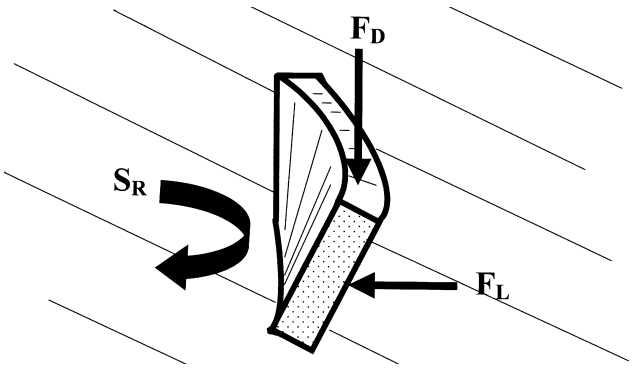

Figure 4 A simple cantilevered beam coming out of the page and with two bending forces applied. A combination of a force downward (F_D) from top and a lateral force from page right to page left (F_L) cause predictable bending in those two planes. However, the resultant strain (S_R) is a combination of bending in those two planes and an additional resultant axial torsion. Thus, even with very regular structures, combinations of even simple loads can induce complex strain behaviors. More complex biological structures like the femur would compound this strain reaction further.

stresses, including torsion along with the initial two simple bending stresses (Fig. 4). This complicating effect is even more apparent in irregularly shaped objects such as a long bone. The measurement of deformation resulting from any of these stresses, when normalized by the original configuration of the specimen, is called *strain:*

$$\begin{aligned}\text{Strain} = \varepsilon &= \text{change in length/original length} \\ &= (\text{deformed length} \\ &\quad - \text{original length})/\text{original length}.\end{aligned}$$

Strain is dimensionless and is therefore expressed as a percentage of change from the original dimensions or angular configuration of the structure. The application of these terms to bone can be made by considering the stresses and strains generated in the diaphysis of the femur (Fig. 5). For this purpose, the assumption must be that a very thin transverse section of bone behaves like a small cube, the top face of which may be designated *A*. Two types of internal forces can act on *A*, a perpendicular force *F* and shear force *S*. The former produces a normal stress gamma (δ), equal to *F/A*, whereas shear force results in a shear stress (*T*), equal to *S/A*. A normal stress might be applied toward the face of the cube, in which case it is called compression, or away from the face of the cube, in which case it is called tension.

The stresses described cause local deformation of the cube (Fig. 6). A normal compressive stress will cause shortening by a distance *L*, and the normal strain in the cube is then defined as the ratio of the change in length of the side of the cube ΔL to the original length *L* (strain = $\Delta L/L$). A shear stress applied to the top face of the cube will cause the front of the face to be deformed, and the resultant shear strain can be defined as the deviation of one side of the cube from its original angle, i.e., strain = $\Delta L/L$.

Considering that the cube represents a section of bone, the normal and shear strains experienced will be influenced

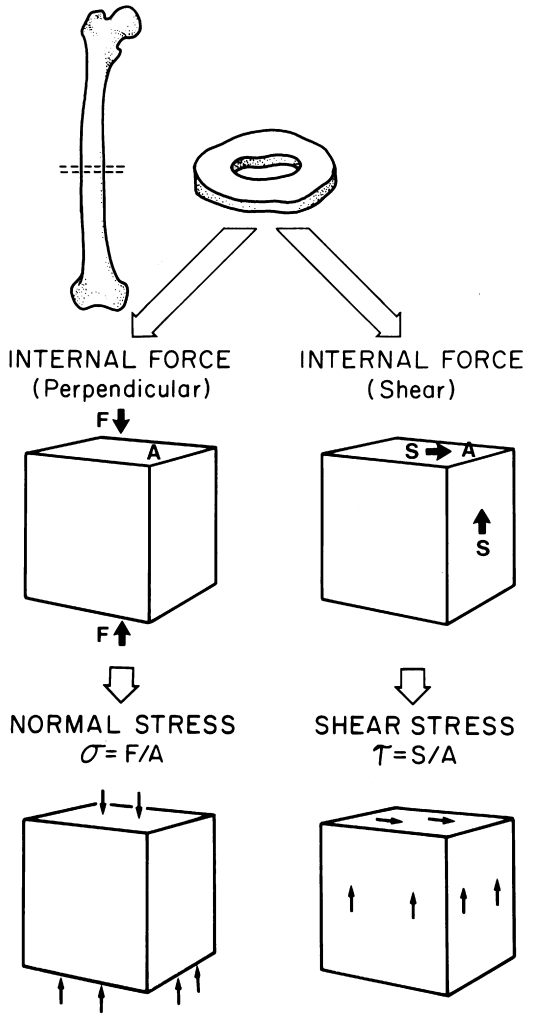

Figure 5 Schematic representation of the stresses acting on the diaphysis of the femur. In this example, a thin transverse section of the femur is considered to behave as if it were a small cube. *F,* perpendicular force; *S,* shear force; *A,* area on which force acts. Reprinted with permission from Einhorn, T. A. (1988). "Biomechanical Properties of Bone. Triangle," p. 28.

not only by the magnitude of the stresses applied, but also by the inherent material and structural properties of the bone. Stresses applied to normal, well-mineralized bone tissue will cause small strains, whereas the same stresses applied to poorly mineralized tissue, such as osteomalacic bone, will produce large strains. Likewise, if a bone experiences a bending stress in a direction in which it has a relatively greater areal moment (*I*), it will experience less strain than when loaded in a direction having a lower areal moment. It must be remembered, however, that in nature stresses are applied to bone not only from perpendicular and horizontal directions, but also from oblique angles and combinations of loads, resulting in a variety of complex mechanical relationships. Although strain is given most commonly in length dimensions, it may also be represented by angular deformation as well as other structural alterations such as volumetric changes.

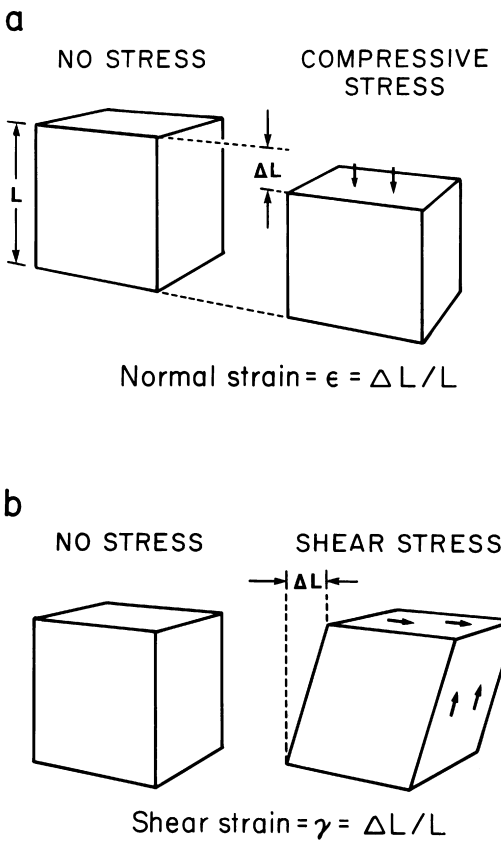

Figure 6 Schematic representation of normal (a) and shear (b) strains through the same section of bone as in Fig. 4. Reprinted with permission from Einhorn, T. A., Azria, M., and Goldstein, S. A. (1992). "Bone Fragility: The Biomechanics of Normal and Pathologic Bone." Sandoza Pharma Ltd. Monograph.

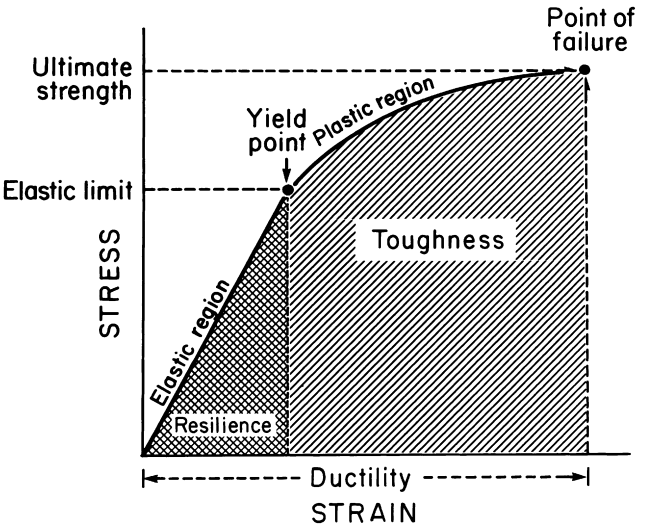

Figure 7 A standard stress–strain curve of bone loaded in bending. The linear portion of the curve represents the elastic region, and the slope of this part of the curve is used to derive the stiffness of the bone. Loading in this region will result in nonpermanent deformation, and the energy returned by the bone when the load is removed is know as its resilience. The nonlinear portion of the curve represents the plastic region in which the bone will be permanently deformed by the load. The junction of these two regions is known as the yield point, and the stress here is known as the elastic limit. The maximum stress at the point of failure is known as the ultimate strength of the bone. The maximum strain at this point is known as the ductility of the bone. The area under the curve is known as the strain energy, and the total energy stored at the point of fracture is known as the toughness of the material. Reprinted with permission from Einhorn, T. A. (1992). Bone strength: The bottom line. *Calcif. Tissue Int.* **51,** 333–339.

Stress–Strain Curve

Under controlled laboratory conditions, the testing of materials involves the application of known forces and measurement of the resulting deformation. For the purposes of this discussion and in order to simplify the mechanical complexities imparted to bone by its geometry, the material properties of bone will first be considered. This involves testing small specimens of uniform geometry under specific loading conditions. Knowing the size of the specimen, the applied force, the area of force application, and the deformation produced, the material properties can be derived from a plot of the stress–strain relationship.

By convention, stress is plotted on the ordinate (*y* axis) and strain on the abscissa (*x* axis; Fig. 7). As three types of stress exist (tension, compression, and shear) and as these stresses are produced by different externally applied forces (tension, compression, bending and torsion), the *y* axis could represent any of these loading conditions and the curve reflect any load versus deformation relationship. Moreover, as in this stress–strain plot for an idealized material the geometric factors are not considered, stress is normalized to unit area, and strain is deformation normalized to unit length. The curve can therefore be applied to a specimen of any size.

The stress–strain curve can be analyzed by breaking it down into different regions and interpolating and extrapolating these points relative to the *x* and *y* axes. Once this is done, it is then possible to derive the material properties of bone. Thus, at physiologic levels of stress there is a linear relationship between the stress applied and the resultant deformation. This proportionality is called the *modulus of elasticity,* or *Young's modulus* (*E*). It is a measure of the slope of the linear portion of the curve and is calculated by dividing the stress by the strain at any point along the linear portion. If this curve were to be generated by testing a whole bone as opposed to an uniform specimen, this measurement of the linear slope would give the *stiffness* or *rigidity* of the bone in that particular loading direction.

The linear portion of the stress–strain curve is known as the *elastic region*. In the elastic region, a material will deform only while the load is being applied to it, returning to its original shape and dimensions when the load is removed. If the slope is straight, the material is said to be linearly elastic. At the point where the curves become nonlinear, the elastic region gives way to the plastic region and the stress at this point is known as the *elastic limit*. The point on the curve where this occurs is known as the *yield*

point. Further loading beyond the yield point will cause permanent deformation of the material. This property is known as plasticity and indicates that a material has undergone some permanent deformation. Up to the elastic limit, energy applied to a material stretches or bends its atomic bonds but does not rearrange its atomic organization. Almost all the energy can be recovered by releasing the applied load (some, for example may, be lost to heat), but once the material has reached its elastic limit, or yield point, removal of the load will no longer cause the material to return to its original dimensions and it will be deformed permanently.

The strength of a bone or specimen of bone tissue is determined by calculating the maximum stress at the point of failure. In tensile loading, this is known as the *ultimate tensile strength* and, similarly, with torsional or compressive loading it is known as the *ultimate torsional* or *ultimate compressive strength.* The strain at the point of failure is known as the *ductility.* Integration of the curve gives the area under the curve and this is a measure of the strain energy. The total strain energy stored at the point of failure is known as the *toughness of the specimen.*

Energy put into deforming an elastic material just prior to reaching the yield point can be recovered by removing the stress. The energy recovered is known as the *resilience* and is a measure of the material's ability to store elastic energy. Although this energy is not always recoverable in a useful form, it will not be lost as long as the material does not undergo permanent deformation. If the area under the stress–strain curve during load removal is smaller than the area during load application, then energy is lost. This energy loss is called *hysteresis.*

Under certain pathological conditions, bone may fail before undergoing permanent deformation, i.e., without exhibiting plasticity. When the yield point and the ultimate failure coincide in this way, bone is said to be *brittle.*

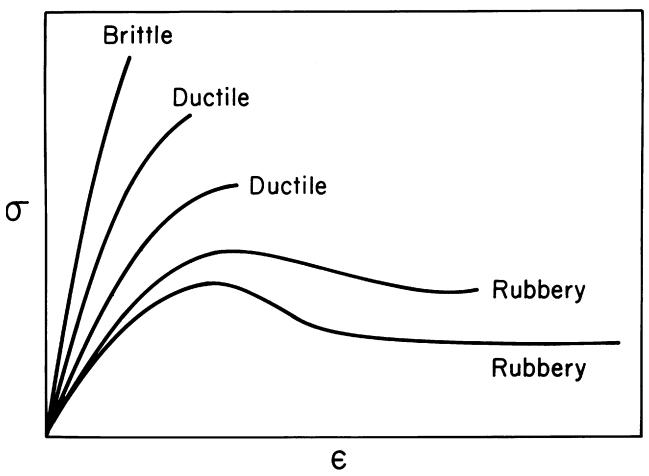

Figure 8 Stress–strain curves for brittle, ductile, and rubbery materials. Reprinted with permission from Einhorn, T. A., Azria, M., and Goldstein, S. A. (1992). "Bone-Fragility: The Biomechanics of Normal and Pathologic Bone." Sandoza Pharma Ltd. Monograph.

However, bones showing normal failure behavior exhibit ductility, which means that some of the energy required to produce the permanent deformation is recoverable and some is not. The molecular structure of the material is reorganized into a new and stable configuration and the external shape of the specimen does not revert to its original configuration. Rubbery materials, such as incompletely healed fracture callus, are capable of withstanding elastic deformation under relatively large strains without yielding: permanent deformation does not occur despite the appearance of the stress–strain curve. The stiffness of this material may be low relative to that of intact bone, but the energy stored prior to failure can in some cases be greater. A brittle material can be tough due to a high modulus and high strength even if it does not show significant deformation prior to failure, while a ductile material can absorb an equal amount of energy by undergoing significant plastic deformation before failing (Fig. 8). Therefore, two materials can have the same toughness with entirely different stiffness, strength, and ductility properties.

Special Properties of Bone

Biological structures generally have orderly structural elements that give them very different material and mechanical properties under different conditions. Thus, the mechanical properties of bone vary not only according to the magnitude of the applied force, but also to its direction and rate of application. Ideal materials are homogeneous and always behave the same way regardless of load orientation. This property is known as *isotropy* and these materials are considered to exhibit isotropic behavior (Turner *et al.,* 1995). Bones, however, have different mechanical properties in different loading directions, a phenomenon known as *anisotropy*. The anisotropy of bone can be illustrated by the application of a load to the femur. As the femur is oriented vertically, it is subjected to a compressive load with every step taken and is therefore capable of resisting high compressive loads (such as jumping from a modest height) without showing permanent deformation. However, the same load applied from a transverse direction, causing bending stresses, will not be as well tolerated by the femur and may result in fracture. Thus, the strength and rigidity of a bone are typically greater in the direction of customary loading. This is particularly true in cortical bone, where osteons are oriented in a longitudinal direction as indicated by the bone's loading history (Hert *et al.,* 1994).

The anisotropic nature of bone is documented by data for the material properties of cortical and trabecular bone in several loading configurations (Table I). Plastic deformation is diminished with transverse loading (Melton *et al.,* 1988), and bone is consequently more brittle in this direction. Cortical bone is stronger in compression than in tension (Burstein *et al.,* 1976), and after maturation the tensile strength and the modulus of elasticity of femoral cortical bone decline by approximately 2% per decade (Burstein

Table I Mean Values for Human Bone Material Parameters

Type of bone	Direction and type of load	Apparent density (g/cm^3)	Ultimate strength	Modulus of elasticity (10^6 MPa)
Cortical (midfemur)	Longitudinal tension	1.85	133	17,000
	Longitudinal compression	1.85	193	17,000
	Longitudinal shear	1.85	68	3,000
	Transverse tension	1.85	51	11,500
	Transverse compression	1.85	33	11,500
Trabecular (vertebral body)	Compression	0.31	6	76

et al., 1976). The ultimate compressive strength of trabecular bone is related to the square of its apparent density so that a decline in the latter due to aging or metabolic bone disease is associated with a reduction in compressive strength. Mathematically, therefore, if the apparent density of a bone were to decline by one-third, there would be a reduction in its compressive strength of the order of one-ninth (Carter and Hayes, 1977).

Trabecular bone also exhibits anisotropy. Compressive strength is greatest along the vertical axis of trabeculae in the lumbar vertebrae (Galante *et al.,* 1970; Mosekilde *et al.,* 1985) but parallel to the trabecular elements in the femoral neck (Brown and Ferguson, 1978). Trabecular bone has a lower modulus of elasticity than cortical bone due to its greater porosity. However, although less stiff, trabecular bone can withstand greater strains, fracturing at deformations of approximately 7%, while cortical bone will fail at strains of only 2% (Nordin and Frankel, 1980).

Creep and Stress Relaxation

Bone, and especially trabecular bone, exhibits phenomena known as *creep* and *stress relaxation* (Bowman *et al.,* 1999). Creep is defined as the change in strain of a mechanically loaded object, over time, whereas stress relaxation is the diminishment of the stress necessary to maintain a given strain over time. This behavior is most evident when bone undergoes a relatively slow rate of load application, which is then maintained statically over time. However, it also contributes to fatigue behavior in cyclically loaded trabecular bone (Bowman *et al.,* 1998). The cause of this behavior can be attributed to several factors, including changes in collagen biochemistry or to trabecular element microcracking. Collagen has been identified as a common denominator in creep behavior in both trabecular and cortical bone (Bowman *et al.,* 1999). Trabecular element microcracking is largely responsible for trabecular bone creep, with up to 94% recovery from the induced strain, and is believed to be a mechanism for energy dissipation (Fyhrie and Schaffler, 1994). Thus, creep can be considered a method by which bone tissue yields to stress without the catastrophic failure of whole-bone fracture.

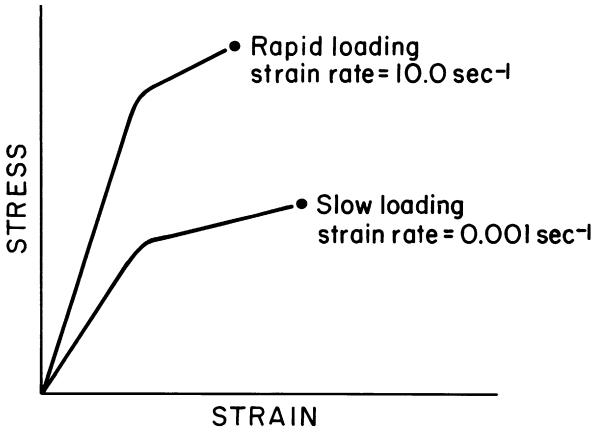

Figure 9 Different stress–strain curve configurations depending on the rate of loading. Reprinted with permission from Einhorn, T. A., Azria, M., and Goldstein, S. A. (1992). "Bone-Fragility: The Biomechanics of Normal and Pathologic Bone." Sandoza Pharma Ltd. Monograph.

Viscoelasticity

Another important mechanical property exhibited by bone is known as *viscoelasticity.* A viscoelastic material is one that undergoes material flow under sustained stress and exhibits different mechanical properties under different rates of loading. If, for example, one slowly places their hand in a tub of water, it will submerge with little resistance, whereas if one slaps their hand down into the tub, it will meet with great resistance. This phenomenon is due to the fact that the material (water in this example) actually flows under an applied load. Thus, an increase in the loading (or strain) rate decreases the time allowed for flow, increasing the modulus of elasticity of the material, while decreasing the ultimate strain (Fig. 9). At low strain rates, bone shows no appreciable elastic deformation but rather flows like a viscous liquid, whereas at high strain rates the same bone can behave like a brittle elastic solid. In normal activities, bone is subjected to strain rates below 0.01/sec, with the modulus of elasticity and the ultimate strength of bone approximately proportional to the strain rate raised to the power of 0.06 (Einhorn *et al.,* 1992). Thus, over a very wide range of strain rates, both the ultimate tensile strength and the modulus of elasticity show a strong linear relationship. Because of these characteristics, the strain rate and the direction of the load applied must be

specified when describing the behavior of bone material (Einhorn *et al.,* 1992).

Bone Structure, Biochemical Composition, and Mechanical Integrity

Bone is comprosed of approximately 70% mineral, 22% protein, and 8% water (Lane, 1979). The viscoelasticity of bone is largely a result of its water content, whereas material properties, such as strength and toughness, come from its solid-phase components. As the major components of bone are mineral and matrix phases, it is possible to describe its material properties as if it were a two-phase composite material (Carter and Hayes, 1977). However, the composition of bone can vary somewhat by species and anatomical location (Currey, 1979; Zioupos *et al.,* 1997), and this variation can influence the mechanical properties of bone. These are important factors to consider when conducting biomechanical research utilizing animal models.

Burstein *et al.* (1977) investigated the individual contributions of collagen and mineral to the elastic and plastic properties of bone. Their findings were consistent with an elastic-perfectly plastic model for the mineral phase of bone tissue in which the mineral contributes the major portion of the tensile yield strength, whereas the slope of the plastic region of the stress–strain curve is solely a function of the matrix. By sequentially demineralizing machined strips of cortical bone and subjecting them to bending loads, these investigators showed that the major determinant of the modulus of elasticity is related to the mineral phase, whereas ultimate yield strength is determined both by the mineral composition and by the integrity of the collagenous matrix (Burstein *et al.,* 1977). Landis (1995) demonstrated that this contribution of the mineral phase to bone strength is a function of the molecular structure and organization of the mineral crystals within the extracellular matrix.

Studies on the contribution of the collagen phase to the mechanical properties of bone have made use of transgenic mice expressing abnormal type I collagen gene products (Bonadio *et al.,* 1993). Because the matrix phase of these bones are rendered biochemically abnormal, the mice represent conditions that might be analogous to human collagen diseases, such as osteogenesis imperfecta. Experiments in young animals have shown that the reduced synthesis of type I collagen leads to a reduction in stiffness and strength properties when static loading tests are performed. Gradual deterioration in the mechanical properties of bones has also been found in conjunction with advancing age where it is suggested that increased bone fragility during aging is accompanied by changes in collagen material properties (Zioupos *et al.,* 1999). Apparent bone density in cancellous bone has likewise been linked to changes in the mechanical properties of aging bone (McCalden *et al.,* 1997). However, with age, adaptive changes in bone geometry leading to cortical expansion, endosteal resorption, and periosteal bone apposition result in the maintenance of structural level mechanical properties due to increases in the areal and polar moment of inertia properties of whole bones (Bonadio *et al.,* 1993). This relationship among structural geometry, bone composition, and biomechanical properties illustrates the importance of the form–function relationship in bone biomechanics.

Approximately 70–80% of the variance in the ultimate strength of bone tissue is accounted for by an age related decrease in bone mineral density (McCalden *et al.,* 1997; Smith and Smith, 1976; Singer *et al.,* 1995), with the remainder possibly due to qualitative changes associated with the altered composition of either the mineral or the matrix phase (Yamada, 1970; Zioupos *et al.,* 1999). Collagen, for example, experiences qualitatives changes with advancing age, but this may only partially affect the ultimate strength of bone and be compensated for by changes in structural properties. Unlike the case with collagen, abnormalities in mineral structure, such as those seen in a variety of metabolic bone diseases, significantly affect both the modulus of elasticity and the ultimate strength. This occurs via factors such as crystal size, crystal structure, and the way in which hormonal changes affect bone resorption and thereby the differential removal of mineral phase components.

Cellular activity plays an important role in determining the mechanical properties of bone. This may account, in part, for the fact that not all patients with low bone mass experience the osteoporotic syndrome (skeletal fragility and a propensity to fracture under minimal load). Although bone has a specific biochemical composition and structural form at any given point in time, the fact that these two properties are in a constant state of dynamic change may affect the ability of bone to respond to an applied load. It is easy to understand how bone, which is constantly undergoing breakdown and repair, may have reduced mechanical properties when compared with bone that exhibits normal homeostasis. The resorptive cavities produced by osteoclastic action may act as stress risers for the initiation of crack propagation, even before osteoblasts have had a chance to fill them in with new bone (Currey, 1962; Goodier, 1993). This is illustrated by the example of two brick walls of identical size and thickness and containing identical quantities of bricks and mortar in exactly the same spatial array. If one wall undergoes continuous degradation and repair (i.e., chipping away of its substance with immediate replacement of any brick or mortar that is lost), it will show reduced mechanical stiffness and strength as compared to the wall that is constantly intact (Fig. 10). Thus, bone that is in a state of high turnover (hyperparathyroidism, high remodeling osteoporosis, Paget's disease) may show a reduction in mechanical properties compared with bone undergoing normal remodeling in response to physiological loads (Einhorn, 1992). It is important to recognize that this phenomenon occurs independent of bone density. Thus, even though bone strength is correlated with density, the remodeling state of bone may be a more important factor with respect to risk of fracture.

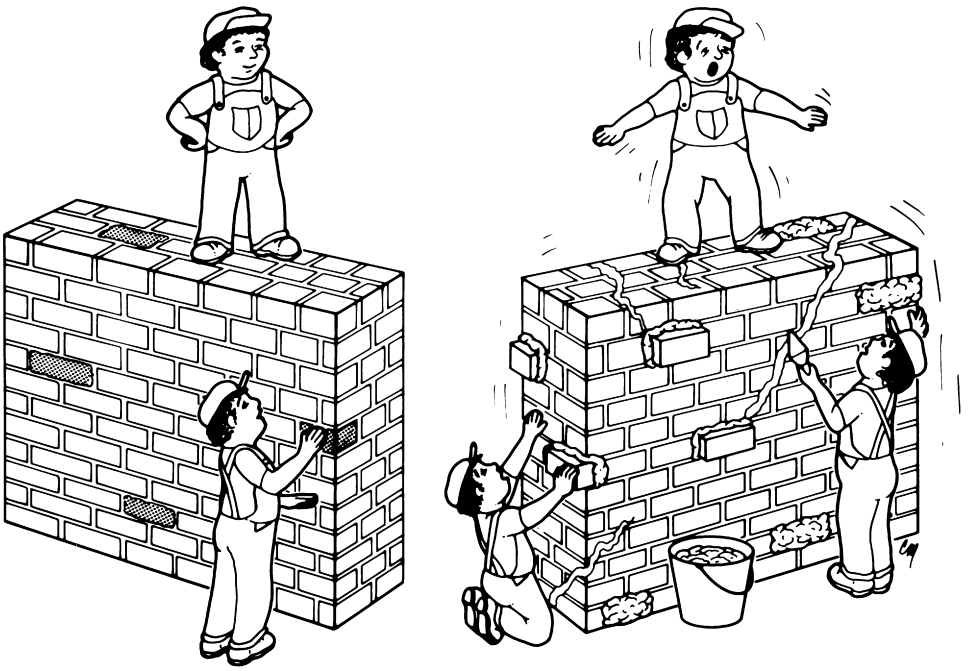

Figure 10 Demonstration that two brick walls of the same mass and dimensions will have different mechanical properties if one is constantly being excavated and patched whereas the other undergoes controlled remodeling. This situation may be analogous to bone that is in a state of high turnover versus bone that experiences normal homeostatic remodeling. Reprinted with permission from Einhorn, T. A. (1992). Bone strength: The bottom line. *Calcif. Tissue Int.* **51,** 333–339.

Mechanisms of Mechanotransduction in Bone

Because bone development, remodeling, and repair are dependent on the cellular responses associated with formation and resorption, it is presumed that mechanical loads applied to bone are transduced through the skeleton and received by a cellular network. That mechanical signal is detected by certain receptor cells, leading to the generation of a secondary, cytogenic signal aimed at target cells that modulate actual bone formation and resorption. However, the mechanisms of mechanotransduction, mechanosensitivity, and response signaling in bone are not completely understood. The currently favored model of mechanotransduction involves osteocytes and their canalicular network (Burger and Klein Nulend, 1999; Cowin *et al.,* 1991; Kufahl and Saha, 1990; Martin, 2000). Readers interested in an excellent review of osteocyte biology should refer to Chapter 6.

Evidence for an osteocyte-mediated mechanotransduction mechanism in bone is growing. Osteocytes are by far the most abundant cell in bone and represent a vast interconnected canalicular network throughout the entire skeleton (Parfitt, 1977). Studies have shown that under mechanical strain, osteocytes increase RNA production and glucose consumption (Pead *et al.,* 1988; Skerry, *et al.,* 1989) as well as produce c-fos, insulin-like growth factor I (IGF-I), and osteocalcin (Mikuni-Takagaki, 1999). The extensive processes of osteocytes, positioned throughout the canalicular network, are linked by gap junctions, which potentially allow a number of cellular signaling mechanisms (Doty, 1981).

This extensive network throughout the skeleton makes osteocytes excellent candidates for mechanical signal reception and transduction, although this is likely not their sole function (Cullinane and Deitz, 2000). Strain derived fluid flow through the canalicular network has been suggested to be the mechanism by which osteocytes receive strain information and then initiate a cellular response to a given load (Burger and Klein-Nulend, 1999; Cowin *et al.,* 1991; Martin, 2000; Owan *et al.,* 2000; Turner *et al.,* 1994). This same canalicular network could then act as a conductance pathway by which osteocyte-generated signal molecules can be distributed throughout the bone network, as well as facilitate the transport of nutritional factors and waste (Burger and Klein-Nulend, 1999). Actual *in vivo* measurements of the efficacy of this canalicular system for molecular transportation demonstrate that a 0.2% strain induced in the forelimb of sheep causes a significant increase in fluid transport (Knothe-Tate and Knothe, 2000). Likewise, Weinbaum *et al*. (1994) suggested that even very small stresses, such as 1 Pa, may be detected by the osteocyte – canalicular network. Microcracking has also been shown to reduce fluid flow downstream of the damage site, increasing osteocyte morbidity (Knothe Tate *et al.,* 2000), and osteocyte morbidity has in turn been linked to the initiation of bone remodeling via osteoclastic activation (Noble *et al.,* 1997; Verborgt *et al.,* 2000). It is even conceivable that osteocyte lacunae act as stress concentration centers within bone, providing a potential mechanism for strain detection (Reilly, 2000). Likewise, osteocyte hypoxia has

been identified as a possible mechanotransduction pathway (Dodd *et al.,* 1999).

Various molecules are potentially involved with this osteocytic signaling system including prostaglandins (Burger and Veldhuijzen, 1993; Noble and Reeve, 2000), nitrous oxide (Noble and Reeve, 2000), and parathyroid hormone (PTH) (Noble and Reeve, 2000; Sekiya *et al.,* 1999), among others. In addition to potential molecular signaling, osteocytes may have the capacity to act as a neural network in bone via a glutamate neural transmission-like mechanism (Noble and Reeve, 2000). Further evidence for a neuroelectric mechanism in osteocyte communication was found in damaged bone where an electrical signal was detected at the site of a fracture (Rubinacci *et al.,* 1998).

The specific nature of the fluid flow to which osteocytes are most likely to respond has been investigated by several authors. Hypothesizing that osteocytes are the mechanosensors in bone, Klein-Nulend *et al*. (1995) tested this hypothesis using intermittent hydrostatic compression and pulsating fluid flow. Osteocytes, but not osteoblasts or periosteal fibroblasts, were shown to react to 1 h of pulsating fluid with the sustained release of prostaglandin E_2. Intermittent hydrostatic compression stimulated prostaglandin production, but to a lesser extent. The investigators concluded that osteocytes are the most mechanosensitive cells in bone and that stress on bone predominantly causes fluid flow in the lacunar–canalicular system, which signals osteocytes to produce factors that stimulate bone metabolism (Klein-Nulend *et al.,* 1995). Evidence also suggests that among steady, pulsing, and oscillating flow, oscillating flow was found to be a much less potent stimulator of osteocytic reaction, and a reduction in responsiveness occurred with increases in dynamic flow frequencies (Jacobs *et al.,* 1998). The authors suggest that a response by bone cells to fluid flow may be dependent on chemotransport effects (Jacobs *et al.,* 1998).

Although not fully investigated in bone cells, an alternate or parallel mechanism for mechanotransduction is directly through the cytoskeleton of cells. Wang *et al*. (1993) have shown that the direct attachment of endothelial cells to their extracellular matrix via integrin interactions with the extracellular matrix provides a mechanism for alterations in cellular responses (Cowin and Weinbaum, 1998; Wang *et al.,* 1993; Zimmerman *et al.,* 2000). These experiments suggested that attachment of cells to their extracellular matrix allows direct mechanical strain in the environment to be transduced across the cell, through its cytoskeleton, and directly to the nucleus.

Fracture Behavior of Bone

When whole bones are subjected to experimental or physiological loads they exhibit structural behavior. This behavior is dependent on the mass of the tissue, its material properties and its geometry, and the magnitude and orientation of the load. A useful way of describing the structural behavior of bone is to consider what happens when a bone fractures. When bones are exposed to severe loads, large stresses are generated. As noted earlier, if the stresses exceed the ultimate strength of the bone tissue (material) in the section being loaded, a fracture will occur. Thus, a fracture is an event initiated at the material level, which ultimately affects the load-bearing capacity of the whole bone at the structural level. To describe these events, a set of biomechanical parameters that specify the behavior of bone is required.

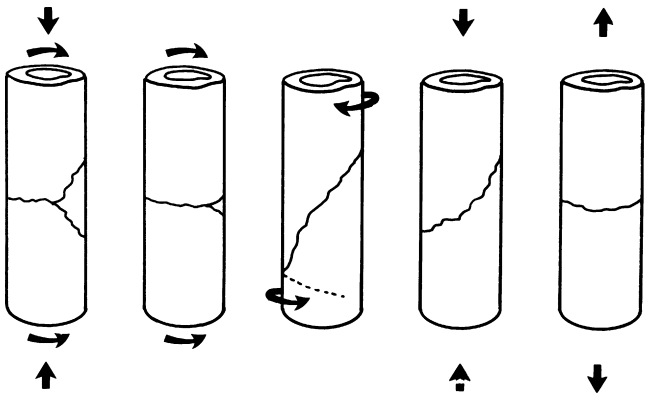

Figure 11 Fracture patterns in a cylindrical section of bone subjected to different complex loading configurations. Bending (a combination of compression and tension) produces an essentially transverse fracture with a small fragment on the concave side; torsion produces a spiral fracture; axial compression causes an oblique fracture; and tension produces a purely transverse fracture. Reprinted with permission from Carter, D. R., and Spengler, D. M. (1982). Biomechanics of fractures. *In* "Bone in Clinical Orthopaedics," pp. 305–334. Saunders, Philadelphia.

As noted previously, four modes of loading occur in whole bones—compression, tension, bending, and torsion—and these modes result in the types of long bone fractures observed clinically (Fig. 11). Axial loading can take place in either tension or compression. Tension is particularly important in the pathogenesis of *avulsion fractures*. These are fractures caused by excessive loads on tendons or ligaments, where the acute tensile force generated actually breaks off a fragment of bone at the point of soft tissue insertion (Fig. 12).

Fractures of the vertebral bodies are of particular interest in metabolic bone diseases. These fractures occur as a result of combinations of compression, bending, and shear. Compression forces arise as loads are transmitted across the spine in the axial direction, but if the cross-sectional area of the vertebral body is reduced due to age-related bone loss, its effective length of vertical trabeculation will be increased. This is a result of the removal of horizontal trabeculae, which act as lateral supports or cross-ties (Mosekilde *et al.,* 1985). The trabeculae then behave like columns and, as such, are subject to critical buckling loads. A 50% reduction in cross-sectional area is associated with a 75% reduction in load-bearing capacity, and a doubling in relative length with a 75% reduction in the critical buckling load (Compston, 1994).

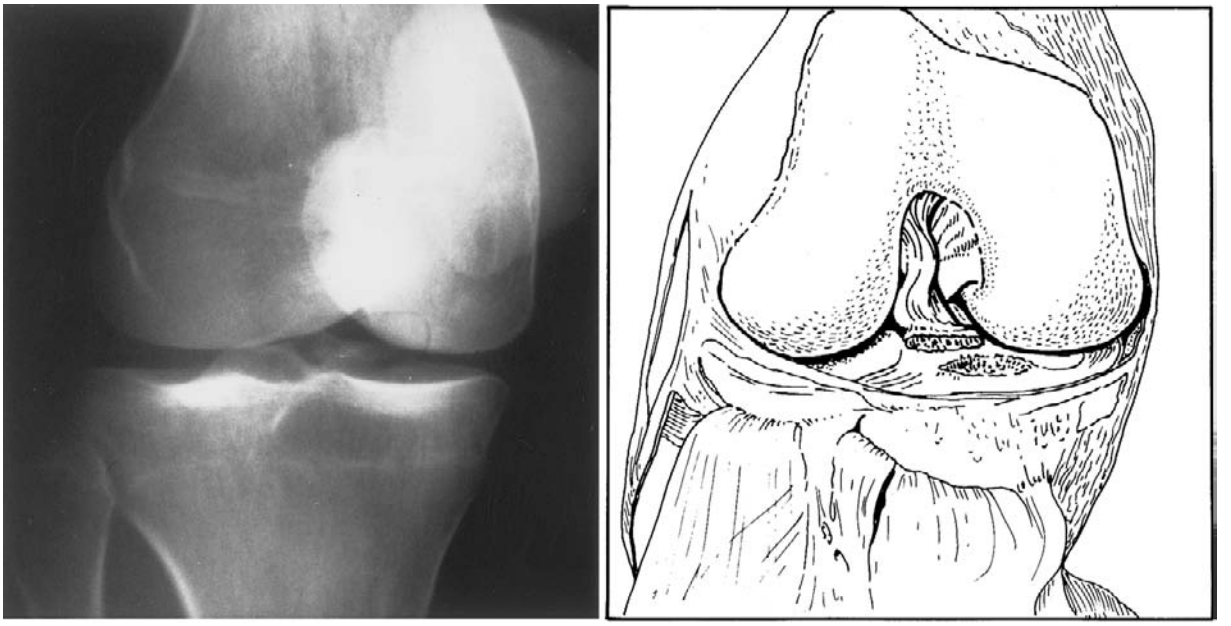

Figure 12 Anterior–posterior radiograph and associated schematic drawing of a fracture produced by a pure tensile force that occurred in a skiing accident. In this patient, an acute tensile force produced by the anterior cruciate ligament caused an avulsion fracture of bone to occur in the tibial plateau. Reprinted with permission from Einhorn, T. A., (1992). Bone strength: The bottom line. *Calcif. Tissue Int.* **51,** 333–339.

Most long bone failures will occur under a combination of axial compression, bending, and torsion, a realistic combination of loads under normal activity. With pure bending, which involves subjecting one side of the cortex to tension and the other to compression, the rigidity of the bone will depend on its cross-sectional shape, its length, and its material properties, as well as on how its ends are fixed. In the case of bending, the cross-sectional area is less important than its distribution with respect to the axis of loading, which, ideally, should be as far from the axis of bending (neutral axis) as possible; the geometric parameter used to describe this is the *areal moment of inertia* (Einhorn *et al.,* 1992; Fig. 13). Similarly, in torsion, deformation is resisted more efficiently the farther bone (or any material) is distributed away from the torsional axis. The geometric parameter used to describe this is the *polar moment of inertia* (Einhorn *et al.,* 1992; Fig. 14).

With aging, the outer cortical diameter of bone increases while the cortical wall thickness declines. This is the result of the combined effects of increased endosteal resorption (due to involutional osteoporosis) and periosteal appositional bone

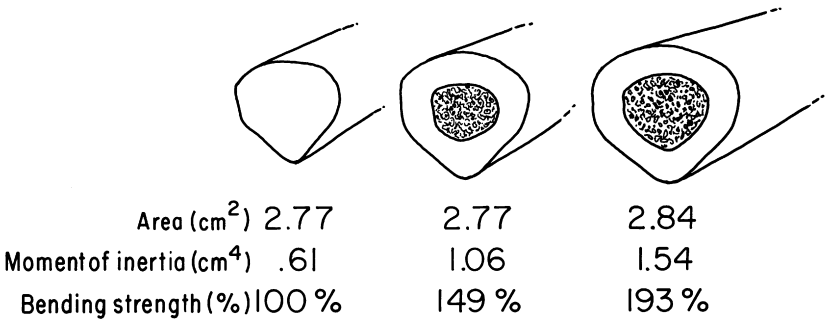

Figure 13 Moment of inertia properties of bone. Although the cross-sectional areas of bone in each of these three bones are roughly equivalent, their bending strengths are very different due to the differences in moments of inertia. This occurs as a result of the way bone is distributed in relation to the central axis of bending or rotation. The solid bone on the left has the same amount of bone (area) as the one in the center, but the latter has a higher moment of inertia because the bone is farther away from the central axis. Thus its bending strength is 50% greater. Similarly, the bone on the right has only slightly more bone area than the one in the center, but its moment of inertia is again 50% higher, making it 30% stronger under bending stress. Reprinted with permission from Einhorn, T. A., Ariza, M., and Goldstein, S. A. (1992). "Bone-Fragility: The Biomechanics of Normal and Pathologic Bone." Sandoza Pharma Ltd. Monograph.

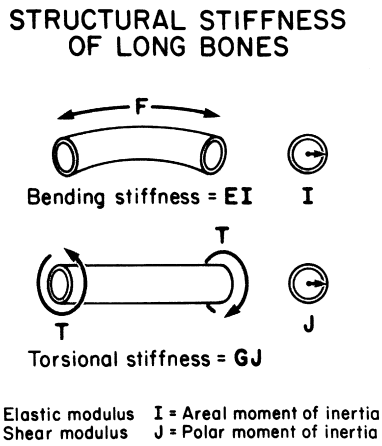

Figure 14 Diagram of the structural stiffness of long bones loaded in bending and torsion. Note that the elastic modulus and the shear modulus, measures of stiffness in bending and torsion, are related to the areal and polar moments of inertia, respectively. These moments of inertia are increased, and thus the structural stiffnesses are increased when bone is distributed farther away from the neutral axis of bending or torsion. Reprinted with permission from Einhorn, T. A. (1992). Bone strength: The bottom line. *Calcif. Tissue Int.* **51,** 333–339.

formation (due to mechanical strain). Although the net effect is cortical thinning, the increased diameter of the bone improves its resistance to bending and torsional loads through its areal and polar moment properties. This may also be sufficient to offset any loss of bone tissue (Melton *et al.,* 1988) and may explain why cortical bone fractures are surprisingly uncommon in osteoporotic patients.

Stress Fractures

By definition, a bone should fail when it is subjected to a load that exceeds its ultimate stress. However, it is not always necessary that the ultimate stress be exceeded in order for a fracture to occur; repeated loading of the bone can cause it to fail even if loads are below this level (Muller *et al.,* 1998; Reilly and Currey, 2000; Yeh and Keaveny, 2000). This phenomenon is known as *fatigue failure,* and fractures that result from this kind of loading are known as *stress fractures* or *fatigue fractures.* Fatigue failure occurs when each loading cycle produces a small amount of microdamage, which accumulates with repetitive loads (Muller *et al.,* 1998; Schaffler *et al.,* 1994). Biological materials such as bone have repair mechanisms for healing the microdamage as it occurs. Under normal conditions, microdamage will occur but will not accumulate because it will be repaired in a timely fashion (Hirano *et al.,* 2000; Tomlin *et al.,* 2000). This process of bone matrix repair has been linked to signals originating via osteocyte apoptosis (Verborgt *et al.,* 2000). However, when the normal repair mechanisms are impaired or attenuated (e.g., in certain metabolic bone diseases or in patients taking certain drugs) or when bones are loaded repetitively over short periods without sufficient time for a reparative process (e.g., during intense basic military training), bones may exhibit fatigue failure after several cycles of loading (Givon *et al.,* 2000; Hirano *et al.,* 2000; Lauder *et al.,* 2000; Stanitski *et al.,* 1978). A high prevalence of stress fractures in patients who are maintained on glucocorticoids is due to the combined effects of osteoporosis and the impaired healing of microfractures. Sodium fluoride may have a similar effect on bone and may account for the increased prevalence of appendicular fractures reported in certain fluoride-treated patients (Boivin *et al.,* 1991).

Stress fractures are most likely to occur when bone is loaded repeatedly in the plastic region (Chamay, 1970). Here, small deformations of the tissue have already occurred and repeated damage will eventually lead to the ultimate point of failure. However, fatigue failure in the elastic region is also possible, particularly in bones that are more brittle. This requires a large number of loading repetitions (Chamay, 1970) or less rest time between loading cycles (Schaffler *et al.,* 1994).

Stress fractures may occur on either the tension side or the compression side of a bone undergoing bending. It should be noted that a stress fracture on the tension side, resulting in a crack in the cortex, is more serious because it may rapidly go on to a complete fracture, as bone is stronger under compression than under tension (Burstein *et al.,* 1976). This phenomenon is especially true if the initial microcracks are created in compression and then subjected to tension, reducing the energy-absorbing capacity of a bone by as much as 40% (Reilly and Currey, 2000). Stress fractures that occur on the compression side of bones may also result from a slower process, in which repair mechanisms may be mobilized more easily, leading to healing before a complete fracture occurs (Baker *et al.,* 1972).

Correlation among Bone Strength, Bone Fragility, and Fracture Risk

Although there is considerable evidence that bone density has an important effect on the risk of fracture, the relationship among bone strength, bone fragility, and fracture risk may depend on several factors. As mentioned previously, the proteinaceous matrix of bone plays an important role in determining its elastic and plastic stiffness. Moreover, the way in which the mineral phase is embedded in the matrix also dictates strength-related properties, as does the spatial distribution of bone tissue.

Bone formation or remodeling can occur in response to conditions such as age (Tanck *et al.,* 2000; Weinans, 1998), mechanical stimulation (Biewener and Bertram, 1994; Fyhrie and Carter, 1986; Hauser *et al.,* 2000), metabolic disorders (Lanyon, 1996), and even as compensation for tumors (Hauser *et al.,* 2000). This adaptive response suggests that reduction in strength due to osteolytic bone defects can be compensated for by adaptive remodeling of

Table II Trabecular Bone Content at Various Skeletal Sites

Vertebrae	66 – 90%
Hip (intertrochanteric)	50%
Hip (femoral neck)	25%
Distal radius	25%
Midradius	1%
Femoral shaft	5%

the cortical bone via thickening of the adjacent cortex, increases in areal moment, or formation of buttress-like septae. Thus, bone is capable of compensating for changes in activity level, age, and disease, and these compensations by the skeleton are an effort to reduce the risk of fracture. However, confounding factors, such as reduction in cell sensitivity due to advancing age, may be difficult to overcome by structural remodeling (Stanford *et al.,* 2000).

Fracture risk is highly site specific, depending on the type of bone involved and the loading to which it is subjected. For example, because the human spine is predominantly under compressive loading and is composed predominantly of trabecular bone, it is the mechanical properties of vertebral trabecular bone that primarily dictate the fracture risk of vertebrae. This is of particular relevance to metabolic bone disease, as turnover in trabecular bone is nearly eight times faster than in cortical bone (Parfitt, 1987). Bones with a high trabecular component are at a much higher risk of fracture in patients with metabolic bone disorders (Table II). Thus, bone loss due to metabolic disorders such as osteoporosis differentially affect the axial skeleton (Grey *et al.,* 1996) leaving it more vulnerable to fracture over the course of the disease.

Consideration of Table II may lead one to wonder why femoral neck fractures are so common in the elderly if trabecular bone content at this site is no higher than the average for the skeleton as a whole. This explanation may lie in the phenomenon of increased periosteal bone apposition leading to age-related cortical expansion and changes in areal and polar moments. Because the femoral neck is an intracapsular structure (within the hip capsule) and is not covered by periosteum (Phemister, 1939; Pankovich, 1975), its external diameter does not increase as the skeleton ages, despite increased mechanical loading. Thus, whereas cortical bone in the rest of the skeleton increases its areal and polar moments of inertia, protecting older persons from sustaining fractures in the diaphysis of their long bones, this biomechanical adaptation is not exhibited by the femoral neck. Endosteal resorption occurs, the cortex becomes thinner, and the femoral neck is then weakened in the absence of a compensatory response via a periosteal increase in bone diameter. Thus, there are peculiarities to location, tissue composition, and systemic metabolic status that must all be incorporated into risk of fracture assessments.

Predicting Fractures

As can be inferred from a discussion of structure – function relationships at hierarchical levels, changes in both material and structural properties of bone may arise from numerous causes. Geometric changes at any structural level will significantly influence the mechanical integrity of the tissue composite. For example, increases in the radius of long-bone diaphyses will increase resistance to torsion or bending loads by a factor raised to the fourth power, as predicted by both polar and bending moments of inertia. Increases in trabecular plate thickness at the microscopic level, however, are more difficult to assess, as the continuum properties of trabecular bone volumes are influenced approximately equally by alterations in bone mass and orientation (Keaveny *et al.,* 2000). Similarly, precise estimates of the effects of other changes in trabecular morphology, such as plate perforations, trabecular reorientation, and increases or decreases in connectivity, cannot be made readily. Use of the statistically based empirical relationships described earlier may provide some insight, but these relationships have not been verified for diseased bone or bone undergoing significant adaptation. Future work should continue to address these relationships.

While current understanding of bone mechanics makes it possible to estimate the effects of geometric changes, as described previously, very little data available for assessing the effects of changes at upper hierarchical levels. At the tissue level, decreases in the mean wall thickness of trabecular packets, as well as potential changes in lamellar thickness, have been reported as a function of age and gender. While it has been suggested that these morphological changes affect global mechanical behavior significantly, no direct evidence of their effects has been presented. Similarly, changes in extracellular matrix ultrastructure, mineralization profiles, and remodeling rate probably have a significant effect on the mechanical integrity of the tissue and its ability to resist or propagate cracks, although properties such as mineral density and tensile strength may not be reflected in fracture toughness (Wang *et al.,* 1998).

The long-term objective of the majority of studies designed to characterize the mechanical behavior of bone is to provide the means for accurate fracture risk prediction. Much of the discussion in this chapter has been devoted to identifying those factors that contribute to the integrity of bone and, more specifically, its resistance to fracture. The complex, anisotropic, heterogeneous properties of bone, its ability to adapt continuously to environmental and metabolic changes, and our limited understanding of specific failure mechanisms associated with crack propagation have severely limited accurate fracture risk prediction. By far the majority of studies to date have tried to relate fracture risk to bone density, but this has only partially succeeded in explaining changes seen *in vivo* (patients) and *in vitro*. More recently, attempts to account for architecture by including density distributions have helped improve estimates of fracture risk, but have not yet been verified in clinical studies.

The use of analytical models, such as finite element analyses, has the advantage of taking both complex geometric and anisotropic tissue properties into account when attempting to predict the occurrence of fractures. These models incorporate specific geometric measures from individual patients but are still dependent on appropriate estimates of material properties. They will continue to increase their predictive capacity as finer resolution, noninvasive imaging techniques become available. In addition, accurate failure analysis is dependent on the selection of loading conditions appropriate to the bony region being modeled, as well as of appropriate failure criteria within the structure. These parameters can be estimated accurately only through careful locomotion analysis and biomechanical testing. These noninvasive fracture assessment methods are currently being investigated and refined for future use.

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CHAPTER 3

Embryonic Development of Bone and the Molecular Regulation of Intramembranous and Endochondral Bone Formation

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Introduction

The skeletal system is multifunctional in that it provides the rigid framework and support that gives shape to the body, serves to protect delicate internal organs, endows the body with the capability of movement, acts as the primary storage site for mineral salts, and functions in hematopoiesis. The vertebrate skeleton is composed of two main subdivisions: axial and appendicular components. The axial skeleton encompasses the skull, spine, sternum, and ribs, whereas the appendicular skeleton defines the bones of the extremities. The skull, in turn, is best regarded as consisting of two units: the chondrocranium whose elements first develop in cartilage and includes the cranial base and capsules surrounding the inner ears and nasal organs, and the cranial vault and most of the upper facial skeleton, which arise from the direct conversion of undifferentiated mesenchymal cells into bone.

Skeletal cells are derived from three distinct embryonic cell lineages: neural crest cells contribute to the craniofacial skeleton; sclerotome cells from somites give rise to the axial skeleton; and lateral plate mesoderm cells form the appendicular component. Cells from these lineages participate in the process of skeletogenesis in four distinct phases: (1) the migration of cells to the site of future skeletogenesis, (2) the epithelial–mesenchymal interaction that leads to (3) the formation of condensations, and (4) the overt differentiation of chondroblasts or osteoblasts (Fig. 1, see also color plate) (Hall and Miyake, 2000).

Bone formation arising from a cartilaginous template is referred to as endochondral ossification. This is a complex, multistep process requiring the sequential formation and degradation of cartilaginous structures that serve as templates for the developing bones. Formation of calcified bone on a cartilage scaffold, however, occurs not only during skeletogenesis, but is also an integral part of postnatal growth, bone modeling, and fracture repair. Intramembranous bone differs from the endochondral component in that it is formed in the absence of a cartilaginous blastema. Rather, it arises directly from mesenchymal cells condensing at ossification centers and being transformed directly into osteoblasts.

The organization and morphology of the developing skeleton are established through a series of inductive interactions. The functional elements in these inductive and morphogenetic processes are not individual cells but rather interacting populations that elaborate an extensive extracellular matrix, which in turn feeds back onto these matrix-producing cells and controls their differentiation potential. Since the early 1990s, considerable insight has been gained

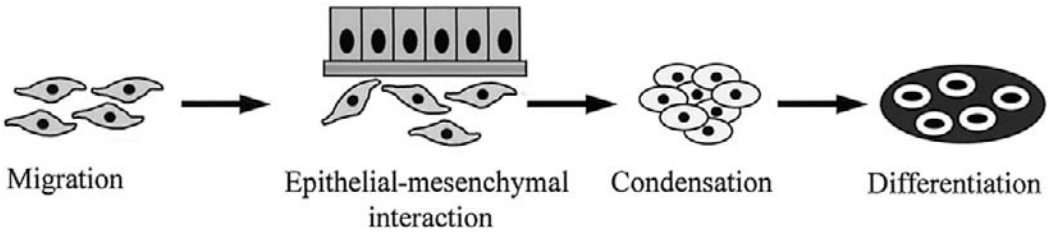

Figure 1 The four phases of skeletal development. Migration of preskeletal cells to sites of future skeletogenesis is followed by these cells interacting with an epithelium. This in turn leads to mesenchymal condensation and subsequent differentiation to chondroblasts or osteoblasts. Adapted from Hall and Miyake (2000). (See also color plate.)

into the molecular mechanisms that control these developmental programs. Genetic and biochemical analyses of human heritable skeletal disorders in concert with the generation of transgenic and knockout mice have provided useful tools for identifying key molecular players in mammalian skeletogenesis. It is the nature and interplay of these signaling cascades controlling skeletal patterning and cellular differentiation that are the focus of this chapter.

Axial Skeleton

Somitogenesis

A defining feature of the vertebrate body plan is metameric segmentation of the musculoskeletal and neuromuscular systems. The origin of this basic anatomic plan during embryogenesis is segmentation of the paraxial mesoderm (for reviews, see Burke, 2000; Christ *et al.,* 1998). Upon gastrulation, paraxial mesoderm cells segregate from axial and lateral mesoderm to form two identical strips of unsegmented tissue (referred to as presomitic mesoderm in the mouse embryo or segmental plate in the avian embryo) on either side of the neural tube. Paraxial mesoderm in vertebrates gives rise to the axial skeleton, as well as all trunk and limb skeletal muscles, and portions of the trunk dermis and vasculature. Through a series of molecular and morphogenetic changes, this unsegmented tissue is converted into a string of paired tissue blocks on either side of the axial organs, called somites (Fig. 2A). The process, referred to as somitogenesis, occurs sequentially by the addition of new somites in a strict craniocaudal (head-to-tail) direction along the body axis with a periodicity that reflects the segmental organization of the embryo. The recruitment of new presometic tissue from the primitive streak into the posterior end of the presometic mesoderm, as well as cell division within it, permits the presomite mesoderm to maintain its longitudinal dimension as somite budding is taking place anteriorly. Somite formation is preceded by epithelialization of the presometic mesoderm so that a new pair of somites is formed when cells are organized into an epithelial sphere of columnar cells en-

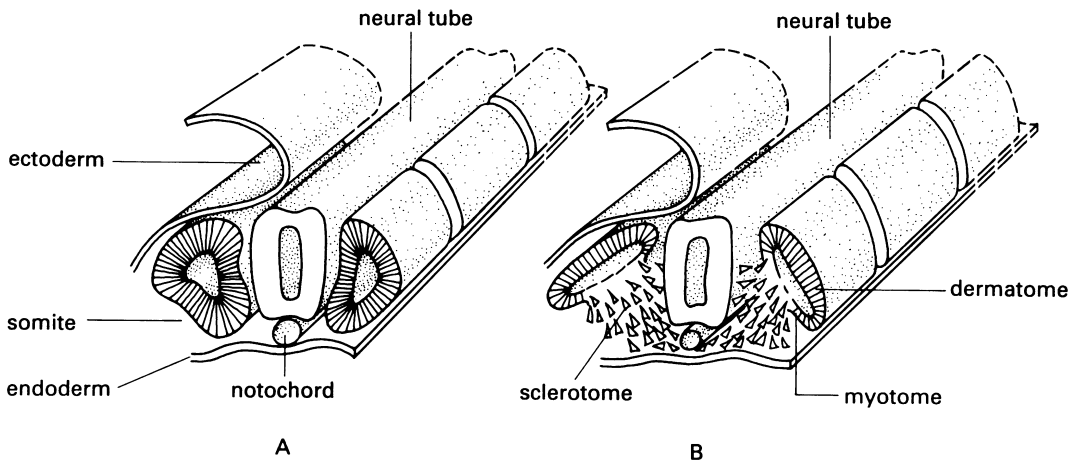

Figure 2 Diagrammatic representation of sclerotome formation. (A) Organization and differentiation of somites in the trunk region of the mouse embryo. (B) Ventral medial somite cells (those further away from the back and closer to the neural tube) undergo mitosis, migrate ventrally, lose their epithelial characteristics, and become mesenchymal cells, which give rise to the sclerotome. They ultimately become the vertebral chondrocytes that are responsible for constructing the axial skeleton (vertebrae and ribs). The notochord provides the inductive signal by secreting SHH. Following formation of the vertebral bodies, the notochordal cells die, except in between the vertebrae where they form the intervertebral discs. Adapted from Hogan *et al.* (1994).

veloping mesenchymal cells within the central cavity, the somitocoel. This epithelial structure, however, is not maintained, as somite maturation is accompanied by a commitment of its cells to different lineages in response to signals that arise from adjacent tissues. Cells on the ventral margin undergo an epitheliomesenchymal transition as they disperse and move toward the notochord, giving rise to the sclerotome, which serves as the precursor of the vertebrae and ribs (Fig. 2B). The dorsal epithelial structure of the somite is maintained in the dermatomyotome, which eventually gives rise to the epaxial muscles of the vertebrae and back (medial myotome), the hypaxial muscles of the body wall and limbs (lateral myotome), and the dermis of the skin of the trunk (dermatome).

Somitogenesis has long been known to be driven by mechanisms intrinsic to the presomitic mesoderm. Two distinct molecular pathways have been implicated in vertebrate segmentation (Fig. 3, see also color plate). The first is referred to as the "segmentation clock" (McGrew *et al.,* 1998; Palmeirim *et al.,* 1997; Pourquie, 1999). This clock corresponds to a molecular oscillator identified on the basis of rhythmic production of mRNAs for *c-hairy1,* the vertebrate homologue of the *Drosophila* pair-rule gene *hairy,* and for *lunatic fringe,* the vertebrate homologue of the fly *fringe* gene. The expression of these genes appears as a wave, which arises caudaly and progressively sweeps anteriorly across the presomitic mesoderm. Although a new wave is initiated once during the formation of each somite, the duration of the progression of each wave equals the time to form two somites. This wave does not result from cell displacement or from signal propagation in the presomitic mesoderm but rather reflects intrinsically coordinated pulses of *c-hairy1* and *lunatic fringe* expression.

The second pathway implicated in somitogenesis is *Notch/Delta* signaling, an essential regulator of paraxial mesoderm segmentation. It centers on a large transmembrane receptor called Notch, which is able to recognize two sets of transmembrane ligands; Delta and Serrate. Upon ligand binding, Notch undergoes a proteolytic cleavage at the membrane level, leading to the translocation of its intracytoplasmic domain into the nucleus, where, together with the transcription factor Su(H)/RBPjk, it activates the expression of downstream genes such as *HES1/HES5* in vertebrates. Many of the genes in this pathway are expressed strongly in the presomitic mesoderm, and mutation studies in the mouse have established their role in the proper formation of rostral–caudal compartment boundaries within somites, pointing to a key role for a *Notch*-signaling pathway in the initiation of patterning of vertebrate paraxial mesoderm (Barrantes *et al.,* 1999; Conlon *et al.,* 1995; Kusumi *et al.,* 1998; Yoon and Wold, 2000). It is now recognized that *lunatic fringe* is the link between the *Notch*-signaling pathway and the segmentation clock, as there is evidence to suggest that it acts downstream of *c-hairy1* and

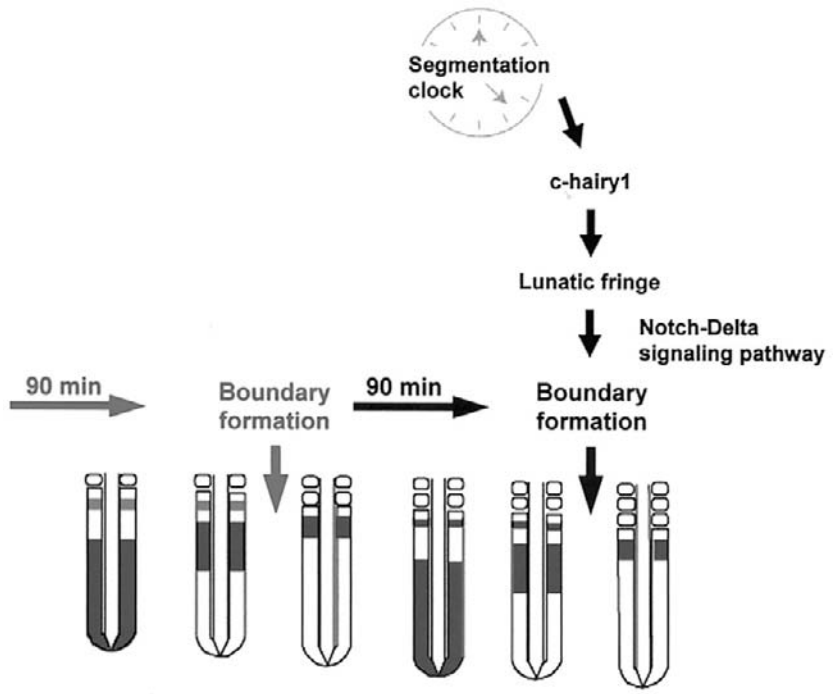

Figure 3 Proposed role of the segmentation clock in somite boundary formation. Waves of *c-hairy* and *Lunatic Fringe* (green) expression arise caudally and get narrower as they move anteriorly. A wave completes this movement in the time required to form two somites. Boundary formation occurs when the wave has reached its most rostral domain of expression where it is associated with rhythmic activation of the Notch signaling pathway that endows cells with setting-of-boundary properties. The purple color indicates a second such wave. Adapted from Pourquié (1999). (See also color plate.)

modifies Notch signaling. Likely, it is *lunatic fringe* that delimits domains of Notch activity, thereby allowing for the formation of the intersomitic boundaries characterized morphologically by the epithelization event (for review, see Pourquie, 1999).

Epithelization and somite formation require the expression of the gene *paraxis*. Paraxis is a basic helix-loop-helix (bHLH) transcription factor expressed in paraxial mesoderm and somites. In mice homozygous for a *paraxis*-null mutation, cells from the paraxial mesoderm are unable to form epithelia and so somite formation is disrupted (Burgess *et al.,* 1996). In the absence of normal somites, the axial skeleton and skeletal muscle form but are patterned improperly. Sosic *et al*. (1997) have shown that *paraxis* is a target for inductive signals that arise from the surface ectoderm, but the nature of these signals remains unknown.

Specifying the Anterior–Posterior Axis

Initially, somites at different axial levels are almost indistinguishable morphologically and eventually give rise to the same cell types such as muscle, bone, and dermis. A great deal of research has therefore focused on identifying factors that dictate the ultimate differentiation fate of somitic cells as well as the overall patterning of the body plan. Burke (2000) has proposed that correct pattern requires two levels of information. At one level, short-range, local signals could dictate to a cell to differentiate into a chondroblast instead of a myoblast. These signals, however, would not contain all the information required to also bestow regional identity to this chondrocyte and ensure that it would, for example, contribute to the development of the appropriate vertebral body, be that cervical, thoracic, or lumbar. In fact, additional information would be needed in order to provide global landmarks and to ensure correct pattern formation.

Patterning of somites extends beyond the formation of distinct epithelial blocks. Early on they acquire cues that dictate anteroposterior as well as dorsoventral position. What signals are important for regional specification of segments along the anterior–posterior dimensions into occipital, cervical, thoracic, lumbar, and sacral domains? This regionalization is, in part, achieved by specific patterns of *Hox* gene expression (for reviews, see Mark *et al.,* 1997; McGinnis and Krumlauf, 1992; Veraksa *et al.,* 2000). All bilateral animals, including humans, have multiple *Hox* genes, but in contrast to the single *Hox* cluster in *Drosophila* and other invertebrates, four clusters of *Hox* genes—*HOXA, HOXB, HOXC,* and *HOXD*—have been identified in vertebrates. Mammalian *HOX* genes are numbered from 1 to 13, starting from the 3′ end of the complex. The equivalent genes in each complex (*HOXA-1, HOXB-1, HOXD-1*) are referred to as a paralogous group. Comprising a total of 39 genes in human, these clusters are arranged such that all the genes in each cluster are oriented in the same 5′ to 3′ direction. Moreover, genes located at the 3′ end of the cluster are expressed prior to and extend more anteriorly in the developing embryo than those at the 5′ end. The high degree of evolutionary conservation of homeotic gene organization and transcriptional expression pattern of these genes in flies and mammals argues strongly for a common scheme in anteroposterior axis formation.

How do *Hox* genes dictate pattern formation? It appears that there is a code of *Hox* gene expression that determines the type of vertebrae along the anterior–posterior axis. For example, in the mouse, the transition between cervical and thoracic vertebrae is between vertebrae 7 and 8, whereas in the chick, it is between vertebrae 13 and 14. In either case, *Hox-5* paralogues are seen in the last cervical vertebra, whereas *Hox-6* paralogues extend up to the first thoracic vertebra, their anterior boundary. Changes in the *Hox* code lead to shifting in the regional borders and axial identities, otherwise known as homeotic transformations. Therefore, *Hox* loss of function results in the affected body structures resembling more anterior ones, whereas gain-of-function mutant phenotypes due to ectopic expression of more posterior *Hox* genes cancel the function of more anterior ones and specify extra posterior structures. The persistent expression of *Hox* genes in discrete zones on the anteroposterior axis is required in order to remind cells of their position identity along the axis.

Hox proteins are all transcription factors that contain a 60 amino acid motif referred to as the homeodomain and exert their effect through the activation and repression of numerous target genes. In mammals, little is known about the upstream mechanisms that initiate *Hox* gene expression (Manzanares *et al.,* 1997; Marshall *et al.,* 1996). More is known about factors involved in the maintenance of *Hox* expression in both flies and mice (see Veraksa *et al.,* 2000 and references therein). Studies with the Trithorax and Polycomb protein groups indicate that the former function as transcriptional activators whereas the latter are transcriptional repressors of the *Hox* genes. In loss-of-function mutants for *Polycomb* genes *Bmi1* and *eed,* the domain of expression of the *Hox* gene is expanded, causing homeotic transformation and, conversely, loss of the *Trithorax* group gene *Mll* results in diminished levels of expression of the *Hox* gene with the phenotype resembling the mutants of the *Hox* gene themselves. Interestingly, axial–skeletal transformations and altered *Hox* expression patterns of *Bmi1*-deficient and *Mll*-deficient mice are normalized when both *Bmi1* and *Mll* are deleted, demonstrating their antagonistic role in determining segmental identity (Hanson *et al.,* 1999). In summary, repeated identical units formed by the action of segmentation genes become different due to *Hox* gene action.

Specifying the Dorsal–Ventral Axis

The newly formed somites are composed of a sphere of columnar epithelial cells and a central cavity, the somatocoel, containing mesenchymal cells. Early somites are characterized by the expression of the *Pax3* gene. Following somite formation, however, expression of the gene is downregulated in the ventral half of the somite epithelium and in

the somatocoel cells whereas as it persists in the dorsal half of the somite. The ventral medial cells of the somite subsequently undergo mitosis, lose their epithelial characteristics, and become mesenchymal cells again. This epitheliomesenchymal transition of the ventral part of the somite is preceded by the expression of *Pax1* in the somitic ventral wall and somatocoel cells as it signals the beginning of sclerotome formation. Mutations in *Pax1* affect sclerotome differentiation, as reported with different mutations in the *undulated* (*un*) locus (Balling *et al.,* 1988). Successful dorsoventral compartmentalization of somites ultimately leads to the development of the sclerotome ventrally and the dorsally located dermomyotome.

In the fourth week of human development, cells from the somites migrate to the most ventral region of the somite in an area surrounding the notochord forming the ventral sclerotome (Fig. 2B). These mesenchymal cells differentiate to prechondrocytes and ultimately form the template of vertebral bodies and ribs. The initiation of sclerotome formation is under control of the notochord. Sonic hedgehog (Shh), a secreted signaling molecule known to play a role in the patterning of the central nervous system and the limb in vertebrates, is expressed in the notochord at that time and has been implicated as the key inductive signal in patterning of the ventral neural tube and initiation of sclerotome formation (Fan and Tessier-Lavigne, 1994; Johnson *et al.,* 1994). Mutations in the gene encoding human SHH are associated with holoprosencephaly 3, an autosomal-dominant disorder characterized by single brain ventricle, cyclopia, ocular hypotelorism, proboscis, and midface hypoplasia (Roessler *et al.,* 1996). In humans, loss of one *SHH* allele is insufficient to cause ventralization defects of sclerotomes. In the mouse, loss of both *Shh* alleles leads to brain abnormalities and a skeletal phenotype typified by a complete absence of the vertebral column and posterior portion of the ribs (Chiang *et al.,* 1996). Formation of the sclerotome, however, does take place, although the sclerotomes are smaller and *Pax1* expression is decreased markedly, suggesting that Shh does not initiate but rather maintains the sclerome program. McMahon and associates (1998) have reported that *Noggin,* which encodes a bone morphogenetic protein (BMP) antagonist expressed in the node, notochord, and dorsal somite, is required for normal Shh-dependent ventral cell fate. In *Noggin*-null mice, somite differentiation is deficient in both muscle and sclerotomal precursors and *Pax1* expression is delayed, whereas the addition of Noggin is sufficient to induce *Pax1*. These findings suggest that different pathways mediate induction and that *Noggin* and *Shh* induce *Pax1* synergistically. Inhibition of BMP signaling by axially secreted Noggin, therefore, is an important requirement for normal induction of the sclerotome.

In contrast to the sclerotome, dorsal signals promote the development of the dermomyotome. These are members of the Wnt family of proteins emanating from the dorsal neural tube and the surface ectoderm necessary for the induction of myogenic precursor cells in the dermomyotome (Wagner *et al.,* 2000). Ectopic *Wnt* expression (*Wnt1, 3a-,* and *4*) is able to override the influence of ventralizing signals arising from the notochord and floor plate. This shift of the border between the two compartments is identified by an increase in the domain of *Pax3* expression and a complete loss of *Pax1* expression in somites close to the ectopic *Wnt* signal. Therefore, Wnts disturb the normal balance of signaling molecules within the somite, resulting in an enhanced recruitment of somitic cells into the myogenic lineage. In contrast, Shh reduces *Wnt* activity in the somitic mesoderm, at least in part, by upregulating *Secreted frizzled-related protein 2 (Sfrp2),* which encodes a potential *Wnt* antagonist (Lee *et al.,* 2000).

In summary, dorsoventral polarity of the somitic mesoderm is established by competitive signals originating from adjacent tissues. Studies suggest that the dorsoventral patterning of somites involves the coordinate action of multiple dorsalizing and ventralizing signals. The ventrally located notochord provides the ventralizing signals to specify the sclerotome, whereas the dorsally located surface ectoderm and dorsal neural tube provide the dorsalizing signals to specify the dermomyotome.

Sclerotome Differentiation

Pax1-expressing cells that arise from the ventromedial end of the sclerotome invade and colonize the perinotochordal space. These cells, expressing additional sclerotome markers such as *twist* and *scleraxis,* proliferate under the influence of *Shh* signaling from the notochord and form the perinotochordal tube from which vertebral bodies and intervertebral discs will develop. Segmentation begins by the condensation of sclerotome cells that represent the intervertebral discs, thereby defining the boundaries of the future vertebral bodies. Notochordal cells die if surrounded by sclerotome cells that form a vertebral body, whereas those that become part of the intervertebral disc form the nucleus pulposus. The ribs, pedicle, and lamina of the neural arch arise from *Pax1*-expressing cells in the lateral sclerotome.

Not all of the sclerotome cells are under the influence of Shh and Noggin emanating from the notochord and consequently express *Pax1*. Cells located in the ventrolateral and dorsomedial angles of the sclerome escape the ventralizing signals. While other sclerotomal cells migrate ventrally to surround the notochord where they form the vertebral body, these cells move dorsomedially to form the dorsal mesenchyme, which is the precursor of the dorsal part of the neural arch and the spinous process. These sclerotome cells express homeobox genes (*Msx1* and *Msx2*) as they are subjected to a different microenvironment, specifically to signals arising from the roof plate of the neural tube and surface ectoderm (Monsoro-Burq *et al.,* 1994). BMP4 is expressed transiently in these structures and likely exerts a positive effect on the induction of dorsalizing gene expression in sclerotome cells (Monsoro-Burq *et al.,* 1996).

The Cranial Vault and Upper Facial Skeleton

In contrast to the obvious segmentation of the axial skeleton, craniofacial development is a poorly understood process. The craniofacial skeleton forms primarily from neural crest cells that migrate from hindbrain rhombomeres into the branchial arches. Neural crest cells are multipotential stem cells that contribute extensively to vertebrate development and give rise to various cell and tissue types, including mammalian craniofacial development. Migrating from the rhombomeric neuroectoderm to the pharyngeal arches, these cephalic neural crest cells proliferate as the ectomesenchyme within the arches, form mesenchymal condensations, and differentiate into cartilage and bone of endochondral and membranous skull, respectively. Little is known about the molecular basis underlying their migration, but it appears that interactions with tissues encountered during migration strongly influence this segmental migratory pattern. Neural crest cells possess integrin receptors that are essential for interacting with extracellular matrix molecules in their surroundings. The aberrant migration of cephalic neural crest cells leads to craniofacial defects, as demonstrated in platelet-derived growth factor-α receptor (Soriano, 1997)-and Shh-deficient mice embryos (Ahlgren and Bronner-Fraser, 1999). Homozygotes die during embryonic development and exhibit incomplete cephalic development. Increased apoptosis is observed on pathways followed by migrating neural crest cells, indicating that these signaling molecules affect their survival.

Interactions between neural crest-derived ectomesenchymal cells and surrounding cells are critical as defects in this process can also lead to craniofacial malformation. The development of the facial primordia is in part mediated by transcription factors that are programmed by an intricate array of intercellular signaling between ectomysenchymal neural crest-derived cells and epithelial and mesodermal cell populations within the arches (Francis-West *et al.,* 1998). *Hox* gene products, including Hoxa1, Hoxa2, and Hoxa3, play a role in the development of craniofacial structures derived from the second and third branchial arches, but they are not involved in the patterning of first arch derivatives. Other homeodomain proteins are expressed in cranial neural crest cells that migrate into the first branchial arch, including *goosecoid* (*Gsc*), *MHox*, and members of the *Dlx* and *Msx* families. A pivotal role in this process has been ascribed to components of the endothelin pathway. The G-protein-coupled endothelin-A receptor (ET_A) is expressed in the ectomesenchyme, whereas the cognate ligand for ET_A, endothelin-1 (ET-1), is expressed in the arch epithelium and the paraxial mesoderm-derived arch core. Absence of either ET-1 (Kurihara *et al.,* 1994) or ET_A (Clouthier *et al.,* 1998) results in numerous craniofacial defects. While neural crest cell migration in the head of ET_A-null embryos appears normal, the expression of transcription factors (*Gsc*, *Dlx-2*, *Dlx-3*, *dHAND*, *eHAND*, and *Barx1*) important in the differentiation of cephalic crest cells in the arches during epithelial–mesenchymal interactions is either absent or reduced significantly in the ectomesenchymal cells (Clouthier *et al.,* 2000). Because *Dlx-1, Hoxa-2,* and *MHox* are normally expressed in these mutants, it would argue that additional pathways work in conjunction with the ET_A pathway in patterning the facial primordia from buds of undifferentiated mesenchyme into the intricate series of bones and cartilage structures that, together with muscle and other tissues, form the adult face.

Limb Initiation and Development

Overview of Limb Development

Not all of the mesoderm is organized into somites. Adjacent to the somitic mesoderm is the intermediate mesodermal region, which gives rise to the kidney, and genital ducts and further laterally on either side is the lateral plate mesoderm. In the second month of human development, the proliferation of mesenchymal cells from the lateral plate mesoderm gives rise to the formation of limb buds (Fig. 4A, see also color plate). *Hox* genes expressed within the lateral plate mesoderm specify the positions at which forelimbs and hindlimbs will be developing (for review, see Ruvinsky and Gibson-Brown, 2000). T-box genes, which encode a family of transcription factors that share a conserved domain with the classical mouse *Brachyury (T)* gene, function as activators or repressors of transcription of downstream target genes involved in the regulation of vertebrate limb development. Specifically, transcripts of two of these genes, *Tbx5* and *Tbx4*, are activated as a result of a "read out" of the *Hox* code for pectoral and pelvic appendages, respectively. This positional information then leads to limb development within the perspective fields.

The vertebrate limb is an extremely complex organ in that its patterning takes place in three distinct axes (for review, see Schwabe *et al.,* 1998): (1) the proximal–distal axis (the line connecting the shoulder and the finger tip), which is defined by the apical ectodermal ridge (AER), a single layer of epidermal cells that caps the limb bud and promotes the proliferation of mesenchymal cells underneath. As the limb elongates, mesenchymal cells condense to form the cartilage anlagen of the limb bones. (2) The posterior–anterior axis (as in the line between the little finger and the thumb), which is specified by the zone of polarizing activity (ZPA), a block of mesodermal tissue near the posterior junction of the limb bud and the body wall. (3) The dorsal–ventral axis (as in the line between the upper and lower surfaces of the hand), which is defined by the dorsal epithelium.

THE PROXIMAL–DISTAL AXIS

A variety of growth factors, patterning morphogens, transcription factors, and adhesion molecules, participate in a highly orchestrated system that dictates the blueprint of the developing mammalian limb. The first step, initiation of the site where the presumptive limb will develop, is critically

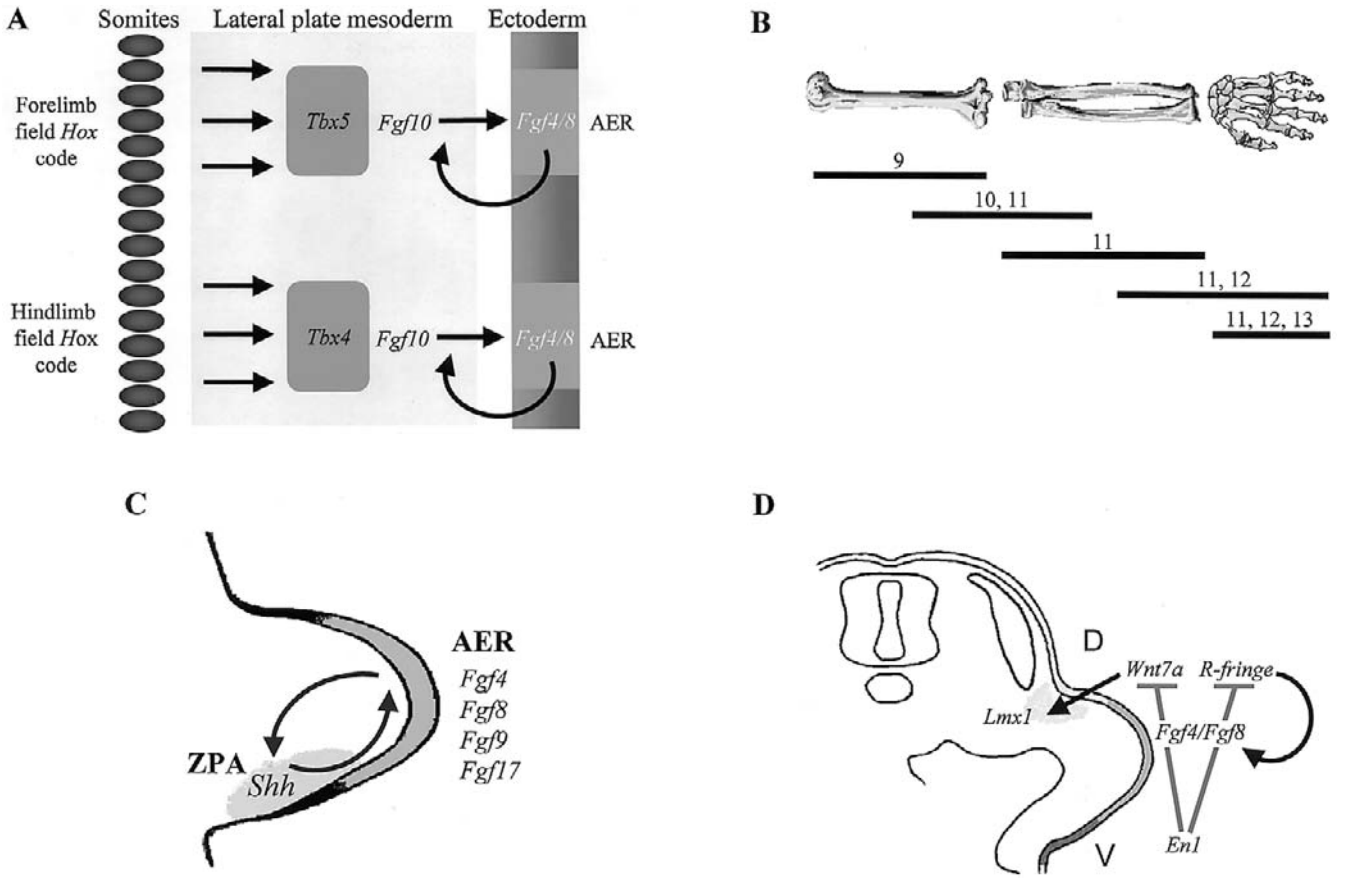

Figure 4 Molecular regulation of limb patterning. (A) Schematic model for specification of limb position. *Hox* genes expressed in the lateral plate mesoderm define the positions where limbs will develop. *Tbx4* and *Tbx5* expression activates the perspective fields and sets up the *Fgf10–Fgf4/8* positive feedback loop implicated in induction of the AER in the overlying ectoderm and initiation of limb bud outgrowth. Adapted and modified from Ruvinsky and Gibson-Brown (2000). (See also color plate.) (B) *HoxA* and *HoxD* genes involved in limb specification. Groups 9 and 10 paralogous genes organize the proximal part of the limb, groups 10, 11, and 12 genes the distal part of the limb, and groups 11, 12, and mostly 13 genes pattern the digits. Adapted and modified form Zakany and Duboule (1999). (C) Specification of the anterior–posterior axis. *Shh* expressed in the ZPA (purple) and *Fgfs* in AER (orange) participate in a positive feedback loop to provide the polarizing signal for anterior–posterior patterning of the limb. (See also color plate.) (D) Specification of the dorsal–ventral axis. *Wnt7a* and *Lmx1* expression correlates with dorsal fate, whereas *En1* expression dictates ventral fate by repressing *Wnt7a* and *R-fhg,* a secreted molecule that directs the formation of the AER (orange) in the boundary between cells that express it in the dorsal ectoderm (yellow) and cells that do not express it in the ventral ectoderm (blue). D, dorsal, V, ventral. Adapted and modified from Niswander (1997). (See also color plate.)

dependent on fibroblast growth factor (FGF) signaling mediated by high-affinity FGF receptors (FGFRs). Expressed in the lateral plate mesoderm, *Fgf10* binds and activates the IIIb splice form of the FGF receptor 2 (FGFR2) in the AER in the overlying ectoderm. This signaling is absolutely crucial for limb bud initiation, as evidenced by the complete absence of limb development in mice homozygous for a null *Fgf10* (Min *et al.,* 1998; Sekine *et al.,* 1999) or *Fgfr2* (Xu *et al.,* 1998) allele. In turn, *Fgf4* and *Fgf8* expressed in the AER act on the underlying mesoderm to maintain *Fgf10* expression, thereby promoting elongation of the limb. As the limb grows, cells directly underneath the AER, in a region termed the progress zone (PZ), maintain their characteristics of undifferentiated mesenchyme while they continue to proliferate. In contrast, the more proximal mesenchymal cells begin to condense and differentiate into the cartilage anlage of the limb. In this scheme, the *Hox* gene expression pattern activates downstream target genes according to the position along the axis. These key signaling pathways control various aspects of limb development, including establishment of the early limb field, determination of limb identity, elongation of the limb bud, specification of digit pattern, and sculpting of the digits. Accumulating evidence indicates that *Hoxa* and *Hoxd* genes are involved in limb specification (for review, see Zakany and Duboule, 1999). In contrast, *Hoxb* and *Hoxc* genes do not participate in limb patterning. Targeted mutations for each gene and compound mutants produced in mice have indicated that genes belonging to groups 9 and 10 determine the length of the upper arm, groups 10, 11, and 12 pattern the lower arm; and groups 11, 12, and mostly 13 organize the digits (Fig. 4B).

THE ANTERIOR–POSTERIOR AXIS

Specification of the anterior–posterior axis is under the control of a small block of mesodermal tissue near the posterior junction of the developing limb bud and the body

wall, referred to as the ZPA. It is now established that *Shh* expressed in the ZPA is the major molecular determinant in the anterior–posterior patterning of the limb (Riddle *et al.,* 1993). The unequivocal requirement for Shh signaling in limb development has been demonstrated by the *Shh* loss-of-function mutation, resulting in the complete absence of distal limb structures (Chiang *et al.,* 1996). The profound truncation of the limbs indicates the existence of a positive feedback loop between the ZPA and the AER. Thus, *Shh* expression in the polarizing region activates *Fgf4* in the AER. The presence of *Formin* (*Fmn*) and *Gremlin* (*Gre*) in the initial mesenchymal response to *Shh* is required to relay this signal to the AER (Zeller *et al.,* 1999; Zuniga *et al.,* 1999). In turn, *Fgf4* maintains *Shh* expression (Niswander *et al.,* 1994), thus providing a molecular mechanism for coordinating the activities of these two signaling centers. This SHH/FGF4 feedback loop model is supported by genetic evidence showing that *Fgf4* expression is not maintained in *Shh*-null mouse limbs. Contradicting this model is the observation that *Shh* expression is maintained and limb formation is normal when *Fgf4* is inactivated in mouse limbs (Moon *et al.,* 2000; Sun *et al.,* 2000). Moreover, expression patterns of *Shh, Bmp2, Fgf8,* and *Fgf10* are normal in the limb buds of the conditional mutants, suggesting that no individual Fgf expressed in the AER is solely necessary to maintain *Shh* expression. Instead, it is the combined activities of two or more AER-Fgfs (*Fgf4, Fgf8, Fgf9,* and *Fgf17*) that function in a positive feedback loop with *Shh* to control limb development (Fig. 4C, see also color plate). *Shh* also regulates BMP gene expression (*Bmp2, Bmp4,* and *Bmp7*) in a gradient through the limb mesoderm. These in turn act to induce *Hox* gene expression in the AER and PZ.

THE DORSAL–VENTRAL AXIS

Dorsoventral patterning is the least understood of the three axes of pattern formation in the limb (for review, see Niswander, 1997). Molecular studies indicate that the signaling molecule *Wnt7a,* a secreted molecule encoded by *Radical fringe* (*R-fng*), and the transcription factor *Engrailed-1* (*En1*) are intimately involved in this process (Fig. 4D, see also color plate). *Wnt7a* is expressed in the dorsal ectoderm and regulates the expression of a LIM homeodomain gene, *Lmx1,* in the dorsal mesenchyme, important for maintaining dorsal structure identity. *R-fng* expression is also localized in the dorsal ectoderm and dictates the location of AER as defined by the boundary between cells that do and cells that do not express *R-fng* (Laufer *et al.,* 1997). However, *En1* expression restricts *R-fng, Wnt7a,* and *Lmx1* to the dorsal ectoderm and mesenchyme and correlates with ventral fate.

The Skeletal Dysplasias

It is implicit from the foregoing discussion that mutations in the genes involved in limb patterning would tend to have profound effects on the final outcome of human limb design. For example, expansion of a polyalanine stretch in the amino-terminal end of the protein product of *HOXD13* is the cause of synpolydactyly, type II, an autosomal dominant disorder characterized by variable syndactyly and insertion of an extra digit between digits III and IV (Muragaki *et al.,* 1996). In homozygous individuals, homeotic transformation of metacarpal and metatarsal bones occurs so that they resemble carpal and tarsal anlages rather than long bones.

Mutations in *GLI3*, a transcription factor involved in the transduction of hedgehog signaling, have been described in four human autosomal dominant disorders: Greig cephalopolysyndactyly syndrome, characterized by a peculiar skull shape, frontal bossing, high forehead, and the presence of (poly)syndactyly (Vortkamp *et al.,* 1991); Pallister–Hall syndrome, a neonatally lethal disorder characterized by hypopituitarism, renal agenesis, cardiac defects, cleft palate, short nose, flat nasal bridge, and short limbs (Kang *et al.,* 1997); postaxial polydactyly type A, a trait typified by the presence of a rather well-formed extra digit, which articulates with the fifth or an extra metacarpal (Radhakrishna *et al.,* 1997); and preaxial polydactyly IV, distinguished by mild thumb duplication, syndactyly of fingers III and IV, first or second toe duplication, and syndactyly of all toes (Radhakrishna *et al.,* 1999).

Heterozygous mutations in the *LMX1B* (LIM homeobox transcription factor 1, β) gene have been described in patients with the nail-patella syndrome, an autosomal dominant disorder encompassing nail dysplasia, hypoplastic patella, decreased pronation and supination, iliac horns, and proteinuria (Dreyer *et al.,* 1998). Functional studies indicate that these mutations either disrupt sequence-specific DNA binding or result in the premature termination of translation. These are the first described mutations in a LIM-homeodomain protein that account for an inherited form of abnormal skeletal patterning.

Mesenchymal Condensation and Skeletal Patterning

Mesenchymal Condensation

The third phase in skeletogenesis, the appearance of mesenchymal condensations that arise as a consequence of epithelial–mesenchymal interactions, originates in areas where cartilage is to appear and where bone is to form by intramembranous ossification. These condensations define not only the position of the skeletal elements they represent, but also their basic shape. Therefore, if a condensation were in the wrong place or of the wrong shape and size, it would be expected to produce a skeletal element that is similarly misplaced or misshapen.

Condensations can be visualized easily *in vivo* as they express cell surface molecules that bind peanut agglutinin lectin (Stringa and Tuan, 1996). Their formation takes place when previously dispersed mesenchymal cells form aggregations and, once again, it is *Shh*, *Bmp* (*Bmp2-5, Bmp7*), *Fgf,* and *Hox* genes that determine the fundamental

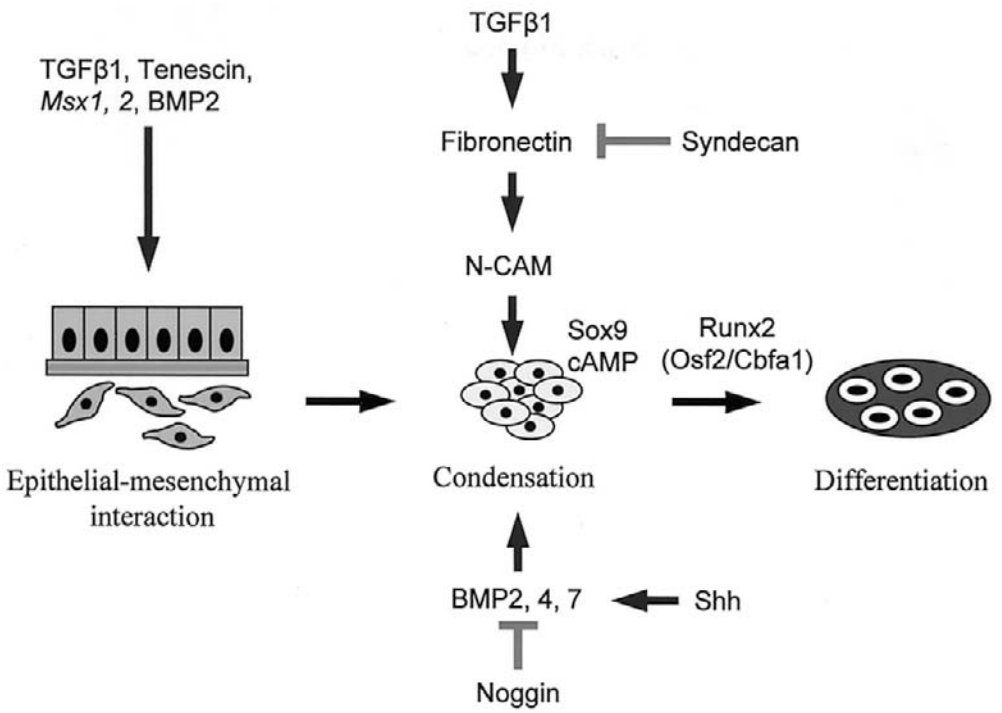

Figure 5 Regulation of mesenchymal condensation. Diagrammatic representation of the network of signaling factors involved in the formation of mesenchymal condensations and their subsequent transition to overt differentiation. Adapted and modified from Hall and Miyake (2000). (See also color plate.)

attributes, such as the timing, position, and shape that they will assume. Mesenchymal condensation can be envisioned as a multistep process involving initiation, setting of boundary, proliferation, adherence, growth, and, finally, differentiation (for review, see Hall and Miyake, 2000) (Fig. 5, see also color plate). Initiation arises as a result of epithelial–mesenchymal interaction upregulating the expression of a number of molecules associated with prechondrogenic and preosteogenic condensations, such as tenascin, fibronectin, N-CAM, and N-cadherin. Transforming growth factor-β (TGFβ) and other members of the TGFβ superfamily that regulate many aspects of growth and differentiation (reviewed in Moses and Serra, 1996) play a pivotal role in this process. This family of signaling molecules, which includes several TGFβ isoforms, the activin and inhibins, growth and differentiation factors (GDFs), and the BMPs, potentiate condensation by promoting the establishment of cell–cell and cell–extracellular matrix interactions (Chimal-Monroy and Diaz de Leon, 1999; Hall and Miyake, 1995). Cell surface adhesion and extracellular matrix proteins contribute to the formation of condensations as they participate in cell attachment, growth, differentiation, and survival. The integrin family of cell surface receptors serves to mediate cell–matrix interactions, thereby providing the link between extracellular matrix and intracellular signaling that can affect gene expression. Integrins that act as receptors for fibronectin ($\alpha5\beta1$), types II and VI collagen ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$), laminin ($\alpha6\beta1$), and vitronectin and osteopontin ($\alpha5\beta3$) are expressed early in the condensation process (Loeser, 2000), although further work is required to precisely define the role of these molecules in the developmental program of the process.

Levels of intracellular cAMP increase during prechondrogenic condensation and, along with the concomitant cell–cell interactions, are thought to mediate the upregulation of chondrogenic genes. The transcription factor Sox9 [SRY (sex-determining region Y)-related HMG box gene 9] is a potent inducer of genes required for cartilage formation, such as type II collagen (*Col2a1*) and aggrecan (*Agc*), and its phosphorylation by protein kinase A (PKA) increases its DNA-binding and transcriptional activity (Huang *et al.,* 2000). *Sox9* expression starts in mesenchymal chondroprogenitor cells and reaches a high level of expression in differentiated chondrocytes. Cells deficient in *Sox9* are excluded from all cartilage but are present as a juxtaposed mesenchyme that does not express the chondrocyte-specific markers (Bi *et al.,* 1999). This exclusion occurs at the condensing mesenchyme stage of chondrogenesis, suggesting that *Sox9* controls the expression of cell surface proteins needed for mesenchymal condensation, thereby identifying *Sox9* as the first transcription factor essential for chondrocyte differentiation and cartilage formation.

Cessation of condensation growth leads to differentiation characterized by the transient expression of the runt-related transcription factor 2, Runx2 (also known as Cbfa1

for core-binding factor, α subunit 1, or Osf2 for osteoblast-specific *cis*-acting element-2), in prechondrogenic as well as preosteogenic condensations. Once overt differentiation takes place, however, its expression is restricted solely to osteogenic cells and is downregulated in chondrogenic lineages (Ducy and Karsenty, 1998). Condensed mesenchymal cells that differentiate into chondroblasts begin to produce a matrix rich in type II collagen, the molecule that best defines the chondroblast/chondrocyte phenotype, and mucopolysaccharides. In contrast, osteogenenic cells produce most notably type I collagen in conjunction with a variety of noncollagenous, extracellular matrix proteins that are deposited along with an inorganic mineral phase. The mineral is in the form of hydroxyapatite, a crystalline lattice composed primarily of calcium and phosphate ions.

The Skeletal Dysplasias

A variety of human skeletal disorders arise as a consequence of gain-of-function and loss-of-function mutations in signaling pathways involved in mesenchymal condensation. *GDF5* (*growth differentiation factor 5*) belongs to the TGFβ superfamily and is expressed predominantly throughout mesenchymal condensations in the developing skeleton. Mutations in *GDF5* are the cause of acromesomelic chondrodysplasia, Hunter–Thompson type (Thomas *et al.,* 1996), an autosomal recessive disorder characterized by short forearms, hands and feet, and very short metacarpals, metatarsals, and phalanges. Mutations in *GDF5* have also been reported in patients with Grebe-type chondrodysplasia, an autosomal recessive disorder characterized by severe limb shortening and dysmorphogenesis with a proximal–distal gradient of severity (Thomas *et al.,* 1997). It is proposed that the mutant GDF5 protein is not secreted and is inactive *in vitro*. It produces a dominant-negative effect by preventing the secretion of other related BMPs, likely through the formation of heterodimers.

Patients with Albright's hereditary osteodystrophy (AHO) have characteristic physical stigmata and skeletal abnormalities consisting of short stature, ectopic calcification and ossification, and short feet and hands, particularly fourth metacarpals. This skeletal disorder has been associated with pseudohypoparathyroidism type Ia (PHP-Ia), which is characterized by parathyroid hormone (PTH)-resistant hypocalcemia and hyperphosphatemia, and other endocrine deficiencies, as well as pseudopseudohypoparathyroidism (pseudoPHP), which encompasses AHO but without the endocrine abnormalities. AHO in PHP-Ia and pseudoPHP arise from heterozygous inactivating mutations in the *GNAS1* gene encoding the α subunit of the stimulatory G protein ($G_s\alpha$) (*Levine et al.,* 1988). Therefore, decreased levels of cAMP arising from a mutated *GNAS1* allele may provide a mechanistic explanation for the impairment in skeletal development associated with this disorder. However, it remains unclear why different parts of the skeleton demonstrate strikingly different phenotypes when the mutant protein is expressed uniformly in all of them.

Defects in *SOX9* are the cause of campomelic dysplasia, a rare, dominantly inherited chondrodysplasia, characterized by craniofacial defects, bowing and angulation of long bones, hypoplastic scapulae, platyspondyly, kyphoscoliosis, 11 pairs of ribs, small thorax, and tracheobronchial hypoplasia (Foster *et al.,* 1994). It is often lethal, soon after birth, due to respiratory distress attributed to the hypoplasia of the tracheobrochial cartilage and restrictive thoracic cage.

Intramembranous Bone Formation

Overview

Intramembranous bone formation is achieved by the direct transformation of mesenchymal cells into osteoblasts, the skeletal cells involved in bone formation. It is the process responsible for the development of the flat bones of the cranial vault, including the cranial suture lines, some facial bones, and parts of the mandible and clavicle. Although the addition of bone within the periosteum on the outer surface of long bones is also described to arise from intramembranous bone formation, current studies suggest that in fact it may be developmentally distinct (see later). With respect to the molecular mechanisms leading to osteoblast differentiation, it can be said that they are rather sketchy. Like cartilage, bone cells are induced initially by specific epithelia (Hall and Miyake, 2000). Here, the cranial sutures will be discussed as intramembranous bone growth sites, which will be followed by a brief description of transcription factors, growth factors, and their receptors associated with normal and abnormal suture development. Intramembranous ossification in the periosteum will be described later on in conjunction with endochondral bone formation.

Cranial Sutures

Cranial vault sutures identify the fibrous tissues uniting bones of the skull and are the major site of bone growth, especially during the rapid growth of the neurocranium. Sutures need to maintain patency while allowing rapid bone formation at the edges of the bone fronts in order to accommodate the rapid, expansile growth of the neurocranium (for review, see Opperman, 2000). The closure of sutures is tightly regulated by growth factors and transcription factors (BMP4, BMP7, FGF9, TWIST, and MSX1 and MSX2) involved in epitheliomesenchymal signaling among the sutural mesenchyme, the underlying dura, and the approaching bone fronts. It is proposed that the approximating bone fronts set up gradients of growth factor signaling between them, which initiates suture formation. For example, a gradient of FGF ligand, from high levels in the differentiated region to low levels in the environment of the osteogenic stem cells, modulates the differential expression of *FGFR1* and *FGFR2*. Signaling through FGFR2 regulates stem cell

proliferation, whereas signaling through FGFR1 promotes osteogenic differentiation (Iseki *et al.,* 1999). As sutures fuse, factors involved in pattern formation (*SHH, MSX1*) are all downregulated, while at the same time *RUNX2* and type I collagen (*COL1A1*) expression is seen at the bone fronts. In the end, the completely fused suture is indistinguishable from bone.

Craniofacial Disorders

As can be inferred from this discussion, the composite structure of the mammalian skull requires precise pre- and postnatal growth regulation of individual calvarial elements. Disturbances of this process frequently cause severe clinical manifestations in humans. The homeobox genes *MSX1* and *MSX2* are of particular interest in that mutated forms are associated with human craniofacial disorders (for review, see Cohen, 2000). An autosomal-dominant form of hypodontia is caused by a mutation in *MSX1* (Vastardis *et al.,* 1996), whereas heterozygous mutations in *MSX2* cause parietal foramina (oval-shaped defects on either side of the sagittal suture arising from deficient ossification around the parietal notch, normally obliterated during the fifth fetal month) (Wilkie *et al.,* 2000). These mutations, which lead to decreased parietal ossification by haploinsufficiency, are in marked contrast to the reported gain-of-function mutation (Pro7His) in *MSX2* associated with premature osseous obliteration of the cranial sutures or craniosynostosis (Boston type) (Jabs *et al.,* 1993). It is likely that *MSX2* normally prevents differentiation and stimulates the proliferation of preosteoblasic cells at the extreme ends of the osteogenic fronts of the calvariae, facilitating expansion of the skull and closure of the suture. Its haploinsufficiency decreases proliferation and accelerates the differentiation of calvarial preosteoblast cells, resulting in delayed suture closure, while its "overexpression" results in enhanced proliferation, favoring suture closure (Dodig *et al.,* 1999).

Osteogenic cell differentiation is influenced by the transcription factor RUNX2. The function of RUNX2 during skeletal development has been elucidated by the generation of mice in which the *Runx2* locus was targeted (Otto *et al.,* 1997). A heterozygous loss of function leads to a phenotype very similar to human cleidocranial dysplasia, an autosomal-dominant inherited disorder characterized by hypoplasia of the clavicles and patent fontanelles that arises from mutations in *RUNX2* (Mundlos *et al.,* 1997). Loss of both alleles leads to a complete absence of bone due to a lack of osteoblast differentiation. *RUNX2,* therefore, controls the differentiation of precursor cells into osteoblasts and is essential for membranous as well as endochondral bone.

Fibroblast growth factor receptors are major players in cranial skeletogenesis, and activating mutations of the human *FGFR1, FGFR2,* and *FGFR3* genes cause craniosynostosis. A C-to-G transversion in exon 5 of *FGFR1*, resulting in a proline-to-arginine substitution (P252R) in the extracellular domain of the receptor, has been reported in affected members of five unrelated families with Pfeiffer syndrome, an autosomal dominant disorder, characterized by mild craniosynostosis, flat facies, shallow orbits, hypertelorism, acrocephaly, broad thumb, broad great toe, polysyndactyly, and interphalangeal ankylosis (Muenke *et al.,* 1994).

Mutations in *FGFR2* (C342Y, C342R, C342S, and a C342W) have been described in patients with Crouzon syndrome (Reardon *et al.,* 1994; Steinberger *et al.,* 1995). This disorder, encompassing craniosynostosis, hypertelorism, hypoplastic maxilla, and mandibular prognathism, is easily distinguishable from Pfeiffer syndrome by the absence of hand abnormalities. Interestingly, the C342Y mutation is also reported in patients with Pfeiffer syndrome and in individuals with Jackson–Weiss syndrome (Tartaglia *et al.,* 1997), an autosomal-dominant disorder characterized by midfacial hypoplasia, craniosynostosis, and cutaneous syndactyly, indicating that the same mutation can give rise to one of several phenotypes. Another conserved cysteine at position 278 is similarly predisposed to missense mutations leading to the same craniosynostotic conditions. However, mutations in S252 and P253 residues have been reported in most cases of Apert syndrome, a condition characterized by craniosynostosis and severe syndactyly (cutaneous and bony fusion of the digits). While the mechanism whereby the same mutation can give rise to distinct phenotypes remains to be clarified, sequence polymorphisms in other parts of the mutant gene may affect its phenotypic expression (Rutland *et al.,* 1995). Finally, mutations within *FGFR2* have also been reported in other rare craniosynostotic conditions (for review, see Passos-Bueno *et al.,* 1999).

In contrast to the propensity of mutations in FGFR1 and FGFR2 affecting craniofacial development, only rarely do mutations in FGFR3 cause craniosynostoses. For the most part, mutations in FGFR3 are associated with dwarfism, suggesting that the primary function of FGFR3 is in endochondral rather than intramembranous ossification. The great majority of FGFR mutations identified to date are inherited dominantly and result in increased signaling by the mutant receptor (Naski and Ornitz, 1999). Altered cellular proliferation and/or differentiation is believed to underlie their pathogenetic effects.

Endochondral Ossification

Overview

The axial and appendicular skeleton develops from cartilaginous blastema, the growth of which arises in a variety of ways (Johnson, 1986). Cartilage is unique among skeletal tissues in that it has the capacity to grow interstitially, i.e., by division of its chondrocytes. This property is what allows cartilage to grow very rapidly. Moreover, cartilage utilizes apposition of cells on its surface, matrix deposition, and enlargement of the cartilage cells as additional means of achieving maximal growth. Appositional growth is the principal function of the perichondrium, which envelops the epiphyses and the cartilaginous diaphysis, serving as

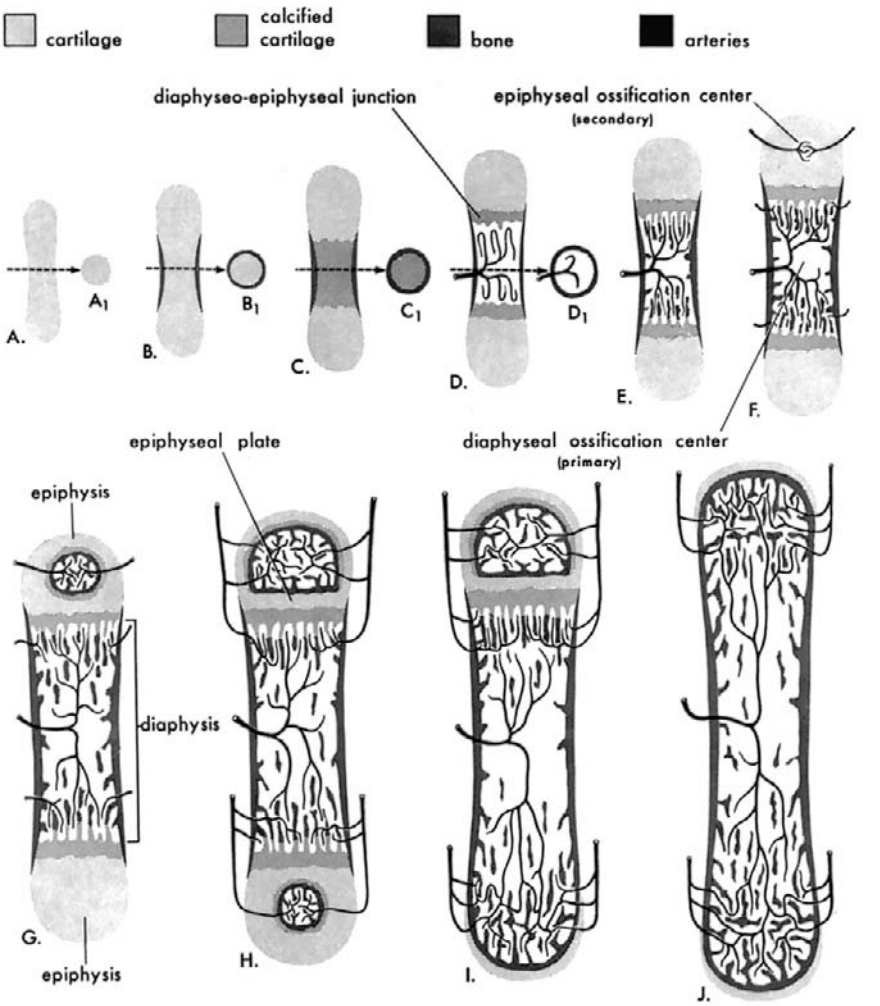

Figure 6 Formation and growth of long bones by endochondral ossification. (A) Mesenchymal condensation leads to the development of a cartilage model. (B) Capillaries invade the perichondrium surrounding the future diaphysis and transform it into the periosteum. (C) Chondrocyte differentiation ensues, just underneath the bone collar, leading to chondrocyte hypertrophy and apoptotic death associated with mineralization of the cartilage matrix. (D and E) Vascular invasion from vessels allows for the migration of osteoblast precursor cells that deposit bone on the degraded matrix scaffold. Chondrogenesis at the ends of the long bone establishes the formation of growth plates. (F) Secondary centers of ossification begin in late fetal life. (G and H) Growth plates serve as a continuous source of cartilage conversion to bone, thereby promoting linear growth. (I and J) Long bones cease growing at the end of puberty, when the growth plates are replaced by bone but articular cartilage persists. Adapted from Recker (1992).

the primary source of chondroblasts. With time, these cells differentiate to chondrocytes that secrete type II collagen, aggrecan, and a variety of other matrix molecules that constitute the extracellular matrix of the hyaline cartilage (Fig. 6). As development proceeds, capillaries invade the perichondrium surrounding the future diaphysis and transform it into the periosteum, while osteoblastic cells differentiate, mature, and secrete type I collagen and other bone-specific molecules, including alkaline phosphatase. This will ultimately mineralize by intramembranous ossification and give rise to the bony collar, the cortical bone.

A predetermined program of chondrocyte differentiation then ensues in the central diaphysis, just underneath the bone collar, leading to chondrocyte hypertrophy, synthesis of type X collagen, and calcification of the cartilage matrix, likely in response to signals emanating from periosteal osteoblasts (Komori *et al.,* 1997; Otto *et al.,* 1997). In turn, matrix mineralization is followed by vascular invasion from vessels originating in the periosteal collar that allows for the migration of osteoblast precursor cells into the cartilaginous blastema (primary ossification center). These cells transform into mature osteoblasts and initiate new bone formation on the degraded matrix scaffolding. The primary growth plates are then established and serve as a continual source of cartilage conversion to bone and linear growth of the long bone during development and postnatally. In late

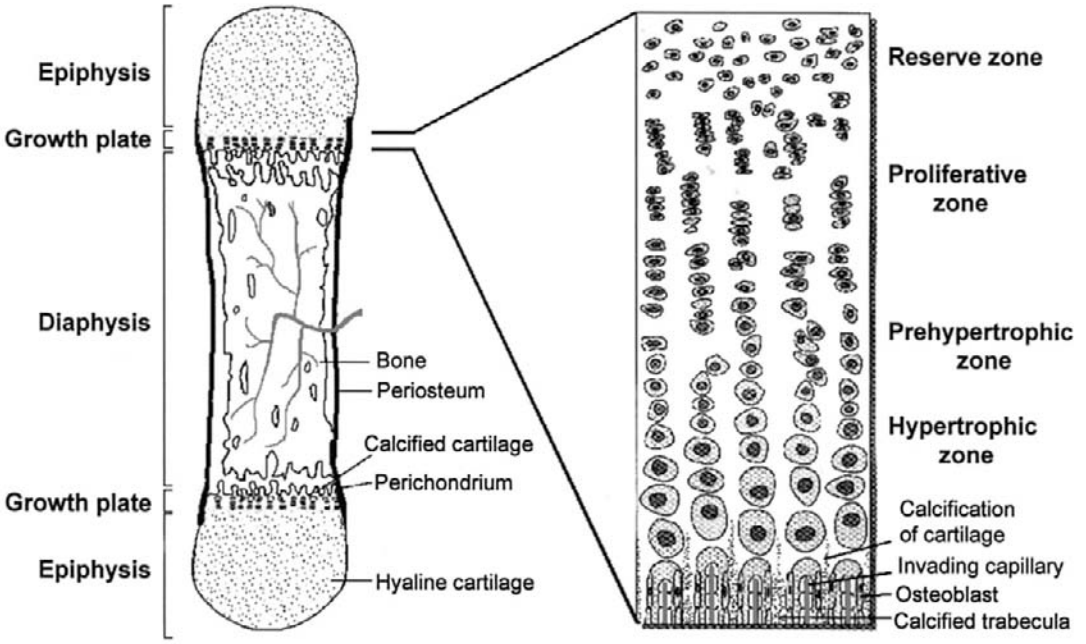

Figure 7 Schematic representation of the organization of the mammalian growth plate. Adapted from Wallis (1996). See text for details. (See also color plate.)

fetal life and early childhood, secondary centers of ossification appear within the cartilaginous epiphyses by a mechanism very similar to that used in the formation of the primary center. Cartilage is retained at the joint surface, giving rise to the articular cartilage, and at the growth plate, extending the full width of the bone and separating epiphysis from diaphysis. Cessation of growth occurs at the end of puberty, when growth plates are replaced by bone.

The Growth Plate

The organization of the mammalian epiphyseal growth plate is represented diagrammatically in Fig.7 (see also color plate). The growth plate conforms to a general basic plan that consists of four zones, which, although distinct, encompass a merging continuum (for reviews, see Johnson, 1986; Stevens and Williams, 1999). In the reserve zone, chondrocytes are nearly spherical in cross section and appear to be arranged randomly, separated by large amounts of matrix consisting largely of type II collagen and proteoglycans. Although parts of this zone are mitotically inert, others function as stem cell sources. Cells from this zone eventually become discoid and are arranged into rather regular columns forming the zone of proliferation. Column formation is in part due to the characteristic division of chondrocytes in that their mitotic axis is perpendicular to the long axis of the bone. Two daughter cells become flattened and are separated by a thin septum of cartilage matrix. Elongation of the blastema occurs mainly at its ends and arises primarily from the division of chondrocytes, as it is here that cell proliferation is maximal. Eventually, chondrocytes from this zone enlarge and lose their characteristic discoidal shape as they enter the zone of maturation (prehypertrophic chondrocytes). Growth here ceases to be due to cell division and continues by increases in the size of the cells. In the midsection, chondrocytes mature, enlarge in size (hypertrophy), and secrete a matrix rich in type X collagen. These cells continue to enlarge to the point that their vertical height has increased nearly five times. Once glycogen stores have been depleted, they undergo programmed cell death or apoptosis (Farnum and Wilsman, 1987), leaving behind longitudinal lacunae separated by septae of cartilaginous matrix that become selectively calcified as well as largely uncalcified transverse septae. In response to these changes, vascular invasion ensues as new blood vessels enter the lower hypertrophic zone from the primary spongiosum and penetrate the transverse septae while calcified cartilage is removed by chondroclasts that accompany this erosive angiogenic process. The remaining longitudinal septae, which now extend into the diaphysis, are used by osteoblasts derived from bone marrow stromal cells to settle on and lay down extracellular matrix (osteoid), which calcifies into woven bone. With time, osteoclasts resorb the woven bone and replace it with mature trabecular bone, thereby completing the process of endochondral ossification.

Mediators of Growth Plate Chondrocyte Proliferation and Differentiation

Proper skeletal formation, growth, and repair are critically dependent on the accurate orchestration of all the processes participating in the formation of endochondral bone at the growth plate. It is only recently, however, that

fundamental insight has emerged into the molecular pathways regulating these processes. This section discusses the major systemic and local influences on growth plate chondrocyte proliferation and differentiation and associated developmental abnormalities arising from their failure to function in the appropriate fashion. In specific situations, the distinction between systemic as opposed to local mediators may not be so apparent, which will be pointed out.

SYSTEMIC MEDIATORS

A variety of systemic hormones, such as the growth hormone (GH)–insulin-like growth factor-1 (IGF1) signaling system, thyroid hormone, estrogens, glucocorticoids, and vitamin D, partake in the regulation of linear growth pre- and postnatally. The importance of these hormones in linear skeletal growth has been highlighted by both genetic studies in animals and "experiments of nature" in humans.

Growth Hormone and Insulin-like Growth Factor 1 GH plays an important role in longitudinal bone growth because bone growth is impaired both in GH-deficient humans (Laron *et al.,* 1966; Rosenfeld *et al.,* 1994) and in the GH receptor-null mouse (Sjogren *et al.,* 2000; Zhou *et al.,* 1997). Homozygous-null mice display severe postnatal growth retardation, disproportionate dwarfism, and markedly decreased bone mineral content. Reduced bone length in GH receptor-negative mice is associated with premature growth plate contraction and reduced chondrocyte proliferation, which is not detectable until 3 weeks of age; before this, bone growth proceeds normally, indicating that GH is not required for normal murine prenatal development or early postnatal growth. While cortical and longitudinal bone growth and bone turnover are all reduced in GH receptor deficiency, many of these effects can be reversed by IGF1 treatment (Sims *et al.,* 2000), suggesting that the main defect relates to reduced IGF1 levels in the absence of GH receptor.

In the original "somatomedin hypothesis," it was proposed that the primary effect of GH was to stimulate IGF1 production by the liver, with circulating IGF1 then stimulating the longitudinal expansion of growth plates in an endocrine fashion. Because longitudinal bone growth is not affected in the liver-specific *Igf1*-knockout mouse (Yakar *et al.,* 1999), locally produced IGF1 and/or direct effects of GH may substitute for deficient systemic IGF1. More recent work suggests that GH acts directly at the growth plate to amplify the production of chondrocytes from germinal zone precursors and then to induce local IGF1 synthesis, proposed to stimulate the clonal expansion of chondrocyte columns in an autocrine/paracrine manner (Ohlsson *et al.,* 1998). Although the actions of GH on a range of cell types are mediated by Stat5 signaling, interestingly, the bone phenotypes in GH receptor- and *Stat5*-knockout animals (Teglund *et al.,* 1998) are different, suggesting that the effects of GH on bone, whether direct or through IGF1, are not mediated by Stat5 transcription factors but by other cytokine or signaling cascades.

IGF1 plays a pivotal role in longitudinal bone growth, as *Igf1* gene deletion results in dwarfism in mice (Liu *et al.,* 1993; Powell-Braxton *et al.,* 1993) and extreme short stature in humans (Woods *et al.,* 1996). In study of longitudinal bone growth in the *Igf1*-null mouse, growth plate chondrocyte proliferation and cell numbers are preserved, despite a 35% reduction in the rate of long bone growth (Wang *et al.,* 1999). The growth defect due to *Igf1* deletion has been traced to an attenuation of chondrocyte hypertrophy, which is associated with *Glut4* glucose transporter expression, glycogen synthesis (GSK3β serine phosphorylation), and ribosomal RNA levels being significantly diminished in *Igf1*-null hypertrophic chondrocytes, resulting in reduced glycogen in these cells. Glycogen stores are normally accumulated by proliferative and early hypertrophic chondrocytes and are depleted during maturation of the hypertrophic chondrocytes. Hypertrophic chondrocytes are highly active metabolically and are dependent on glycolysis to fuel their expansive biosynthetic activity. The decrease in ribosomal RNA in *Igf1*-null hypertrophic chondrocytes may reflect cellular "starvation" for fuel and building blocks for protein synthesis.

Thyroid Hormones Thyroid hormone deprivation has deleterious effects on bone growth. The observed delay in bone development is mediated by a direct effect of thyroid hormone on bone and an indirect effect of the hormone on GH release and IGF1 action (Weiss and Refetoff, 1996). Thyrotoxicosis, however, accelerates growth rate and advances bone age. In euthyroid human (Williams *et al.,* 1998) and rat cartilage (Stevens *et al.,* 2000), thyroid hormone receptor α1 (TRα1), TRα2, and TRβ1 proteins are localized to reserve zone progenitor cells and proliferating chondrocytes. When animals are rendered hypothyroid, growth plates become grossly disorganized and hypertrophic chondrocyte differentiation fails to progress (Fraichard *et al.,* 1997; Stevens *et al.,* 2000). In thyrotoxic growth plates, histology is essentially normal, but mRNA for parathyroid hormone-related protein (*Pthrp*) and its receptor are undetectable. PTHrP signaling exerts potent inhibitory effects on hypertrophic chondrocyte differentiation (see later), suggesting that the dysregulation of local mediators of endochondral ossification may be a key mechanism that underlies growth disorders in childhood thyroid disease. Although thyroid hormone may also act directly on osteoblasts (Abu *et al.,* 2000), these effects have received much less attention.

Estrogens The biosynthesis of estrogens from testosterone in the ovary, adipose tissue, skeletal muscle, skin, hair follicles, and bone is catalyzed by the enzyme aromatase, the product of the *CYP19* gene. In recent years, a number of patients, two men and five women, have been described suffering from aromatase deficiency due to mutations in *CYP19,* resulting in the synthesis of a nonfunctional gene product and the failure to synthesize estrogens (reviewed in Faustini-Fustini *et al.,* 1999). Males with this

condition have sustained linear growth into adulthood as a consequence of failed epiphyseal closure. Reduced bone mineral density and bone age are also characteristic. The women show absence of a growth spurt and delayed bone age at puberty as well as unfused epiphyses later on, despite evidence of virilization.

The biological effects of estrogens are mediated by two estrogen receptors (ER), ERα and ERβ which regulate transcription through direct interaction with specific binding sites on DNA in promoter regions of target genes (for review, see Pettersson and Gustafsson, 2001). Smith and associates (1994) have described a man with a biallelic inactivating mutation of the ERα gene. This patient had normal genitalia but suffered from osteoporosis and was still growing at the age of 28 because the epiphyseal plates were unfused.

These two "experiments of nature" (aromatase and ERα deficiency), supported by the recent identification of ERα and ERβ expression in chondrocytes (Ushiyama *et al.,* 1999) and osteogenic cells of trabecular and cortical bone (Rickard *et al.,* 1999), have firmly established that estrogens exert direct effects on the growth plate and are crucial for peripubertal growth and epiphyseal growth plate fusion at the end of puberty in both women and men. Moreover, they have revealed a greater appreciation for the importance of estrogens in bone mass maintenance in both sexes.

Glucocorticoids Glucocorticoids have well-documented effects on the skeleton, as pharmocological doses cause stunted growth in children (Canalis, 1996). The skeletal actions of glucocorticoids are mediated via specific receptors, which are widely distributed at sites of endochondral bone formation. Studies now indicate that glucocorticoids are involved in chondrocyte proliferation, maturation, and differentiation earlier in life, whereas at puberty they are implicated primarily in chondrocyte differentiation and hypertrophy. Further investigation is required, however, to clarify the physiologic actions of glucocorticoids on cartilage.

Glucocorticoid receptors are also highly expressed in rodent and human osteoblastic cells both on the bone-forming surface and at modeling sites (Abu *et al.,* 2000). Pharmacologic doses of glucocorticoids in mice inhibit osteoblastogenesis and promote apoptosis in osteoblasts and osteocytes, thereby providing a mechanistic explanation for the profound osteoporotic changes arising from their chronic administration (Weinstein *et al.,* 1998).

Vitamin D 1,25$(OH)_2$-vitamin D_3 exerts its effects on growth plate chondrocytes through classical vitamin D (VDR) receptor-dependent mechanisms, promoting mineralization of the extracellular matrix (Boyan *et al.,* 1989). Vitamin D deficiency is the major cause of rickets in children and osteomalacia in adults. Inactivating mutations in the coding sequences of 25-hydroxyvitamin D_3 1α-hydroxylase (*CYP27B1*) (Fu *et al.,* 1997) and *VDR* (reviewed in Hughes *et al.,* 1991) genes are associated with rickets. In both conditions, there is expansion of the hypertrophic zone of the growth plate, coupled with impaired extracellular matrix calcification and angiogenesis. Also, a direct role of vitamin D on bone is suggested, as *VDR* is expressed in osteoblasts and osteoclast precursors (Johnson *et al.,* 1996; Mee *et al.,* 1996).

The 25-hydroxyvitamin D-24-hydroxylase enzyme (24-OHase; *CYP24*) is responsible for the catabolic breakdown of 1,25$(OH)_2$-vitamin D_3. The enzyme can also act on the 25(OH)-vitamin D_3 substrate to generate 24,25$(OH)_2$-vitamin D_3, a metabolite whose physiological importance remains unclear. Although earlier studies in *Cyp24*-knockout mice had suggested that the 24-hydroxylated metabolite of vitamin D, 24R,25$(OH)_2$-vitamin D_3, exerts distinct effects on intramembranous bone mineralization (St-Arnaud, 1999), more recent work has concluded that this metabolite is dispensable during bone development (St-Arnaud *et al.,* 2000).

LOCAL MEDIATORS

TGFβ *TGFβ1*, *2*, and *3* mRNAs are synthesized in the mouse perichondrium and periosteum from 13.5 days *postcoitus* until after birth (Millan *et al.,* 1991). As discussed previously, TGFβ promotes chondrogenesis in early undifferentiated mesenchyme (Leonard *et al.,* 1991), but in high-density chondrocyte pellet cultures or organ cultures it inhibits terminal chondrocyte differentiation (Ballock *et al.,* 1993). TGFβs signal through heteromeric type I and type II receptor serine/threonine kinases. To delineate the role of TGFβs in the development and maintenance of the skeleton *in vivo*, Serra *et al*. (1997) generated transgenic mice that express a cytoplasmically truncated, functionally inactive TGF−type II receptor under the control of a metallothionein-like promoter, which can compete with the endogenous receptors for complex formation, thereby acting as a dominant-negative mutant. Loss of responsiveness to TGFβ promoted chondrocyte hypertrophy, suggesting an *in vivo* role for TGFβ in limiting terminal differentiation. In mouse embryonic metatarsal bone rudiments grown in organ culture, TGFβ inhibited several stages of endochondral bone formation, including chondrocyte proliferation, hypertrophic differentiation, and matrix mineralization (Serra *et al.,* 1999).

Parathyroid Hormone-Related Protein (PTHrP) and Indian Hedgehog (Ihh) Parathyroid hormone-like hormone (PTHLH), or PTH-related protein (PTHrP), as more commonly recognized, is a major determinant of chondrocyte biology and endochondral bone formation. PTHrP was discovered as the mediator of hypercalcemia associated with malignancy but is now known to be expressed by a large number of normal fetal and adult tissues (Philbrick *et al.,* 1996; Wysolmerski and Stewart, 1998). The amino-terminal region of PTHrP reveals limited but significant homology with the parathyroid hormone (PTH), resulting in the interaction of the first 34 to 36 residues of either protein with a single seven transmembrane-spanning G-protein-linked receptor

termed the PTH/PTHrP receptor, or PTH receptor type 1 (PTHR1). Both PTH and PTHrP, through their interaction with this receptor, activate cAMP and calcium second messenger signaling pathways by stimulating adenylate cyclase and/or phospholipase C activity, respectively (Abou-Samra *et al.,* 1992; Juppner *et al.,* 1991). Targeted inactivation of *Pthrp* and *Pthr1* has established a fundamental role for this signaling pathway in chondrocyte proliferation, differentiation, and apoptotic death (Amizuka *et al.,* 1994, 1996; Karaplis *et al.,* 1994; Lanske *et al.,* 1996; Lee *et al.,* 1996). Mice homozygous for *Pthrp*- or *Pthr1*-null alleles display a chondrodysplastic phenotype characterized by reduced chondrocyte proliferation and premature and inappropriate hypertrophic differentiation, resulting in advanced endochondral ossification. Conversely, targeted expression of PTHrP (Weir *et al.,* 1996) or a constitutively active form of PTHR1 (Schipani *et al.,* 1997) to the growth plate leads to delayed mineralization, decelerated conversion of proliferative chondrocytes into hypertrophic cells, and prolonged presence of hypertrophic chondrocytes with delay of vascular invasion.

Correlation of these findings to human chondrodysplasias arose initially from studies in patients with Jansen-type metaphyseal dysplasia. This autosomal-dominant disorder is characterized by short stature, abnormal growth plate maturation, and laboratory findings indistinguishable from primary hyperparathyroidism, despite low normal or undetectable levels of PTH and PTHrP. Schipani and associates (1995, 1996, 1999) reported heterozygous missense mutations in *PTHR1* that promote ligand-independent cAMP accumulation, but with no detectable effect on basal inositol phosphate accumulation. These activating mutations have therefore provided an explanation for the observed biochemical abnormalities and the abnormal endochondral ossification characteristic of Jansen metaphyseal chondrodysplasia. In contrast to *PTHR1*-activating mutations in this disorder, Blomstrand chondrodysplasia arises from the absence of a functional PTHR1 protein (Jobert *et al.,* 1998; Karaplis *et al.,* 1998; Karperien *et al.,* 1999; Zhang *et al.,* 1998). This is a rare autosomal recessive chondrodysplasia characterized by skeletal abnormalities that bear a remarkable resemblance to the phenotypic alterations observed in *Pthr1* knockout mice.

The pivotal role of PTHrP signaling in the growth plate has served as the impetus for subsequent studies aiming to identify and characterize upstream and downstream molecular components regulating chondrocyte proliferation and differentiation. Indian hedgehog (Ihh) is a member of the vertebrate homologs of the *Drosophila* segment polarity gene, hedgehog (*hh*). Although only one *hh* gene has been identified in *Drosophila*, several *hh* genes are present in vertebrates. The mouse Hedgehog (Hh) gene family consists of *Sonic* (*Shh*), *Desert* (*Dhh*), and *Indian* (*Ihh*) *hedgehog*, all encoding secreted proteins implicated in cell–cell interactions. Signaling to target cells is mediated by a receptor that consists of two subunits; Patched (Ptc), a 12 transmembrane protein, which is the binding subunit (Marigo *et al.,* 1996; Stone *et al.,* 1996); and Smoothened (Smo), a 7 transmembrane protein, which is the signaling subunit. In the absence of Hh, Ptc associates with Smo and inhibits its activities. In contrast, binding of Hh to Ptc relieves the Ptc-dependent inhibition of Smo (Nusse, 1996). Signaling then ensues and includes downstream components such as the Gli family of transcriptional factors. The three cloned *Gli* genes (*Gli1, Gli2,* and *Gli3*) encode a family of DNA-binding zinc finger proteins with related target sequence specificities.

Ihh is expressed in prehypertrophic chondrocytes of the mouse embryo. Earlier studies using Ihh overexpression and misexpression in the developing cartilage demonstrated that Ihh delays the hypertrophic differentiation of growth plate chondrocytes (Vortkamp *et al.,* 1996). A number of *in vitro* as well as *in vivo* studies now indicate that the capacity of Ihh to slow chondrocyte differentiation is mediated by PTHrP. Ihh upregulates *Pthrp* expression in the growth plate. This expression, however, is abolished by the targeted disruption of *Ihh* and leads to premature chondrocyte differentiation (St-Jacques *et al.,* 1999), thereby implicating PTHrP as the mediator of Ihh actions on chondrocyte hypertrophy. These observations, among others, have led to the proposal that an Ihh/PTHrP feedback loop regulates the pace of chondrocyte differentiation in the growth plate (Fig. 8, see also color plate) (Chung and Kronenberg, 2000). As chondrocytes differentiate into the prehypertrophic state, they express Ihh, which stimulates the expression of PTHrP. In turn, PTHrP binds and activates PTHR1 in proliferating chondrocytes to delay their differentiation into prehypertrophic chondrocytes, which make Ihh. In so doing, this negative feedback loop serves to regulate the rate of chondrocyte differentiation.

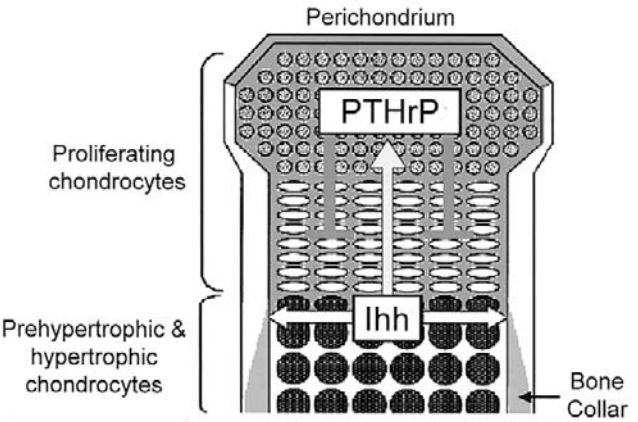

Figure 8 Ihh and PTHrP interaction in the growth plate. Ihh and PTHrP participate in a negative feedback loop to regulate the rate of chondrocyte differentiation. Ihh, expressed in chondrocytes in the prehypertrophic zone, stimulates indirectly (yellow arrow) the synthesis of PTHrP in the growth plate, which in turn acts on proliferating chondrocytes to delay their differentiation (red T bars). Ihh is also implicated in the induction of bone collar formation (white arrows). Adapted from Chung and Kronenberg (2000). (See also color plate.)

What is the mechanism by which Ihh stimulates *Pthrp* expression? Although it is possible that Ihh interacts directly with *Pthrp*-expressing cells in the growth plate, it is more likely, given the number of restrictions imposed on Ihh diffusion (Chuang and McMahon, 1999), that the action is indirect. BMPs have been proposed to serve as a secondary signal downstream of Ihh. For example, viral expression of a constitutively active form of the BMP receptor IA increased *Pthrp* mRNA expression in embryonic chicken limbs and blocked chondrocyte differentiation in a similar manner as misexpression of Ihh without inducing *Ihh* expression (Zou *et al.,* 1997). Further studies have indicated that BMP2 and BMP4 are the likely secondary signals, which act through the BMP receptor IA to mediate the induction of *Pthrp* expression (Pathi *et al.,* 1999). It is of interest to note that TGFβ also stimulates *Pthrp* expression in mouse embryonic metatarsal bone rudiments grown in organ culture (Serra *et al.,* 1999). Furthermore, terminal differentiation is not inhibited by TGFβ in metatarsal rudiments from *Pthrp*-null embryos, supporting the model that TGFβ acts upstream of PTHrP to regulate the rate of hypertrophic differentiation. Whether it is TGFβ or other members of the BMP family of proteins that serve as the intermediary relay that links the Ihh and PTHrP signaling pathways remains to be determined. Other studies, however, have failed to support a role for BMPs in this process (Haaijman *et al.,* 1999). For now, it would be prudent to conclude that the mechanism transmitting Ihh signaling to PTHrP-expressing chondrocytes remains, for the most part, uncertain.

What are the downstream molecular mechanisms that convey the inhibitory action of PTHrP on chondrocyte differentiation? Transgenic studies have attempted to address this question by assessing the significance of the cyclic AMP/PKA and phospholipase C/PKC signal transduction pathways on the cartilage differentiation program. In the first scenario, a PTH receptor with normal phospholipase C signaling, but deficient $G_s\alpha$ signaling was expressed in chimeric mice (Chung *et al*. 2000). Cells with deficient $G_s\alpha$ signaling underwent premature maturation in the growth plate, whereas wild-type cells had a normal rate of differentiation. In the second scenario, mice expressing a mutant PTHrP receptor with normal $G_s\alpha$ signaling, but deficient phospholipase C signaling were shown to be of normal size and did not have reduced rates of chondrocyte differentiation (Guo *et al.,* 2000). These genetic experiments, as well as more recent work using cultured chondrocytes (Ionescu *et al.,* 2001), support the contention that the cyclic AMP/PKA signal transduction pathway is involved intimately with chondrocyte differentiation, whereas PKC signaling appears less relevant for these events both *in vivo* and *in vitro*.

Given that PKA-phosphorylated SOX9 is present in the prehypertrophic zone of the growth plate, the same location where the gene for PTHR1 is expressed, then SOX9 is a likely target for PTHrP signaling (Fig. 9, see also color plate). What is the evidence to support this conclusion? SOX9 phosphorylated at serine 181 (S181), one of two consensus PKA phosphorylation sites, is detected almost

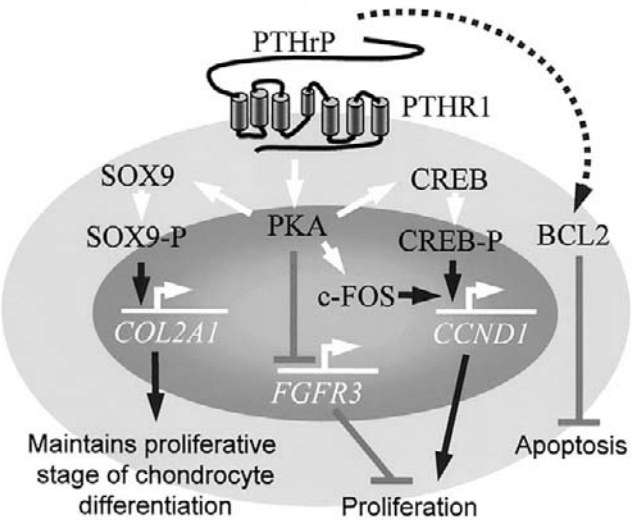

Figure 9 PTHrP and chondrocyte biology. Signaling pathways proposed to mediate the effects of PTHrP on differentiation, proliferation, and apoptotic death of chondrocytes. The dashed line depicts putative PTHrP actions that may not be mediated by PTHR1 (intracrine effects). (See also color plate.)

exclusively in chondrocytes of the prehypertrophic zone in wild-type mouse embryos (Huang *et al.,* 2000). Moreover, no phosphorylation of SOX9 is observed in prehypertrophic chondrocytes of the growth plate or any chondrocytes of *Pthr1*-null mutants. Phosphorylation of SOX9 by PKA enhances its transcriptional and DNA-binding activity (Huang *et al.,* 2000). PTHrP greatly potentiates the phosphorylation of SOX9 (S181) and increases the SOX9-dependent activity of chondrocyte-specific enhancers in *Col2a1,* the gene for type II collagen. These findings indicate that SOX9 is a target of PTHrP signaling in prehypertrophic chondrocytes in the growth plate and that the PTHrP-dependent increased transcriptional activity of SOX9 helps maintain the chondrocyte phenotype of cells in the prehypertrophic zone, thereby delaying their maturation to the hypertrophic state.

While the effects of PTHrP on chondrocyte differentiation are generally considered only in terms of its interaction with the cell surface receptor (PTHR1), studies *in vitro,* as well as *in vivo,* now indicate that the capacity of PTHrP to influence this process must also be assessed in relation to its intracrine actions at the level of the nucleus/nucleolus (Henderson *et al.,* 1995). Nucleolar localization of the protein has been associated with the inhibition of differentiation and delay in the apoptotic death of chondrocytes (Henderson *et al.,* 1996), likely by increasing *Bcl2* gene expression in these cells (Amling *et al.,* 1997). However, the events that determine the timing and degree of PTHrP nucleolar translocation or the role that it may serve in the *in vivo* biology of chondrocytes remain, for the most part, undefined.

Mechanisms underlying the molecular regulation of chondrocyte proliferation have been investigated, notably using transgenic mice that carry either gain- or loss-of-function mutations. From these studies, it has become

evident that one of the most potent inducers of chondrocyte proliferation is Ihh. In support of this contention is the observation that the most striking feature of the *Ihh*-null endochondral skeleton is a profound decrease in limb length arising as a consequence of severe reduction in growth plate chondrocyte proliferation (St-Jacques *et al.,* 1999). Interestingly, this effect, unlike that on differentiation, is for the most part independent of PTHrP (Karp *et al.,* 2000), although PTHrP likely exerts its own unique influence on this process (Amizuka *et al.,* 1994; Karp *et al.,* 2000). What factors, if any, are involved in mediating the proproliferative effect of Ihh, however, remain to be defined.

More is known about the signaling pathways that mediate the role of PTHrP on chondrocyte proliferation. Many of the transcriptional effects of cAMP are mediated by the cAMP response element (CRE)-binding protein CREB, which binds to the CRE element in the upstream region of a variety of genes. CREB is a member of the CREB/activating transcription factor (ATF) family of transcription factors and is phosphorylated by PKA following increases in intracellular cAMP levels. Phosphorylation permits its interaction with p300/CBP and other nuclear coactivators, leading to gene transcription (Montminy, 1997). A role for CREB in skeletal development was not suggested initially by the phenotype of the *Creb*-knockout mice perhaps due in part to the functional compensation by other CREB family members (Rudolph *et al.,* 1998). In keeping with this supposition is the observation that targeted overexpression of a potent dominant-negative inhibitor for all CREB family members to the murine growth plate causes a profound decrease in chondrocyte proliferation, resulting in short-limbed dwarfism and perinatal lethality due to respiratory compromise (Long *et al.,* 2001). Similarly, disruption of the gene encoding the transcription factor ATF2, another member of the CREB/ATF family, also inhibits the proliferation of chondrocytes (Reimold *et al.,* 1996).

Because the genes of the cell cycle machinery execute the intracellular control of proliferation, it is likely that these genes play a pivotal role during endochondral ossification. This view is supported by targeted disruption in mice of the CDK inhibitor p57Kip2 (Yan *et al.,* 1997) and the pRb-related p107 and p130 genes (Cobrinik *et al.,* 1996). In both cases, chondrocytes display delayed exit from the cell cycle and differentiation, leading to severe skeletal defects. A large body of experimental evidence now indicates that the major regulatory decisions controlling cell cycle progression, and hence proliferation, of mammalian cells take place during G_1 (reviewed in Sherr, 1993). Because cell cycle genes play an important role in proliferation, it is reasonable to speculate that they might be involved in the biological responses of chondrocytes to PTHrP. The cyclin D1 gene (*Ccnd1*) is a key regulator of progression through the G_1 phase of the cycle and has been identified as a target for the transcription factor ATF2 (Beier *et al.,* 1999). ATF2 is present in nuclear extracts from chondrogenic cell lines and binds, as a complex with a CRE-binding protein (CREB)/ CRE modulator protein, to the cAMP response element (CRE) in the cyclin D1 promoter. Moreover, site-directed mutagenesis of the cyclin D1 CRE causes a reduction in the activity of the promoter in chondrocytes, whereas overexpression of ATF2 in chondrocytes enhances activity of the cyclin D1 promoter. Inhibition of endogenous ATF2 or CREB by the expression of dominant-negative inhibitors of CREB and ATF2 significantly reduces the activity of the promoter in chondrocytes through the CRE. Finally, levels of cyclin D1 protein are reduced drastically in chondrocytes of ATF2-negative mice. These data identify the cyclin D1 gene as a direct target of ATF2 in chondrocytes and suggest that the reduced expression of cyclin D1 contributes to the defective cartilage development of these mice. Homozygous deletion of *Ccnd1* in mice results primarily in reduced postnatal growth (Sicinski *et al.,* 1995). It is likely that alterations in the proliferation of chondrocytes may have contributed to this phenotype. However, the skeletal defects of these mice are clearly less severe than those of ATF2-null mice, possibly because of the presence of intact cyclins D2 and D3. This advocates that additional target genes of ATF2 are involved in the reduction of chondrocyte proliferation in ATF2-deficient mice. In particular, it will be of interest to determine whether other D-type cyclin genes (cyclin D2 and D3) are regulated by ATF2 in chondrocytes. Finally, it remains to be seen whether PTHrP induces cyclin D1 expression through activation of CREB and how this impacts on chondrocyte proliferation. Alternatively, such a response may be mediated by the transcription factor AP-1, which is also central to the action of PTHrP in chondrocytes (Ionescu *et al.,* 2001). Signaling by PTHR1 activates AP-1, a complex formed through interactions between c-Fos and c-Jun family members, by inducing the expression of c-Fos in chondrocytes. The protein complex binds to the phorbol 12-myristate 13 acetate (PMA) response element (TRE), a specific *cis*-acting DNA consensus sequence in the promoter region of target genes, like cyclin D1.

Fibroblast Growth Factor Receptor 3 (FGFR3) Another major molecular player in growth plate chondrocyte biology is fibroblast growth factor receptor 3 (FGFR3). FGF1, FGF2, and FGF9 bind FGFR3 with relatively high affinity (Ornitz and Leder, 1992); however, the ligands of FGFR3 *in vivo* and their downstream effects in individual tissues have not been defined precisely. Gain-of-function mutations in *FGFR3* have been linked to several dominant skeletal dysplasias in humans, including achondroplasia (Bellus *et al.,* 1995; Rousseau *et al.,* 1994; Shiang *et al.,* 1994), thanatophoric dysplasia (TD) types I (Rousseau *et al.,* 1996; Tavormina *et al.,* 1995) and II (Tavormina *et al.,* 1995), and hypochondroplasia (Bellus *et al.,* 1995). This group of disorders is characterized by a continuum of severity, from hypochondroplasia exhibiting a lesser degree of phenotypic severity, to achondroplasia, and to TDs, two lethal neonatal forms of dwarfism distinguished by subtle

differences in skeletal radiographs. Achondroplasia is the most common genetic form of dwarfism in humans and results from a mutation in the transmembrane domain (G380R) of FGFR3, whereas thanatophoric dysplasia is the most common neonatal lethal skeletal dysplasia in humans and results from any of three independent point mutations in *FGFR3*. Nearly all reported missense mutations in families with TDI were found to cluster in two locations: codon 248 involving the substitution of an arginine for a cysteine residue (R248C) and the adjacent codon 249 causing a serine to a cysteine change (S249C). In all patients with TDII, a lysine to glutamic acid substitution at position 650 (K650E) was described in the tyrosine kinase domain of the FGFR3 receptor. Heterozygous *FGFR3* mutations have also been reported in patients with hypochondroplasia. In 8 out of 14 alleles examined, a single C-to-A transversion causing an asparagine-to-lysine substitution at position 540 (N540K) of the protein was demonstrated.

Clinically, all of these mutations result in a characteristic disruption of growth plate architecture and disproportionate shortening of the proximal limbs. The mechanism by which *FGFR3* mutations disrupt skeletal development has been investigated extensively. Outside of the developing central nervous system, the highest level of *FGFR3* mRNA is found in the cartilage rudiments of all bones, and during endochondral ossification, *FGFR3* is restricted to the resting and proliferating zones of cartilage in the growth plates (Peters *et al.,* 1993). Inactivation of FGFR3 signaling in mice leads to an increase in the size of the hypertrophic zone, as well as a coincident increase in bone length postnatally, suggesting that FGFR3 functions as a negative regulator of bone growth (Colvin *et al.,* 1996; Deng *et al.,* 1996). *In vitro* studies indicate that *FGFR3*-associated mutations confer gain-of-function properties to the receptor by rendering it constitutively active (Naski *et al.,* 1996). Ligand-independent receptor tyrosine phosphorylation then leads to inhibition of cell growth and differentiation in cartilaginous growth plates (Naski *et al.,* 1998; Segev *et al.,* 2000). While the molecular mechanisms that underlie these processes remain sketchy at present, expression of the master chondrogenic factor Sox9 was shown to be upregulated in chondrocytes following FGF treatment (Murakami *et al.,* 2000). FGF stimulation of Sox9 expression was mediated by the mitogen-activated protein kinase (MAPK) cascade, a signal transduction pathway activated by growth factors such as FGF. It would be anticipated, therefore, that in skeletal disorders caused by activating mutations in FGFR3, chondrocyte *SOX9* expression would be abnormally high (de Crombrugghe *et al.,* 2000), thereby helping to maintain the phenotype of cells in the prehypertrophic zone and delaying their maturation to the hypertrophic state.

Interplay of Local Mediators

During the process of proliferation and differentiation, chondrocytes integrate a complex array of signals from both local and systemic factors. Understanding the specific role of one signaling pathway requires an appreciation of how it integrates with other signals participating in bone development. What is known about the interplay among FGFR3, PTHrP, and Ihh signaling in the growth plate? The overlapping expression of FGFR3 and PTHR1 in the growth plate would suggest that these signaling pathways interact. Inactivation of either PTHrP or the PTHR1 in mice results in a marked decrease in the size of the proliferative zone, a phenotype resembling that seen with the constitutive activation of FGFR3 signaling. It is likely therefore that one pathway by which PTHrP can stimulate chondrocyte proliferation may involve downregulation of *Fgfr3* expression. In fact, work by McEwen *et al*. (1999) suggests a model whereby PKA signaling, by effectors such as the PTHR1, attenuates chondrocytic expression of *Fgfr3* and thus serves to regulate endochondral ossification (Fig. 9). Moreover, *in vivo* studies indicate that FGFR3 signaling can repress *Ihh* and *Pthr1* expression in the growth plate (Chen *et al.,* 2001; Naski *et al.,* 1998). This would link Ihh/PTHrP signaling to the FGFR3 pathway in the epiphyseal growth plate and hence complete a potential feedback loop that orchestrates endochondral bone growth.

Articular Cartilage

Little is known about the factors that control the differentiation of chondrocytes located at the epiphyseal tip of long bones to articular cartilage. In contrast to chondrocytes in the shaft, which tend to undergo maturation, hypertrophy, mineralization, and subsequent replacement by bone, these cells resist differentiation and produce abundant extracellular matrix in order to maintain normal joint function throughout life. The mechanisms that drive chondrocytes to this alternative fate are only now beginning to be unveiled. Endogenous TGFβs likely maintain cartilage homeostasis by preventing inappropriate chondrocyte differentiation, as expression of a dominant-negative form of the transforming growth factor type II receptor in skeletal tissue results in increased hypertrophic differentiation in the growth plate as well as articular chondrocytes (Serra *et al.,* 1997).

Studies by Iwamoto *et al.* (2000) have identified C-1–1, a novel variant of the ets transcription factor ch-ERG, which lacks a 27 amino acid segment upstream of the ets DNA-binding domain. C-1–1 expression has been localized in the developing articular chondrocytes, whereas ch-ERG is particularly prominent in prehypertrophic chondrocytes in the growth plate. Virally driven overexpression of C-1–1 in developing chick leg chondrocytes blocks their maturation into hypertrophic cells and prevents the replacement of cartilage by bone. It also induces the synthesis of tenascin-C, an extracellular matrix protein that is unique to developing articular chondrocytes. In contrast, the expression of ch-ERG stimulates chondrocyte maturation. This work identifies C-1–1 as a transcription factor instrumental in the genesis and maintenance of epiphyseal articular

chondrocytes and provides a first glimpse into the mechanisms that dictate alternative chondrocyte developmental pathways.

Coupling Chondrogenesis and Osteogenesis

Formation of Bone Collar

As illustrated in Fig. 6B, the bone collar that forms in the perichondrium is the precursor of the cortical region of long bones. Hypertrophic chondrocytes have been proposed to play a critical role in coordinating growth plate chondrogenesis and perichondrial osteogenesis, although the molecular parameters that regulate these processes remain for the most part undefined. In earlier work, it was noted that *Ihh*-null mice have no bone collar (St-Jacques *et al.,* 1999), whereas overexpression of Ihh induces bone collar formation (Vortkamp *et al.,* 1996). Follow-up observations made in growth plates from genetically altered mice have identified *Ihh* expression by prehypertrophic chondrocytes as the critical determinant in the site of bone collar formation and in the induction of mature osteoblasts in the adjacent perichondrium (Chung Ui *et al.,* 2001). The presence of mature osteoblasts in membranous bones of *Ihh* mutants suggests that the bone collar, which is often referred to as being similar to intramembranous ossification, is in fact developmentally distinct.

Vascular Invasion of the Growth Plate

Ossification begins by the invasion of calcified hypertrophic cartilage. If ossification is to occur successfully, vascular invasion of the growth plate must take place. This process presents a challenge to the system because cartilage, a tissue highly resistant to vascularization, is replaced by bone, one of the most vascular tissues in the body. As such, this process would require the coordination of expression of factors that promote neovascularization and/or removal of factors that inhibit it, along with the proteolysis of the cartilage extracellular matrix that allows for vascular invasion to take place. In support of this concept is the observation that avascular cartilage expresses potent angiogenic inhibitors such as chondromodulin I (Hiraki *et al.,* 1997), whereas a number of factors that promote neovascularization are being produced by hypertrophic chondrocytes.

Matrix metalloproteinases (MMPs), a family of extracellular matrix-degrading enzymes, have been implicated in this process (for review, see Vu and Werb, 2000). MMPs are produced as latent proenzymes and can be inhibited by specific tissue inhibitors of metalloproteinases (TIMPs). Gene-targeting studies have implicated two particular MMPs in bone development: MMP9/gelatinase B (MMP9) and MT1-MMP (MMP14). MMP9 is highly expressed in multinucleated osteoclasts localized along the mineralized longitudinal septae and chondroclasts at the nonmineralized transverse septae of the cartilage–bone junction that lead the vascular invasion front. Endothelial cells, however, which are also abundant at the invasion front of the growth plate, do not express MMP9. Targeted disruption of *Mmp9* in mice leads to the development of abnormal growth plates in the long bones characterized by a nearly doubling in the length of the hypertrophic zone at birth with no changes noted in the reserve or proliferating zones (Vu *et al.,* 1998). By 3 weeks of age, the zone has enlarged to six to eight times the normal length. Because these cells appear normal and the matrix calcifies normally, alterations in the hypertrophic zone are attributed to a delay in the apoptosis of hypertrophic chondrocytes coupled with an impediment in vascular invasion. Because *Mmp9*-null hypertrophic cartilage exhibits a net decrease in angiogenic activity, the model for MMP9 action at the growth plate is attributed to the release of angiogenic factors sequestered in the extracellular matrix.

A variety of angiogenic factors are expressed in the growth plate, including members of the FGF family, IGF1, EGF, PDGF-A, members of the TGF family, Cyr61, and transferrin. However, the importance of these factors in growth plate angiogenesis is still uncertain. Vascular endothelial growth factor (VEGF) is one angiogenic protein that is expressed in hypertrophic chondrocytes and binds to extracellular matrix. When made bioavailable, VEGF binds to its respective tyrosine kinase receptors, Flt-1 (VEGFR1) and Flk-1/KDR (VEGFR2), both of which are expressed on endothelial cells (Ferrara and Davis-Smyth, 1997). Strong experimental evidence now links receptor activation to VEGF-induced mitogenesis, angiogenesis, and endothelial cell survival (Fong *et al.,* 1995; Gerber *et al.,* 1998; Shalaby *et al.,* 1995). Blockade of VEGF action through the systemic administration of a soluble receptor chimeric protein (Flt-(1–3)-IgG) recapitulates the phenotype of the *Mmp9*-null bones by impairing invasion of the growth plate (Gerber *et al.,* 1999). It appears that MMP9 releases VEGF from the extracellular matrix (Bergers *et al.,* 2000), which in turn recruits endothelial cells and thus induces and maintains blood vessels. These blood vessels bring in not only nutrients but also chondroclasts, osteoclasts, and osteoblasts, as well as a proapoptotic signal(s) (Engsig *et al.,* 2000). VEGF-mediated blood vessel invasion is therefore essential for coupling resorption of cartilage with bone formation. In the absence of blood vessel invasion, hypertrophic chondrocytes fail to undergo cell death, resulting in thickening of the growth plate. Therefore, the vasculature conveys the essential signals required for correct growth plate morphogenesis.

Vascular Invasion of the Epiphysis

MT1-MMP (MMP14) is a membrane-bound matrix metalloproteinase capable of mediating the pericellular proteolysis of extracellar matrix components. Its role in skeletal development was also recognized following its targeted inactivation (Holmbeck *et al.,* 1999; Zhou *et al.,* 2000). In contrast to *Mmp9* mice, these animals display craniofacial

dysmorphism and dwarfism, the former arising likely from impaired intramembranous bone formation while the latter reflects defects in endochondral ossification of the epiphyseal (secondary) centers of ossification. In the epiphysis, hypertrophic cartilage is formed in the center, as chondrocyte maturation progresses inward. The formation of vascular canals involves the degradation of uncalcified cartilage to clear a path for invading vessels that bring in osteogenic precursor cells for the ensuing ossification process (Fig. 6F). In *Mmp14*-null mice, invasion of the uncalcified epiphyseal hyaline cartilage by vascular canals, which represents a critical early step in the development of the secondary centers of ossification, fails to occur, leading to a delay in ossification. For reasons that are not exactly clear, this delay has profound consequences on the growth of the epiphyseal plate, including thinning, disorganization, and lack of chondrocyte proliferation. It is speculated that the delay of epiphyseal vascularization results in a shortage of chondrocyte precursors and subsequent growth plate atrophy. In addition to skeletal deformities, these mice display severe osteopenia, arthritis, and generalized soft tissue abnormalities, all of which are speculated to arise from loss of its collagenolytic activity or conceivably from loss of its activity on growth factors that influence the biology of resident cells in these tissues.

Summary

In each phase of skeletal development, it is the appropriate interplay of a number of gene products that will determine the final phenotypic outcome. This chapter, reviewed the developmental biology of the skeleton, the complex array of signals that influence each developmental stage, and finally have touched upon a number of inherited disorders of the skeleton arising from mutant gene products that influence primarily, although not exclusively, one of these specific phases. Knowledge of how specific gene defects contribute to bone pathophysiology will offer insight into the molecular etiology of inherited and metabolic skeletal disorders and will guide further efforts in their treatment.

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CHAPTER 4

Mesenchymal Stem Cells and Osteoblast Differentiation

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Introduction

Despite the known close physiological interactions of the two main cellular systems in bone, there are effectively separate and distinct origins of osteoclasts (hemopoietic cell origin) and stromal/osteoblast lineages from the developing fetus onward in mammalian development (Waller *et al.,* 1995). We have little detail of the early phenotypic stages of the osteogenic cells, the basic mechanisms governing the stem cell cycle, or the activation mechanisms relating to their physiological recruitment. We do know, however, that they are present at all bone surfaces. The old suggestion that osteoblast, and related, stem cells circulate systemically has been reincarnated recently with negligible evidence, however, for normal physiological relevance in bone anabolic processes.

There is still no general consensus of nomenclature for the stem cells that give rise to a number of tissues, including bone and cartilage. Such stem cells have been termed connective tissue stem cells, stromal stem cells, stromal fibroblastic stem cells, and mesenchymal stem cells. Current terminology of the stem cell giving rise to the osteoblast and related cell lineages (Fig. 1) appears to have favored the use of "mesenchymal stem cell" (MSC) introduced by Caplan (1991). Just as the term "stromal stem cell" coined earlier (Owen, 1985), MSC may be considered to be an ambiguous term and not a strictly correct usage. Generally, names for stem cells include the resultant end cells of the lineage they spawn (e.g., hemopoietic stem cells, HSCs) and not their embryonic tissue origin. Embryologically, components of the mesenchyme give rise not only to bone but also to the blood and other cells. Considering the functionally separate origins of the hemopoietic and stromal cell types in physiologically relevant systems, from an early distinct point in fetal development (Waller *et al.,* 1995), the term MSC conveys an inaccurate situation, particularly postnatally. Furthermore, cells termed MSC do not give rise to all components of the mesenchyme, as the name "mesenchymal stem cell" suggests, but they are derived developmentally from the mesenchyme. A more accurate definition for such cells would be mesenchyme-derived stem cells (MDSC), but it seems likely given its expanding usage that MSC, with its indication of cellular origins rather than developmental potential, will probably continue to be used extensively. Thus, we will use MSC in the context of MDSC, even though we believe that a more appropriate alternative name would be preferable. Within this system there may be a hierarchy of stem cells (Aubin, 1998), as there is in the hemopoietic system, of which the ones yielding bone and cartilage may be termed osteogenic cells. Their development into osteoblasts is through a sequence of cellular transitions, which have been described using morphological and molecular criteria (Aubin and Liu, 1996).

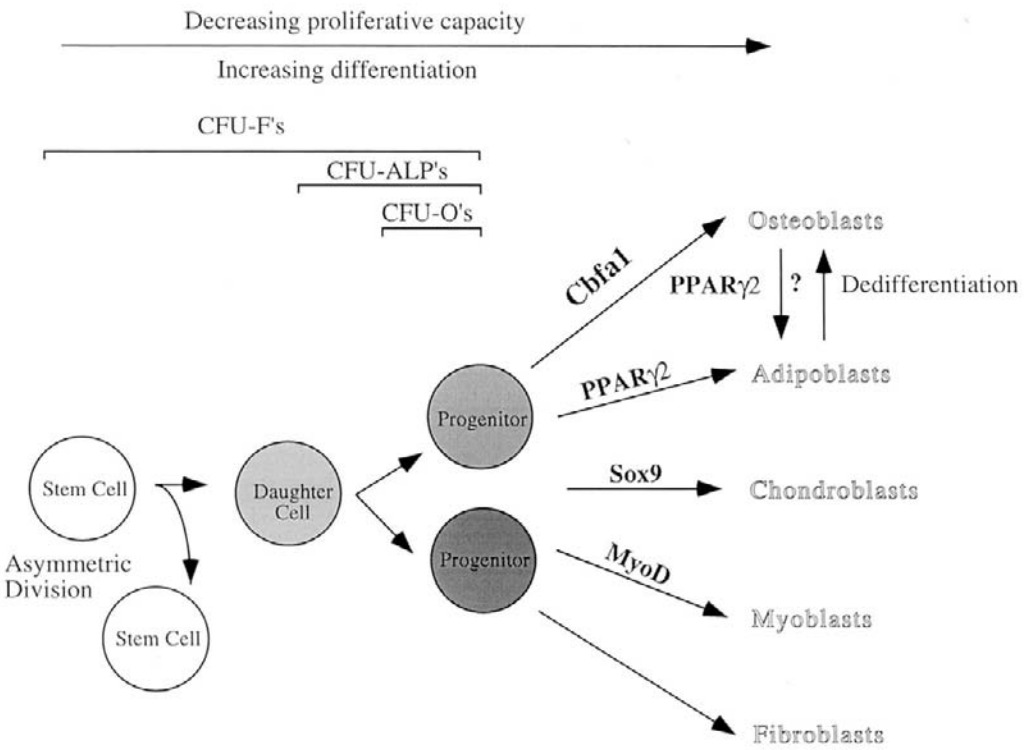


Figure 1 Schematic of stem cell commitment to various end-stage mesenchymal cell types, with known regulatory transcription factors indicated.

Ontogeny of Osteoblasts and Control of Osteoblast Development

CFU-F Assays and Osteogenic Cell Lineage Hierarchies

Friedenstein first showed that bone marrow stroma contains cells that have both significant proliferative capacity and the capacity to form bone when transplanted *in vivo* in diffusion chambers. Subsequently, he and others demonstrated that, in addition to bone, cartilage, marrow adipocytes, and fibrous tissue also formed *in vivo* and that all the tissues could arise from single colonies or CFU-F (Friedenstein, 1990; summarized in Bianco *et al.,* 1999; Owen, 1998; Prockop, 1997). *In vivo* analyses of stromal cells have been augmented by functional assays *in vitro* that show formation of a range of differentiated cell phenotypes and have led many to identify stromal populations as MSCs. However, the kinds of experiments needed to address whether marrow stroma contains a definitive stem cell—by the definition of self-renewal capacity and ability to repopulate all the appropriate differentiated lineages or even by less stringent definitions (Morrison *et al.,* 1997)—are only beginning to be done. For example, while expanded *populations* of human stromal cells are routinely now reported to express capacity to undergo differentiation along multiple mesenchymal lineages, a recent attempt to assess individual colonies showed that, among a small number (only six were reported) of individual colonies, only two appeared to express multilineage capacity and none were tested explicitly for self-renewal capacity (Pittenger *et al.,* 1999). This supports previous and more recent studies that clearly show that CFU-F are heterogeneous in size, morphology, and potential for differentiation (Friedenstein, 1990; Kuznetsov *et al.,* 1997b), consistent with the view that they belong to a lineage hierarchy in which only some of the cells are multipotential stem or primitive progenitors whereas others are more restricted (Aubin, 1998). This is also consistent with studies that show, by limiting dilution or by very low density plating, that only a proportion of CFU-F are CFU alkaline phosphatase (CFU-ALP) and further that only a proportion of these are CFU osteogenic (CFU-O, clonogenic bone colonies or bone nodules) with some variation reported between different species; CFU adipocytic (CFU-A) also comprise a subset of CFU-Fs (Aubin, 1999; Wu *et al.,* 2000) (see later) (Fig. 1). What would help advance the field are assays comparable to those achievable for hemopoietic stem cells, long-term culture-initiating cells (LTC-IC), and HSC/LTC-IC capable of long-term repopulating ability detected by their ability to serially repopulate lethally irradiated mice at limiting dilution (reviewed in Eaves *et al.,* 1999). While such assays may be difficult to achieve for MSCs, especially *in vivo*, clear quantitation of and understanding of the clonality of mesenchymal cell progeny, the ratios of stem to other more restricted progenitors in various stromal populations, the identifiable commitment and restriction points in the stromal cell hierarchy, the self-renewal capacity, and the repopulation capacity of individual precursor cells should be goals. Attempts to combine retrospective assays for specific prog-

enitor cell types with quantitative approaches *in vitro* and *in vivo* [e.g., gene marking, reviewed in Prockop (1997), limiting dilution (Aubin, 1999), and single cell-sorting experiments (Waller *et al.,* 1995)] are beginning to aid in the determination of the frequency and biological properties of various mesenchymal precursor cell populations and concepts where HSC and MSC biology may overlap and diverge. These issues may become increasingly important as work on stromal populations increases based on their proposed utility for tissue regeneration and as vehicles for gene therapy (see later).

Differentiation analyses of clonally derived immortalized (e.g., spontaneously or via large T antigen expression) cell lines derived from stroma, bone-derived cells, or other mesenchymal/mesodermal tissues, such as the mouse embryonic fibroblast line C3H10T1/2, the fetal rat calvaria-derived cell lines RCJ3.1 and ROB-C26, and the mesodermally derived C1 line, have also provided evidence for the existence of multipotential mesenchymal progenitor or stem cells capable of giving rise to multiple differentiated cell phenotypes, including osteoblasts, chondroblasts, myoblasts, and adipocytes (Aubin and Liu, 1996). Studies on these cell lines have led to suggestions of two different kinds of events underlying MSC commitment: a stochastic process with an expanding hierarchy of increasingly restricted progeny [e.g., RCJ3.1 (Aubin, 1998); see also that recent stromal cell clonal analysis (Pittenger *et al.,* 1999) would fit this model] and a nonrandom, single step process in which multipotential progenitors become exclusively restricted to a single lineage by particular culture conditions [an (environment of soluble inducers, substrate, and/or cell density) (e.g., C1 (Poliard *et al.,* 1995)] have been proposed to underlie mesenchymal stem cell restriction (see discussion in Aubin, 1998). These models may be different end points on a single continuum, as particular culture restraints or environmental or local conditions *in vivo* may shift the frequency or probability of what might otherwise be random or stochastic commitment/restriction events to favor particular outcomes.

Committed osteoprogenitors, i.e., progenitor cells restricted to osteoblast development and bone formation, at least under standard conditions, can also be identified by functional assays of their differentiation capacity *in vitro* or, as so-called earlier, the CFU-O assay in not only stromal cell populations but also populations derived from calvaria and other bones. However, under some conditions and from some tissues, mixed colony types can also be seen. A number of studies on human bone-derived cells, both populations derived from human trabecular bone and clonally derived lines of human bone marrow stromal cells, have supported the observations on rodent marrow stromal populations that a bipotential adipocyte–osteoblast precursor cell exists (reviewed in Aubin and Heersche, 1997; Nuttall and Gimble, 2000). It has also been suggested that the inverse relationship sometimes seen between expression of the osteoblast and adipocytic phenotypes in marrow stroma (e.g., in osteoporosis or in some culture manipulations) may reflect the ability of single or combinations of agents to alter the commitment or at least the differentiation pathway these bipotential cells will transit. In many cases, individual colonies are seen in which both osteoblast and adipocyte markers are present simultaneously. However, whether a clearly distinguishable bipotential adipo-osteoprogenitor can be identified or other developmental paradigms, such as transdifferentiation, underlie expression in these two lineages needs to be analyzed further. For example, dedifferentiation has been proposed to account for observations in some cultures of marrow stroma in which highly differentiated adipocytes are thought to revert to a less differentiated, more proliferative fibroblastic precursor phenotype and then to osteogenic phenotype (Park *et al.,* 1999). However, osteoblasts, differentiated to the point of already expressing osteocalcin (OCN), are able to undergo rapid differentiation events that lead to essentially 100% of the formerly osteoblastic cells expressing adipogenesis when they are transfected with the nuclear receptor family member peroxisome proliferator-activated receptor $\gamma 2$ or PPAR$\gamma 2$ (Nuttall *et al.,* 1998) (Fig. 1). Thus, although OCN is a very late marker of osteoblast maturation, data are consistent with osteoblastic cells being able to transdifferentiate into an adipogenic phenotype, an outcome of significant clinical interest in osteoporosis and the aging or immobilized skeleton (summarized in, Aubin and Heersche, 1997; Nuttall and Gimble, 2000).

A variety of observations have suggested that a bipotential osteochondroprogenitor may also exist. However, as raised earlier for adipocyte–osteoblast phenotypes, the ability to transdifferentiate or change expression profiles may also characterize osteoblast–chondroblast lineages (summarized in Aubin, 1998). Such observations and their cellular and molecular basis have led to considerable interest in the concept of "plasticity" of stromal and other cell types (Bianco and Cossu, 1999; Perry and Rudnick, 2000; Williams and Klinken, 1999). The possible presence of undifferentiated/uncommitted stem cells and multi- and bipotential progenitors in cultures that may also contain monopotential but plastic progenitors at higher frequencies often complicates the ability to unambiguously discriminate the nature of the cells being affected. Difficulties in establishing unambiguous evidence for multipotentiality, together with certain discrepancies between results in calvaria versus stromal and other populations, underscore the need for more markers and experiments to distinguish the molecular mechanisms underlying the ability of cells to express multipotentiality, the number and nature of commitment steps to a restricted phenotype(s), and both physiological and pathological mechanisms that may govern plasticity.

Control of Osteoblast Development

Significant strides have been made in identifying the regulatory mechanisms underlying lineage restriction, commitment, and/or differentiation within some of the mesenchymal lineages (Fig. 1). Master genes, exemplified by

the MyoD, myogenin, and Myf-5 helix-loop-helix transcription factors in muscle lineages, are one paradigm in which one transcription factor is induced and starts a cascade that leads to the sequential expression of other transcription factors and of phenotype-specific genes (Perry and Rudnick, 2000). A factor of a different transcription factor family, PPARγ2 mentioned earlier, together with other transcription factors, including the CCAAT/enhancer-binding (C/EBP) protein family, plays a key role in adipocyte differentiation (Rosen and Spiegelman, 2000). Sox9, a member of yet another transcription factor family, is essential for chondrocyte differentiation, expression of various chondrocyte genes, and cartilage formation (de Crombrugghe *et al.,* 2000).

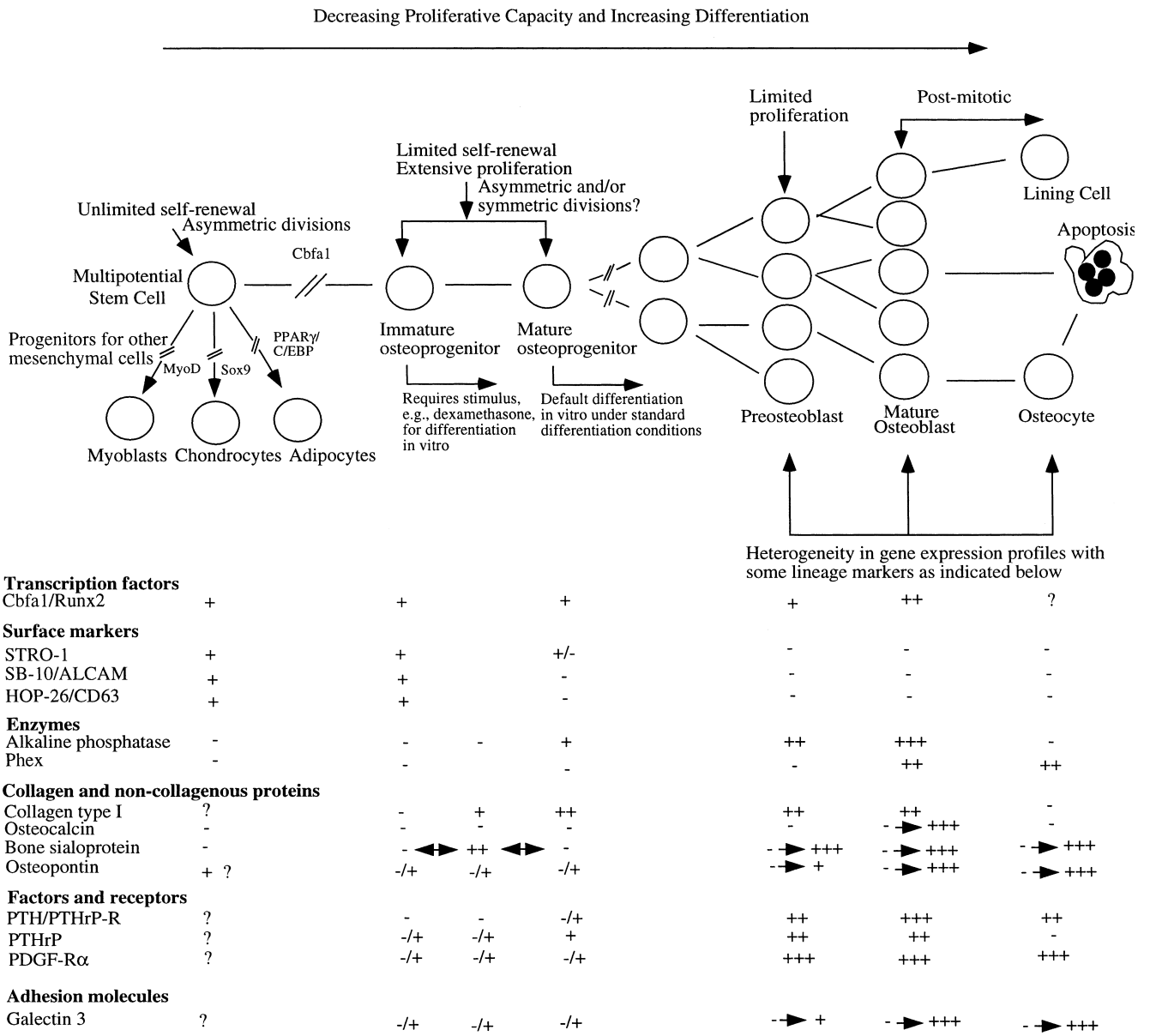
With respect to osteoblasts, Cbfa1 (recently renamed Runx2), a member of the runt homology domain transcription factor family, plays a crucial role in osteoblast development. Cbfa1/Runx2 was identified in part based on its ability to regulate OCN (Banerjee *et al.,* 1997; Ducy *et al.,* 1997), the ectopic expression of Cbfa1 in nonosteoblastic cells leads to the expression of osteoblast-specific genes, including OCN, and, strikingly, deletion of Cbfa1 in mice leads to animals in which the skeleton comprises only chondrocytes and cartilage without any evidence of bone (Komori *et al.,* 1997) and in the amount of bone formed postnatally (Ducy *et al.,* 1999). Haploinsufficiency in mice (Komori *et al.,* 1997) and humans leads to the cleidocranial dysplasia phenotype (Mundlos *et al.,* 1997; Otto *et al.,* 1997; reviewed in Ducy, 2000). Cbfa1/Runx2 is the earliest of osteoblast differentiation markers currently known, its expression during development and after birth is high in osteoblasts, and it is upregulated in cultures treated with bone morphogenetic proteins (BMPs) and other factors that stimulate bone formation (reviewed in Yamaguchi *et al.,* 2000). However, in contrast to MyoD and PPARγ2, Cbfa1 is not a master gene; it is necessary but not sufficient to support differentiation to the mature osteoblast phenotype (Lee *et al.,* 1999; Wang *et al.,* 1999). Interestingly, studies have suggested that at least some hypertrophic chondrocytes express Cbfa1 and that the development/maturation of at least some chondrocytes is aberrant in Cbfa1-deficient mice, although the skeletal site-specific nature of the defects suggests that much more information is needed (reviewed in Ducy, 2000; Komori, 2000). With respect to the issue of plasticity, it is interesting that PPARγ2, which, as discussed earlier, is able to transdifferentiate osteoblastic cells to adipocytes, may do so via its ability to downregulate Cbfa1 (Lecka-Czernik *et al.,* 1999).

Ablation of Indian hedgehog (Ihh), a member of the Hedgehog family of secreted growth factors, leads to mice with a disorganized growth plate, as expected based on data that Ihh regulates chondrocyte differentiation. However, Ihh-null mice also have no osteoblasts in bone formed by endochondral ossification (St-Jacques *et al.,* 1999). Because the aberrant chondrocyte differentiation phenotype can be mimicked by hedgehog interacting protein (HIP) overexpression in transgenic mice that have osteoblasts (Chuang and McMahon, 1999), data suggest that the failure of osteoblast development in endochondral sites is due specifically to lack of Ihh rather than a secondary effect due to aberrant chondrocytes. Interestingly, Cbfa1 expression, which is normally high in osteoblasts in endochondral bones, is absent in Ihh -/- mice. Also, consistent with the fact that Ihh is not normally expressed in intramembranous bones, osteoblast differentiation occurs normally in these bones in Ihh -/- animals. These data support the concept that Ihh may be a regulator of Cbfa1 and osteoblast development, but in a skeletal site-specific manner.

Elucidation of the osteoprotegerin–RANK–RANKL pathway underlying stromal cell–hemopoietic cell interactions regulating osteoclast formation and activity (see elsewhere in this volume) also makes it tempting to speculate that a reciprocal or related pathway may regulate osteoblast formation and activity. The fact that hemopoietic cells influence CFU-O/osteoblast development in the bone marrow stromal cell model *in vitro* (Aubin, 1999) (see also later) lends some support for this hypothesis. Also, the fact that other apparently osteoblast-specific *cis*-acting elements have been found in several genes suggests that there are other important osteoblast-associated or -specific transcription factors to be elucidated. We will address further a variety of other transcription factors and receptor signaling pathways that clearly also influence the rate and amount of bone formed.

Stem Cell Immunophenotyping

In hemopoiesis and immune cell biology, the importance of large panels of stem and progenitor cell antibodies has been invaluable (Herzenberg and De Rosa, 2000). In oncology, it is realized that the characterization of reliable stem cell markers should be an immediate aim (Bach *et al.,* 2000). This has not received as much attention in the MSC system, but some attempts have given us a few monoclonal antibody markers to evaluate, although many recognize the more mature cells in the lineage (Aubin and Turksen, 1996). In addition, however, not enough emphasis has been placed on the rigorous definition of reactivities of MSCs and other related primitive osteogenic cells with the many other existing monoclonal antibody marker molecules developed for use in other cell systems, although some characteristics are now available. With respect to the development of specific markers, it must be realized that no such moiety can be considered to identify solely or unambiguously a cell stage or lineage. This is particularly evident for markers developed by the screening of tissues with normal physiology, as in pathological states, or in *in vitro* culture, the probability for alternative gene activation and expression of other cell phenotypic characteristics is high. This further emphasizes the need, known for many years, for confirmation of all the potentials for cell development and activity seen in culture with normal *in vivo* physiological responses.



Transcription factors						
Cbfa1/Runx2	+	+	+	+	++	?
Surface markers						
STRO-1	+	+	+/-	-	-	-
SB-10/ALCAM	+	+	-	-	-	-
HOP-26/CD63	+	+	-	-	-	-
Enzymes						
Alkaline phosphatase	-	-	+	++	+++	-
Phex	-	-	-	-	++	++
Collagen and non-collagenous proteins						
Collagen type I	?	-	++	++	++	-
Osteocalcin	-	-	-	-	- → +++	-
Bone sialoprotein	-	- ↔ ++ ↔	-	- → +++	- → +++	- → +++
Osteopontin	+ ?	-/+	-/+	→ +	- → +++	- → +++
Factors and receptors						
PTH/PTHrP-R	?	-	-/+	++	+++	++
PTHrP	?	-/+	+	++	++	-
PDGF-Rα	?	-/+	-/+	+++	+++	+++
Adhesion molecules						
Galectin 3	?	-/+	-/+	→ +	- → +++	- → +++

Figure 2 Postulated steps in the osteoblast lineage implying recognizable stages of proliferation and differentiation as detectable from *in vitro* and *in vivo* experiments. Superimposed on this scheme are several well-established markers of osteoblastic cells with an indication of when during the differentiation sequence they are expressed, but also denoting heterogeneous expression of many of the markers. The list is not exhaustive, but does show some important categories of molecules in the lineage and their utility to help define transitions in osteoblast differentiation. It should be recognized that little is known about the abruptness of turn on or turn off of these markers, in many cases, expression levels may vary as changes to a continuum. −, no detectable expression; −/+–+++, expression ranging from detectable to very high, → +++, heterogeneous expression in individual cells.

A few interesting monoclonal antibodies that react with surface antigens on human MSCs *in vitro* have been generated by a number of research groups. These include the antibodies STR0-1 (Simmons and Torok-Storb, 1991), SH-2, SH-3, SH-4, SB10 (Bruder *et al.,* 1997, 1998), and HOP-26 (Joyner *et al.,* 1997) (Fig. 2). None of them is absolutely lineage and cell stage specific, as may be expected but sometimes receives too little attention. One of the first antibodies that identified the CFU-F in adult human bone marrow was STRO-1 (Simmons and Torok-Storb, 1991). The cell surface antigen recognized by this antibody is still unknown but its expression is restricted to a minor subpopulation of cells in fresh human bone marrow, including the CFU-F. The STRO-1^+ fraction of adult human bone marrow has been shown to contain the osteogenic precursors (Gronthos *et al.,* 1994).

Bruder and colleagues (1997) raised a series of monoclonal antibodies by immunizing mice with human MSCs that had been directed into the osteogenic lineage *in vitro*. Three hybridoma cell lines referred to as SB-10, SB-20, and SB-21 were isolated by screening against osteogenic cells *in vitro* and human fetal limbs. SB-10 was shown to react with marrow stromal cells and osteoprogenitors, but not with more differentiated cells, i.e., those already

expressing alkaline phosphatase (ALP) (see later). By flow cytometry, culture-expanded human MSCs were all found to express SB-10, and these cells appear homogeneous in this respect. In contrast, SB-20 and SB-21 do not react with the progenitor cells *in vivo*, but bind to a subset of ALP-positive osteoblasts. None of these antibodies stains terminally differentiated osteocytes in sections of developing bone. SB-10 has been identified as an activated leukocyte cell adhesion molecule (ALCAM) (Bruder *et al.,* 1998), and orthologues of ALCAM with 90% identity in peptide sequences have been found in rat, rabbit, and canine MSCs. Clearly, ALCAM is not restricted to MSCs or osteoprogenitor cells: ALCAM is an immunoglobulin (Ig) superfamily ligand for the CD6 antigen (Bowen *et al.,* 2000), which is present in lymphoid tissue and may be involved in the homing of hemopoietic cells (Bowen and Aruffo, 1999). Nevertheless, it may be useful combined with other reagents for cell subfractionation and it is worth considering its role in osteoblast development in more detail, as treating human MSCs *in vitro* with SB-10 accelerated osteogenic differentiation, implicating ALCAM as a regulator of this process (Bruder, *et al.,* 1998).

The SH-2 antibody is reported to immunoprecipitate endoglin (CD105), which is the transforming growth factor-β (TGFβ) receptor III present on connective tissue stromal cells, endothelial cells, syncytiotrophoblasts, and macrophages (Barry *et al.,* 1999). This molecule is potentially involved in TGFβ signaling and control of chondrogenic differentiation of MSCs and in interactions between these cells and hematopoietic cells in the bone marrow, as well as in dermal embryogenesis and angiogenesis (Fleming *et al.,* 1998).

The HOP-26 antibody raised against human bone marrow fibroblasts at an early stage of cell culture has been shown to react with cells close to newly forming bone in the periosteum and in trabeculae of the developing human fetal limb (Joyner *et al.,* 1997). In adult trabecular bone, HOP-26 reactivity is much diminished, and a minor proportion of cells within the bone marrow spaces show reactivity. No similar relatively high levels of activity are seen in a variety of soft tissues by immunocytochemistry. By immunopanning, cells with the highest levels of expression of the HOP-26 epitope were shown to be the majority of the CFU-F, and HOP-26 is thus a useful antibody for selecting these cells to enrich osteoprogenitor populations from marrow (Oreffo, 2001). In addition, the reactivity of this antibody with histological specimens fixed routinely with formaldehyde also indicates its value for histopathology investigation. Further studies with histopathological specimens indicate that high HOP-26 expression is also seen in certain populations of mast cells in samples from mastocytoma and in Paget's disease (Joyner *et al.,* 2000).

HOP-26 (Zannettino *et al.,* 2001) was shown by expression cloning to recognize the cell surface and lysosomal enzyme CD63, identical to the melanoma-associated antigen ME491, associated with early melanoma tumour progression (Metzelaar, 1991). The latter is a member of the superfamily of tetraspan glycoproteins (TM4SF), which also includes CD9, CD37, CD53, and CD151. CD63 is expressed on activated platelets and endothelium and is a lysosomal membrane glycoprotein translocated to the cell surface following activation (Vischer and Wagner, 1993). It is also present on monocytes, macrophages, and, at lower levels, on granulocytes and B and T lymphocytes. The initial report on the distribution of HOP-26 used mainly fixed tissues or paraffin-embedded tissues, but other studies with freshly isolated or viable, cultured cells have shown that HOP-26 identifies a range of cell types in preparations of fresh marrow. Histological fixation appears to render the epitope unreactive with the HOP-26 antibody in most cells, except osteoprogenitor cells, perhaps because of the higher level of expression of the epitope in the latter cell types. Studies with blood cells have shown inhibitory effects of CD63 antibodies on cell adhesion, and CD63 has also been shown to interact specifically with $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins in a variety of cell types and therefore may be important in cell signaling pathways (Schwartz and Shattil, 2000). Osteoblastic cells also express many of these integrin heterodimers (reviewed in, Damsky, 1999). Compared with another CD63 antibody, 12F12 (Zannettino *et al.,* 1996), there are subtle differences in reactivities of cells with HOP-26, which may be related to glycosylation, but the preservation of this epitope on osteoprogenitors following fixation, unlike those reacting with other CD63 antibodies, suggests that HOP-26 is a valuable reagent for further studies on bone metabolism.

Combinations of antibodies and flow cytometry procedures have been used to subfractionate marrow stromal cells. Most will react with the CFU-F, which include the progenitors with most stem cell-like characteristics, found by Friedenstein (1990) to constitute about 15% of total colonies. Such methods are likely to be valuable for critical studies on cell developmental potential, but any advantage in superselection of primitive progenitors for practical use in cell therapy regimens must be considered carefully. For example, any specificity gained by selection must be offset against the lower manipulation of cell populations when total CFU-F cell progeny are used and the likelihood that the most primitive cells will mainly contribute eventually to the expanded cell population that develops.

Gronthos *et al*. (2001) used STRO-1 and an antibody directed against vascular cell adhesion molecule 1 (VCAM-1/CD106) with magnetic-activated cell sorting followed by fluorescence-activated cell sorting to isolate a highly enriched population of human marrow stromal precursor cells. These cells were homogeneous in phenotype, being STRO-1 bright and VCAM-1-positive, and were large COLL-I-positive cells lacking the phenotypic characteristics of leukocytes or vascular endothelial cells. Selected cells in the STRO-1 bright fraction were noncycling *in vivo* and with expression of constitutive telomerase activity *in vitro.* Data demonstrate that these cells have stem cell characteristics, as defined by extensive proliferative capacity and retention of differentiation capacity for osteogenesis and adipogenesis.

Expression of the bone/liver/kidney isoform of ALP has been used in a number of different studies with and without combination with other antibodies (Aubin and Turksen, 1996) (Fig. 2). Expression of ALP has been studied in relation to STRO-1 with dual-color fluorescence-activated cell sorting in osteogenic cells (Gronthos *et al.,* 1999). Human trabecular bone-derived cells expressing STRO-1 antigen exclusively with no ALP appeared to be early osteoblast precursors with absence of bone-related proteins bone sialoprotein (BSP), osteopontin (OPN), and parathyroid hormone/parathyroid hormone-related protein receptor (PTH1R) by RT-PCR analysis (see also later). The STRO-1-/ALP+ and STRO-1-/ALP- cell phenotypes were considered to represent differentiated osteoblasts, whereas the STRO-1+/ALP+ subset was an intermediate developmental stage. All STRO-1/ALP subpopulations expressed multiple isoforms of Cbfa1, suggesting the presence of cells committed to osteogenesis. With reculture of the four different STRO-1/ALP-selected subpopulations, only the STRO-1+/ALP- subpopulation yielded all of the four subsets with the same proportions of STRO-1/ALP expression as seen in the original primary cultures. Similar studies were performed by others using human marrow fibroblastic populations (Stewart *et al.,* 1999) in which an inverse association was found between the expression of STRO-1 and ALP. Both studies suggest that these antibody combinations permit the identification of cells of the osteoblast lineage at different stages of differentiation and support previous studies suggesting a hierarchy of marker expression during osteoblastic development *in vitro* (reviewed in Aubin and Liu, 1996) (see also later).

Other combinations of antibodies and cell surface characteristics have also been used (for earlier work, see Aubin and Turksen, 1996). For example, osteogenic cells were sorted from mouse bone marrow based on light scatter characteristics, Sca-1 expression, and their binding to wheat germ agglutinin (WGA) (Van Vlasselaer *et al.,* 1994). Cells from the Sca-1 + WGA(bright) gate, but not from other gates, synthesized bone proteins and formed a mineralized matrix, but lost this capacity when subcultured. Further immunophenotypic characterization showed that FSC(high)SSC(high)Lin-Sca-1 + WGA(bright) cells expressed stromal (KM 16) markers and endothelial (Sab-1 and Sab-2) markers, but not hemopoietic cell markers, such as c-kit and Thy1.2. Sorted FSC(high)SSC(high)Lin-Sca-1+WGA(bright) cells formed bone nodules. Somewhat in contradiction to the absence of OPN expression in the STRO-1+/ALP- population, just defined as comprising stem cells, OPN expression combined with cell size and granularity was used to sort rat calvaria and bone marrow stromal cells to attempt to enrich for cells responsive to BMP-7; these were said to have stem-like properties (Zohar *et al.,* 1997, 1998).

Better understanding of the surface chemistry profiles and their temporal changes with the development of osteogenic cells would advance the field. Further, while all the studies summarized have clearly fractionated populations into subpopulations with different characteristics and differentiation potentials, few attempts have yet been made to quantify the most primitive progenitors or stem cells in these populations and their self-renewal, proliferative, and differentiation potentialities at single cell or colony levels so that clear lineage relationships and hierarchies can be established analogous to those that have been achieved in hemopoietic populations (see, however, Pittenger *et al.,* 1999) (see later). In addition, more of the existing antibodies produced against other cell types need to be used and new ones produced that have specificity to restricted developmental stages during osteogenic differentiation.

Osteoprogenitor Cells and Regulation of Osteoblast Differentiation and Activity

Osteoprogenitor Cells

The morphological and histological criteria by which osteoblastic cells, including osteoprogenitors, preosteoblasts, osteoblasts, and lining cells or osteocytes, are identified have been reviewed extensively and will not be reiterated in detail here (Aubin, 1998). Morphological definitions are now routinely supplemented by the analysis of expression of cell- and tissue-specific macromolecules, including bone matrix molecules [type I collagen (COLL-I), OCN, OPN, and BSP, among others] and transcription factors that regulate them and commitment/differentiation events (e.g., Cbfa1, AP-1 family members, Msx-2, Dlx-5) (see also later). Committed osteoprogenitors, i.e., progenitor cells restricted to osteoblast development and bone formation under default differentiation conditions, can be identified in bone marrow stromal cell populations and populations derived from calvaria and other bones by functional assays of their proliferation and differentiation capacity *in vitro* or, as often designated, the CFU-O assay. As mentioned earlier, CFU-O appear to comprise a subset of CFU-F and CFU-ALP (Aubin, 1999; Herbertson and Aubin, 1995, 1997; Wu *et al.,* 2000). Cells morphologically essentially identical to cells described *in vivo* and subject to many of the same regulatory activities can be identified, and the deposited matrix contains the major bone matrix proteins.

Much has been learned from the *in vitro* bone nodule assay in which both the nature of the osteoprogenitors and their more differentiated progeny have been investigated by functional (the nature of the colonies they form, e.g., mineralized bone nodules), immunological (e.g., immunocytochemistry, Western analysis), and molecular (e.g., Northerns, PCR of various sorts, *in situ* hybridization) assays. CFU-O/bone nodules represent the end product of the proliferation and differentiation of osteoprogenitor cells present in the starting cell population. Estimates by limiting dilution have indicated that these osteoprogenitor cells are relatively rare in cell populations digested from fetal rat calvaria (i.e., $<1\%$) (Bellows and Aubin, 1989) and rat (Aubin, 1999) and mouse (Falla *et al.,* 1993) bone marrow stroma (i.e.,

$0.5 - 1 \times 10^{-5}$ of the nucleated cells of unfractionated marrow or <1% of the stromal layer) under standard isolation and culture conditions. The number of nodules or colonies forming bone can be counted for an assessment of osteoprogenitor numbers recoverable from fetal calvaria or other bones [e.g., vertebrae (Ishida *et al.,* 1997; Lomri *et al.,* 1988) or the primary spongiosa of femur metaphysis (Onyia *et al.,* 1997)] and bone marrow stroma (reviewed in Owen, 1998) under particular assay conditions. However, evidence from rat calvaria cell bone nodule assays suggests the existence of at least two populations of osteoprogenitors. One population appears capable of constitutive or default differentiation *in vitro*, i.e., in standard differentiation conditions (ascorbic acid, β-glycerophosphate, fetal calf serum) differentiation leading to the mature osteoblast phenotype appears to be a default pathway, whereas the other population, apparently more primitive based on cell sorting and immunopanning with ALP antibodies (Turksen and Aubin, 1991), undergoes osteoblastic differentiation only following the addition of specific inductive stimuli (Fig. 2). Thus the addition of glucocorticoids [often dexamethasone but natural corticosteroids have also been used (Bellows *et al.,* 1987)], other steroids [e.g., progesterone (Ishida and Heersche, 1997)], or other kinds of factors [e.g., BMPs (Hughes *et al.,* 1995)] increases the number of bone nodules or bone colonies in calvaria-derived and bone marrow stromal cell cultures, suggesting the presence of "inducible" osteoprogenitor cell populations as well. Whether other precursor stages in addition to the multipotential or committed progenitors discussed already can also be identified by combinations of assays *in vitro* remains to be explicitly tested.

Whether all progenitors that differentiate to osteoblasts and make bone belong to the same unidirectional lineage pathway (i.e., immature progenitors induced by a variety of agents to undergo differentiation to mature osteoblasts), whether osteoprogenitor cells must transit all recognizable differentiation stages (or may skip steps under appropriate conditions) under all developmental situations, or whether recruitment from other parallel lineages and pathways can result in functional osteoblasts remains to be established (see also later). However, as discussed already, plasticity between mature cell phenotypes normally considered indicative of terminal differentiation can contribute to osteoblast pools, at least *in vitro*. It is also worth considering whether the osteoprogenitors in calvaria or other bones and bone marrow stroma or other tissues [e.g., pericytes (Bianco and Cossu, 1999; Doherty and Canfield, 1999; Proudfoot *et al.,* 1998; Shanahan *et al.,* 2000)] are the same. As discussed in more detail later, they do appear to reach *similar* end points with respect to the ability to make and mineralize a bone matrix, but may not be *identical*. Data have also indicated that, in rat stromal populations, as in rat calvaria-derived populations, there are two pools of osteoprogenitors: ones that differentiate in the absence of added glucocorticoids (assumed to be more mature) and ones that do so only in its presence (assumed to be more primitive), although the number of the former type is relatively low and so detectable only at relatively high plating cell densities and the latter comprise the majority in stromal populations (Aubin, 1999). Whether the two sorts or stages of progenitors are identical in other features to the progenitors in calvaria remains to be assessed rigorously, as must their relationship to multilineage CFU-F. It is also worth noting that, in rat stroma, unlike in rat calvaria, limiting dilution analysis indicates that more than one cell type is limiting for nodule formation *in vitro*, suggesting a cell nonautonomous aspect to differentiation of the stromal CFU-O; osteogenic differentiation is enhanced, for example, when the nonadherent fraction of bone marrow or its conditioned medium is added to the adherent stromal layer (Aubin, 1999). A role for accessory cells in the osteogenesis of human bone marrow-derived osteoprogenitors has also been shown (Eipers *et al.,* 2000). The relationship of inducible osteoprogenitors that apparently reside in the nonadherent fraction of bone marrow and are assayable under particular culture conditions, e.g., in the presence of PGE_2 [rat (Scutt and Bertram, 1995)] or as colonies in soft agar or methylcellulose [human (Long *et al.,* 1995)], also remains to be determined. Direct and unambiguous comparisons have not yet been done but should be advanced as more markers for the most primitive progenitors, including stem cells, become available.

Morphologically recognizable osteoblasts associated with bone nodules appear in long-term bone cell cultures at predictable and reproducible periods after plating. Recent time-lapse cinematography of individual progenitors forming colonies in low-density rat calvaria cultures indicated that primitive (glucocorticoid-requiring) osteoprogenitors divide ~8 times prior to overt differentiation, i.e., to achieving cuboidal morphology and matrix deposition (Aubin and Liu, 1996; Malaval *et al.,* 1999). Interestingly, however, the measurement of large numbers of individual bone colonies in low density cultures shows that the size distribution of fully formed bone colonies covers a large range but is unimodal, suggesting that the coupling between proliferation and differentiation of osteoprogenitor cells may be governed by a stochastic element, but distributed around an optimum, corresponding to the peak colony size/division potential (Malaval *et al.,* 1999). Osteoprogenitors measurable in functional bone nodule assays also appear to have a limited capacity for self-renewal in both calvaria (Bellows *et al.,* 1990a) and stromal (McCulloch *et al.,* 1991) populations, consistent with their being true committed progenitors with a finite life span (reviewed in Aubin, 1998). However, in comparison to certain other lineages, most notably hemopoietic cells, relatively little has been done to assess the regulation of self-renewal in different osteogenic populations beyond the effects of glucocorticoids. According to signaling threshold models, the differentiation of hemopoietic stem cells is suppressed when certain receptor–ligand (soluble or matrix-bound) interactions are kept above a particular threshold and is increased or more probable when levels are reduced (Eaves *et al.,* 1999); this regulation is

sometimes a proliferation–differentiation switch, but in some cases it is independent of proliferation (Ramsfjell *et al.,* 1999). Very little has been done in osteoblast lineage to assess comparable pathways, yet the differential expression of a variety of receptors for cytokines, hormones, and growth factors during osteoblast development and in different cohorts of osteoblasts predicts that similar mechanisms may play a role in bone formation (see also later).

Differentiation of Osteoprogenitor Cells to Osteoblasts

One fundamental question of osteoblast development remains how progenitors progress from a stem or primitive state to a fully functional matrix-synthesizing osteoblast. Based on bone nodule formation *in vitro*, the process has been subdivided into three stages: (i) proliferation, (ii) extracellular matrix development and maturation, and (iii) mineralization, with characteristic changes in gene expression at each stage; some apoptosis can also be seen in mature nodules. In many studies, it has been found that genes associated with proliferative stages, e.g., histones and protooncogenes such as c-fos and c-myc, characterize the first phase, whereas certain cyclins, e.g., cyclins B and E, are upregulated postproliferatively (Stein *et al.,* 1996). Expression of the most frequently assayed osteoblast-associated genes COLLI, ALP, OPN, OCN, BSP, and PTH1R is upregulated asynchronously, acquired, and/or lost as the progenitor cells differentiate and the matrix matures and mineralizes (Aubin, 1998; Stein *et al.,* 1996). In general, ALP increases and then decreases when mineralization is well progressed; OPN peaks twice during proliferation and then again later but prior to certain other matrix proteins, including BSP and OCN; BSP is expressed transiently very early and is then upregulated again in differentiated osteoblasts forming bone; and OCN appears approximately concomitantly with mineralization (summarized in Aubin, 1998) (Fig. 2). Notably, however, use of global amplification poly(A) PCR, combined with replica plating and immunolabeling, showed that all these osteoblast-associated markers are upregulated prior to the cessation of proliferation in osteoblast precursors except OCN, which is upregulated only in postproliferative osteoblasts; in other words, differentiation is well progressed before osteoblast precursors leave the proliferative cycle. Based on the simultaneous expression patterns of up to 12 markers, osteoblast differentiation can be categorized into a minimum of seven transitional stages (Aubin and Liu, 1996; Candeliere *et al.,* 1999; Liu and Aubin, 1994), not just the three stages mentioned earlier (Fig. 2). An interesting issue is whether osteoprogenitor cells in all normal circumstances must transit all stages or can "skip over" some steps under the action of particular stimuli or regulatory agents.

While many cell systems have been reported to follow the general proliferation–differentiation just outlined some discrepancies exist that are not always noted in detail. At least some of the variations may reflect inherent differences in the populations being analyzed, e.g., species differences or different mixtures of more or less primitive progenitors and more mature cells. However, there is growing evidence from both *in vitro* and *in vivo* observations that somewhat different gene expression profiles for both proliferation and differentiation and regulatory markers may underlie developmental events in different osteoblasts (Aubin *et al.,* 1999). For example, in a study of ROS cells differentiating and producing mineralizing bone matrix in diffusion chambers *in vivo*, neither proliferation nor most differentiation markers followed the pattern described previously (Onyia *et al.,* 1999). One possible explanation is that different subpopulations of cells within the chambers were undergoing different parts of the proliferation–death–matrix synthesis cycle at different times, such that activities of some subpopulations may have been obscured among larger subpopulations engaged in other activities in the chamber at the same time. At least some data supported this view, which may also account for the discrepancies in some reported observations *in vitro*. It is also possible that protein levels may not match the mRNA levels detected. Another possibility is that there are differences in proliferation–differentiation coupling and/or the nature of the matrix and process of mineralization in osteosarcoma cells versus normal diploid osteoblasts. In addition, however, it is growing clear that high levels of genes typical of some normal osteoblasts may not be required in others, i.e., that some pathways by which mineralized matrix can be formed *in vivo* or *in vitro* are different from others and that there is heterogeneity among osteoblast developmental pathways and/or the resulting osteoblasts.

The possibility that marked intercellular heterogeneity in expressed gene repertoires may characterize osteoblast development and differentiation is an important concept (Aubin *et al.,* 1999) (Fig. 2). As already discussed, Ihh is expressed in and required for the development of osteoblasts associated with endochondral bones but not other osteoblast populations (St-Jacques, *et al.,* 1999). It has been evident for some time that not all osteoblasts associated with bone nodules *in vitro* are identical (Liu *et al.,* 1994; Malaval *et al.,* 1994; Pockwinse *et al.,* 1995). Single cell analysis of the most mature cells in mineralizing bone colonies *in vitro* showed that the heterogeneity of expression of markers by cells classed as mature osteoblasts is extensive and appears not to be related to cell cycle differences (Liu *et al.,* 1997). That this extensive diversity is not a consequence or an artifact of the *in vitro* environment was confirmed by the analysis of osteoblastic cells *in vivo*. When individual osteoblasts in 21-day fetal rat calvaria were analyzed, only two markers of nine sampled, ALP and PTH1R, appeared to be "global" or "ubiquitous" markers expressed by all osteoblasts *in vivo*. Strikingly, all other markers analyzed (including OPN, BSP, OCN, PTHrP, c-fos, Msx-2, and E11) were expressed differentially at both mRNA and protein levels in only subsets of osteoblasts, depending on the maturational state of the bone, the age of the osteoblast, and on the environment

(endocranium, ectocranium) and the microenvironment (adjacent cells in particular zones) in which the osteoblasts reside (Candeliere *et al.,* 2001). The biological or physiological consequences of the observed differences are not known, but they support the notion that not all mature osteoblasts develop via the same regulatory mechanisms nor are they identical molecularly or functionally. They predict that the makeup of different parts of bones may be significantly different, as suggested previously by the observations that the presence of and amounts of extractable noncollagenous bone proteins are different in trabecular versus cortical bone and in different parts of the human skeleton (for discussion, see Aubin *et al.,* 1999; Candeliere *et al.,* 2001). They also suggest that the global or ubiquitously expressed molecules COLL-I, ALP, and PTH1R serve common and nonredundant functions in all osteoblasts and that only small variations in the expression of these molecules may be tolerable; e.g., all bones display mineralization defects in ALP knockout mice (Fedde *et al.,* 1999; Wennberg *et al.,* 2000). Differentially expressed lineage markers, however, e.g., BSP, OCN, and OPN, vary much more, both between osteoblasts in different zones and between adjacent cells in the same zone and may have specific functions associated with only some positionally or maturationally defined osteoblasts. In this regard, it is striking that all of the noncollagenous bone matrix molecules are extremely heterogeneously expressed and that ablation of many of those studied to date, e.g., OCN (Ducy *et al.,* 1996), OPN (Yoshitake *et al.,* 1999), and BSP (Aubin *et al.,* 1996b) does not result in a complete failure of osteoblast differentiation and maturation, although the amount, quality, and remodeling of the bone formed may differ from normal.

The nature of the signals leading to the diversity of osteoblast gene expression profiles is not known (for discussion, see Aubin *et al.,* 1999; Liu *et al.,* 1997); however, the fact that the heterogeneity is apparently controlled both transcriptionally and posttranscriptionally implies that regulation is complex. The observations also suggest that it will be important to analyze the expression of regulatory molecules, including more transcription factors, not only globally but at the individual osteoblast level, e.g., as mentioned earlier for the Ihh regulation of osteoblast development or, for example, for the homeobox transcription factor Dlx-5, whose levels modify bone formation *in vivo* (see later) and which appears more highly expressed in periosteal compared to endosteal osteoblasts (Acampora *et al.,* 1999). Another unanswered question remains whether the striking diversity of marker expression in different osteoblasts is nonreversible or reversible in a stochastic manner, governed by changes in a microenvironmental signal or receipt of hormonal or growth factor cues, or both. Because the heterogeneity observed extends to the expression of regulatory molecules such as cytokines and their receptors, it also suggests that autocrine and paracrine effects may be elicited on or by only a subset of cells at any one time and that the responses to such stimuli could themselves be varied (Aubin *et al.,* 1999). We also cannot rule out the possibility that the heterogeneity can be subdivided further, a scenario that seems likely as new markers for the lineage are identified.

The observed differences in mRNA and protein expression repertoires in different osteoblasts may also contribute to the heterogeneity in trabecular microarchitecture seen at different skeletal sites (Amling *et al.,* 1996), to site-specific differences in disease manifestation such as seen in osteoporosis (reviewed in Byers *et al.,* 1997; Riggs *et al.,* 1998), and to regional variations in the ability of osteoblasts to respond to therapeutic agents. A highly pertinent example of potential site-specific cellular responses concerns the estrogen receptors (ERs). Studies support the notion that ERα and ERβ are expressed differentially in different parts of bones, e.g., ERβ was reported to be highly expressed in the cancellous bone of lumbar vertebrae and distal femoral metaphysis but expressed at much lower levels in the cortical bone of the femur (Onoe *et al.,* 1997). These data offer a possible mechanism by which the estrogen deficiency caused by ovariectomy induces bone resorption preferentially in cancellous bone and in vertebrae. Further analysis of differential expression profiles for a variety of receptors in different skeletal sites, and at different maturational age of the cells and skeleton, should provide further insight into site-specific effects of not only estrogen, but other treatments, including fluoride, calcitonin, PTH, and even calcium. In this regard, the observations of Calvi *et al*. (2001) on transgenic mice overexpressing constitutively active PTH1R are interesting. The opposite effects observed in trabecular and endosteal osteoblast populations versus periosteal osteoblast populations are reminiscent of the differential effects of PTH in trabecular versus cortical bone in primary hyperparathyroidism (Parisien *et al.,* 1990; Calvi *et al.,* 2001). However, consistent with our own observations on the global expression of PTH1R in all osteoblast populations (Candeliere *et al.,* 2001), Calvi *et al*. (2001) found similar levels of transgene mRNA expression in both compartments, suggesting that in this case, differential receptor expression cannot account for the different responses elicited by the ligand/PTH. These data predict exquisite control of a signaling threshold as discussed earlier, other intrinsic differences downstream of the PTH1R in these different osteoblast populations, or that the different bone microenvironments in these sites modulate the osteoblast response.

As summarized previously (Aubin and Liu, 1996), many other molecules are now known to be made by osteoblast lineage cells, often with differentiation stage-specific changes in expression levels, but sometimes without known function yet in bone. Several molecules have been identified to be particularly highly expressed in osteoblasts and osteocytes, suggesting that they may play roles in mechanosensing among other activities in bone. For example, a novel osteocyte factor, termed OF45, has been identified by subtractive hybridization based on its high expression in bone marrow stromal cells (Petersen *et al.,* 2000). Northern blot analysis detected the mRNA in bone, but not other

tissues, and immunohistochemistry revealed that the protein was expressed highly in osteocytes within trabecular and cortical bone. The cDNA was predicted to encode a serine/glycine-rich secreted peptide of 45 kDa containing numerous potential phosphorylation sites and one RGD sequence motif, which may suggest a role for this new protein in integrin binding, and osteoblast (or osteoclast) recruitment, attachment, and differentiation. The mRNA for Phex, a phosphate-regulating gene with homology to endopeptidases on the X chromosome, which is mutated in X-linked hypophosphatemia (XLH), is expressed differentially in osteoblasts as they differentiate (Ecarot and Desbarats, 1999; Guo and Quarles, 1997; Ruchon *et al.,* 1998), and the protein is detectable only in osteoblasts and osteocytes (Ruchon *et al.,* 2000). Two cell surface multifunctional molecules that are regulated by osteotropic hormones and expressed throughout osteoblast differentiation, but most highly in osteoblasts and osteocytes, are galectin-3 (Aubin *et al.,* 1996a) and CD44 (Jamal and Aubin, 1996). Consistent with one of its earlier names, i.e., Mac-2, and role as a macrophage marker, ablation of galectin-3, which is thought to have pleiotropic effects in cells in which it is expressed, with diverse roles in adhesion, apoptosis, and other cellular functions, alters monocyte–macrophage function and survival (Hsu *et al.,* 2000). In addition, growth plate and chondrocyte defects with altered coupling between chondrocyte death and vascular invasion have been reported in galectin-3 null mice (Colnot *et al.,* 2001); notably, most hypertrophic chondrocyte–osteoblast markers studied (PTH1R, OPN, Cbfa1/Runx2) appeared to be distributed relatively normally, although Ihh expression was altered. Further work will be required to determine whether osteoblasts and bone metabolism are changed in these animals. In contrast, simultaneous ablation of all known CD44 isoforms, some of which are known to bind OPN, altered the tissue distribution of myeloid progenitors, with evidence for defective progenitor egress from bone marrow, and highly tumorigenic fibroblasts, but no bone abnormalities have been reported yet (Schmits *et al.,* 1997).

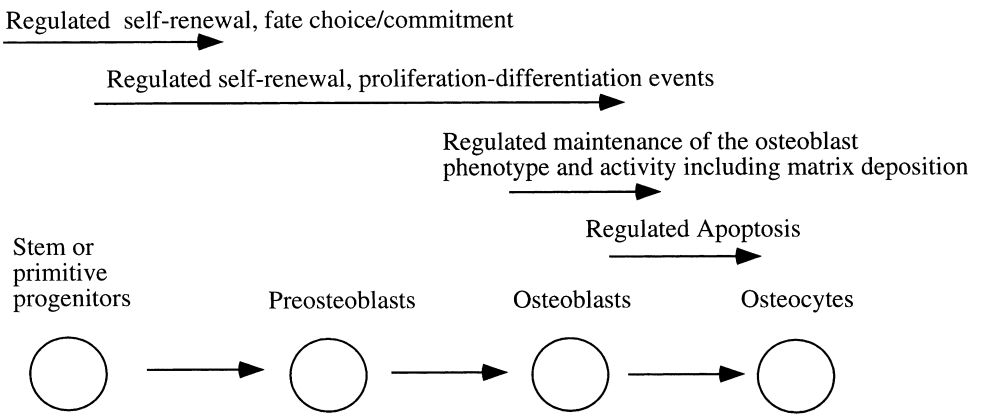
With completion of the human genome sequence project and those of other species and significant progress on the mouse and rat, together with novel new methods for cell-specific gene identification and functional genomics, we can expect to see in a short time a startling increase in known osteoblast gene products (Carulli *et al.,* 1998). For example, in a small-scale cDNA fingerprinting screen from globally PCR-amplified osteoblast colonies, Candeliere *et al*. (1999) identified several new markers with differential expression during osteoblast differentiation, including glycyl tRNA synthetase and cystatin C, among other previously unknown molecules. A high throughput serial analysis of gene expression (SAGE) and microarray hybridization of MC3T3-E1 cells at different times after the induction of osteoblast differentiation yielded a large number of known and novel osteoblast markers (Seth *et al.,* 2000). Rab24, calponin, and calcyclin, among other mRNAs, were coordinately induced, whereas levels of MSY-1, SH3P2, fibronectin, α-collagen, procollagen, and LAMPI mRNAs decreased with differentiation. Among unexpected mRNAs identified was the TGFβ superfamily member Lefty-1, which preliminary blocking studies showed appears to play a role in osteoblast differentiation (Seth *et al.,* 2000).

Transcription Factor, Hormone, Cytokine, and Growth Factor Regulation of CFU-F and Osteoprogenitor Cell Proliferation and Differentation

Transcription factors, hormones, cytokines, growth factors, and their receptors can serve both as markers and as stage-specific regulators of osteoblast development and differentiation (Figs. 2 and 3). It is beyond the scope of this chapter to review every factor known to influence osteoblast differentiation and bone formation at some level, as many will be covered elsewhere in other chapters. However, we have chosen examples that emphasize other issues discussed in this chapter, including heterogeneity of osteoblast response, proliferation–differentiation coupling, and differentiation stage-specific regulatory mechanisms.

Regulation by Transcription Factors

It is already evident from the preceding summary that Cbfa1/Runx2 is necessary for osteoblast development. However, several other issues related to Cbfa1 are of interest. For example, the role of at least three and perhaps more (see, e.g., Gronthos *et al.,* 1999) Cbfa1 isoforms that have been described needs to be clarified (reviewed in Yamaguchi *et al.,* 2000). All three appear to be able to regulate OCN expression (Xiao *et al.,* 1999) and osteoblast differentiation in *in vitro* models (Harada *et al.,* 1999), but with different efficacies/activities. Although it is regulated by BMPs, BMPs are unlikely to be the direct regulators of Cbfa1 expression *in vivo*, and one important area of work is investigation of the regulatory molecules lying upstream of Cbfa1. Cbfa1 regulates itself directly via binding on its own promoter (Drissi *et al.,* 2000; Ducy *et al.,* 1999). As already mentioned earlier, regulation of Cbfa1 by other transcription factors is yielding interesting information on skeletal site-specific regulatory mechanisms for Cbfa1 specifically and bone development generally. For example, downregulation of Cbfa1 expression was observed in both Msx2-deficient (Satokata *et al.,* 2000) and Bapx1-deficient (Tribioli and Lufkin, 1999) mice. A particularly interesting aspect of the phenotypes observed was that Msx-2 deficiency caused delayed growth and ossification of the skull and long bones, whereas the axial skeleton was affected in Bapx-1-deficient mice. In addition, Hoxa-2-deficient mice exhibit ectopic bone formation associated with ectopic expression of Cbfa1 in the second branchial arch (Kanzler *et al.,* 1998), suggesting that Hoxa-2 may normally inhibit the expression of Cbfa1 and bone formation in this area.



Transcription factors altering commitment, proliferation-differentiation choices and/or osteoblast activity

Cbfa-1
Msx-2, Dlx-5
Fra-1, deltaFosB
c-Abl
RORα
PPARγ2

Hormones, growth factors and cytokines or receptors altering self-renewal, commitment, proliferation-differentiation choices, activity and/or apoptosis

Glucocorticoids and other steroids
PTH, PTH1R
TGFβ, TGFβ type II receptor
PGE2, EP receptors
Leptin
BMPs

Antiproliferative proteins altering proliferation-differentiation choices and/or activity

Tob

Matrix proteins altering osteoblast activity

OCN

Figure 3 Regulated events and classes of regulatory molecules in osteoblast development and differentiation. The list is not exhaustive, but highlights some of the known levels of regulation that have been dissected in significant detail in the recent post.

With respect to other mechanisms of Cbfa1 regulation, relatively little is known, but inhibitory cofactors (TLE2/Groucho; HES1), phosphorylation via the MAPK pathway, and cAMP-induced Cbfa1 proteolytic degradation through a ubiquitin/proteosome-dependent mechanism have all been described *in vitro* (reviewed in Ducy, 2000).

Factors that appear to regulate osteoblast recruitment, osteoblast number, and the rate and duration of osteoblast activity are growing in number. Ducy *et al.* (1999) used a dominant-negative strategy in transgenic mice to show that Cbfa1 plays a role beyond osteoblast development in that it appears to regulate the amount of matrix deposited by osteoblasts in postnatal animals. It was also found that mice with leptin or leptin receptor deficiency had increased bone formation, suggesting that leptin may normally be an inhibitor of bone formation acting through the central nervous system (Ducy *et al.,* 2000). While it has been studied most frequently in the context of craniofacial development, Msx-2 appears to play roles in osteoblast differentiation in other bones, as evidenced by the broadly distributed (but not universal, see earlier discussion) bone abnormalities described in Msx-2 -/- mice (Satokata *et al.,* 2000). Msx-2 functional haploinsufficiency also causes defects in skull ossification in humans (Wilkie *et al.,* 2000). However, not only Msx-2 loss-of-function but also gain-of-function studies have been informative. Mutations in Msx-2 that increase its DNA-binding activity (Jabs *et al.,* 1993; Ma *et al.,* 1996) and overexpression of Msx-2 under the control of a segment of the mouse Msx-2 promoter that drives expression in a subpopulation of cells in the skull and the sutures (Liu *et al.,* 1999) result in enhanced calvarial bone growth and craniosynostosis. These latter data, together with findings of Dodig *et al.* (1999) on the effect of forced over- or underexpression of Msx-2 on osteogenic cell differentiation *in vitro*, are consistent with the hypothesis that the enhanced expression of Msx-2 keeps osteoblast precursors transiently in a proliferative state, delaying osteoblast differentiation, resulting in an increase in the osteoblast pool and ultimately in an increase in bone growth. More generally, these studies show that perturbations in the timing of proliferation

and differentiation of osteoprogenitors have profound consequences on the number and activity of osteoblasts and bone morphogenesis.

It was found that osteosclerosis results when either of two different members of the AP-1 subfamily of leucine zipper-containing transcription factors are overexpressed in transgenic mice: Fra-1 (Jochum *et al.,* 2000), a Fos-related protein encoded by the c-Fos target gene *Fosl1* (referred to as *fra-1*), or ΔFosB, a naturally occurring splice variant of FosB (Sabatakos *et al.,* 2000). In both cases, transgenic mice appear normal at birth, but with time, much increased bone formation is evident throughout the skeleton (endochondral and intramembranous bones). The osteosclerotic phenotype derives from a cell autonomous modulation of osteoblast lineage cells that is characterized by accelerated and more osteoblast differentiation and bone nodule formation *in vitro*. Interestingly, the further truncated Δ2ΔFosB isoform, rather than ΔFosB itself, appears to be responsible for the increased bone formation seen in ΔFosB transgenic mice. This suggests that further study of differentiation stage-specific aspects of the mechanism may shed light on the underlying lineage perturbation, as differentiation stage-specific alternative splicing of fosB mRNA and selective initiation site use of ΔFosB appear to be involved in the regulation of osteoblast development and the increased bone formation seen in ΔFosB transgenic mice (Sabatakos *et al.,* 2000). Notably, because neither Fra-1 nor ΔFosB (or Δ2ΔFosB) possesses the typical C-terminal transactivation domain, it seems likely that the transcriptional potential of either resides in or depends on specific heterodimerizing partners or coactivators—either activators or repressors—which remain to be elucidated.

Tob, a member of the emerging family of antiproliferative proteins, has been shown to be a negative regulator of osteoblast production through the regulation of BMP/Smad signaling (Yoshida *et al.,* 2000). Bone histomorphometry showed that the number of osteoclasts in tob -/- mice is equivalent to those in wild-type littermates, but that the osteoblast surface and bone formation rate are increased significantly. These data are consistent with the view that Tob may normally function to inhibit the proliferation of osteoblast precursors and their differentiation into mature ALP-positive osteoblasts, although further studies will be needed to address the possibility that Tob inhibits the function of mature osteoblasts. Mice homozygous for mutations in the gene (AIM) encoding the nonreceptor tyrosine kinase c-Abl also have a bone phenotype, including osteoporotic (both thinner cortical and reduced trabecular) bones and reduced mineral apposition rates (Li *et al.,* 2000), apparently reflecting no change in osteoclast number or activity but an osteoblast defect manifested by delayed maturation *in vitro*. Whether a cell autonomous defect in osteoblasts is responsible for the osteopenia seen in the spontaneous mouse mutant staggerer (sg/sg) (Meyer *et al.,* 2000), which carries a deletion within the retinioic acid receptor-related orphan receptor α (RORα) gene, remains to be determined, but because RORα appears to regulate BSP and OCN transcriptionally, this is clearly one possibility, given the increased bone formation seen earlier in OCN null mice (Ducy *et al.,* 1996). Interestingly, another steroid orphan receptor, estrogen receptor-related receptor α (ERRα), appears to play a positive regulatory role in both proliferation and differentiation of osteoprogenitor cells, at least *in vitro* (Bonnelye *et al.,* 2000).

It is also clear that further analysis of other homeodomain transcription factors expressed in osteoblasts is required. For example, Dlx-5 is expressed differentially as osteoblasts differentiate *in vitro* (Newberry *et al.,* 1998; Ryoo *et al.,* 1997) and in different cohorts of osteoblasts *in vivo* (Acampora *et al.,* 1999). The phenotype of Dlx-5 -/- mice is complex with many tissue abnormalities, but with respect to bone is characterized by craniofacial abnormalities affecting derivatives of the first four branchial arches, delayed ossification of the roof of the skull, and abnormal bone formation in endochondral bones, with the latter perhaps reflecting the differential expression of Dlx-5 at different skeletal sites (Acampora *et al.,* 1999).

It is beyond the scope of this chapter to review all the genes in which mutations or ablations appear to perturb bone patterning or differences in the formation of craniofacial, appendicular, and axial skeletons or differences in the amount of bone formed, but data available already indicate regulatory paradigms of significant complexity and are likely to become more complex as the roles of other related factors are elucidated. Some of these may include a novel Runx1 (Pebp2alphaB/Cbfa2/AML1b) splice variant identified and found to be expressed in bone and the osteoblast-like cell line MC3T3E1 (Tsuji and Noda, 2000); Dlxin-1, which is capable of forming multimers with Dlx-5 and participating in its transactivaton activity (Masuda *et al.,* 2001); and MINT, the Msx-2-binding protein that is expressed differentially as osteoblasts differentiate and modify Msx-2 transcriptional activity on the OCN promoter (Newberry *et al.,* 1999).

Regulation by Hormones, Growth Factors, and Cytokines

In experimental models, bone marrow injury associated with local bleeding, clotting, and neovascularization recapitulates a process similar to callus formation during fracture repair, with the induction of an environment rich in growth factors (e.g., PDGF, FGF, TGFβ, VEGF) followed by a process of very active bone formation (reviewed in Khan *et al.,* 2000; Rodan, 1998). To elucidate the target cells responding (stem cells, mesenchymal precursors, committed progenitors) and the precise nature of the responses in bone and nonbone cells in such an environment, these and a growing list of other systemic or local growth factors, cytokines, and hormones are also being tested in many models *in vitro*. The factors controlling cell lineage development and proliferation of marrow stromal CFU-F and CFU-O from stroma and bone (e.g., calvaria) are under intense study in many laboratories, often with differing

requirements proposed depending on the species studied (Gordon *et al.,* 1997; Kuznetsov *et al.,* 1997a) and opposite results depending on the model cell system under study (e.g., bone marrow stroma versus calvariae-derived populations), whether total CFU-F or specific subpopulations (e.g., CFU-ALP, CFU-O) are quantified, and the presence or absence of other factors. Nevertheless, because of increasingly careful documentation of proliferation and differentiation stages underlying the formation of CFU-F and bone nodules/CFU-O *in vitro*, these models are providing strong support for several concepts proposed earlier and are helping to clarify the nature of perturbations in a normally, carefully orchestrated proliferation–differentiation activity sequence.

VEGFs are suggested to play an important role in the regulation of bone remodeling by attracting endothelial cells and osteoclasts and by stimulating osteoblast differentiation (Deckers *et al.,* 2000). Interferon α has been shown to inhibit human osteoprogenitor cell proliferation, CFU- F formation, HOP-26 expression, and ALP-specific activity and to modulate BMP-2 gene expression, suggesting a role for interferon α in local bone turnover through the modulation of osteoprogenitor proliferation and differentiation (Oreffo *et al.,* 1999). The known anabolic actions of PGE_2 on bone formation may be at least in part via the recruitment of osteoblast precursors from mesenchymal precursor cells (Scutt and Bertram, 1995). While most often considered as osteoclast regulatory factors, interestingly, GM-CSF and IL-3, as well as M-CSF, also appear to stimulate the proliferation and/or differentiation of bone marrow fibroblastic precursors (Yamada *et al.,* 2000).

There is growing evidence that at least some of the actions of growth and differentiation factors are dependent on the relative stage of differentiation (either more or less mature) of the target cells, with stimulatory/mitogenic or inhibitory responses when test factors are added to proliferative/progenitor stages and stimulation or inhibition of differentiation stage-specific precursors and mature osteoblasts when the same factors are added later. This is true, for example, for the inflammatory cytokine IL-1, which is stimulatory to CFU-O formation when calvaria-derived cultures are exposed transiently during proliferative culture stages, and inhibitory when cells are exposed transiently during differentiation stages (Ellies and Aubin, 1990) and able to regulate a variety of osteoblast-associated genes when cells are treated acutely for short (hours) periods of time [e.g., PGHS-2 (Harrison *et al.,* 2000)]; the inhibitory effects dominate when cells are exposed chronically through proliferation and differentiation stages in culture (Ellies and Aubin, 1990). Many other factors of current interest similarly have biphasic or multiphasic effects *in vitro*, including EGF, TGFβ, and PDGF.

Another example of clinical significance is the reported catabolic versus anabolic effects of PTH. PTH1R is expressed throughout osteoblast differentiation, although the levels of expression and activity appear to increase as osteoblasts mature (reviewed in Aubin and Heersche, 2001). Chronic exposure to PTH inhibits osteoblast differentiation in an apparently reversible manner at a relatively late preosteoblast stage (Bellows *et al.,* 1990b). However, when rat calvaria cells were treated for 1-hr versus 6-hr pulses in 48-hr cycles during a 2- to 3-week culture period, either inhibition (1-hr pulse; apparently related to cAMP/PKA pathways) or stimulation (6-hr pulse; apparently related to cAMP/PKA, Ca2+/PKC, and IGF-I) in osteoblast differentiation and bone nodule formation was seen (Ishizuya *et al.,* 1997). In mice deficient in PTH1R, not only is a well-studied defect in chondrocyte differentiation seen (as also seen in PTHrP knockout mice), but also increased osteoblast number and increased bone mass [a phenotype not seen in PTHrP-deficient mice (Lanske *et al.,* 1999)], supporting the view that PTH plays an important role in the regulation of osteoblast number and bone volume. Consistent with this latter view are studies showing that PTH may increase osteoblast lifetime by decreasing osteoblast apoptosis (Jilka *et al.,* 1999). As mentioned, previously, when Calvi *et al.* (2001) expressed constitutively active PTH1R in bone, the osteoblastic function was increased in the trabecular and endosteal compartments, but decreased in the periosteum of both long bones and calvaria; interestingly, an apparent increase in both osteoblast precursors and mature osteoblasts was seen in trabecular bone.

Because of their ability to induce *de novo* bone formation at ectopic sites, BMPs, which are members of the TGFβ superfamily, have been studied extensively *in vivo* and *in vitro* as regulators of osteoblast development (reviewed in Yamaguchi *et al.,* 2000). While many reports document a stimulatory effect of BMPs on osteoblast differentiation, a few show inhibitory effects. Interestingly, while mouse knockout experiments have clearly indicated a role for BMPs in skeletal patterning and joint formation, ablation experiments have not yet provided evidence for a role of BMPs in osteoblast differentiation *in vivo* (for a review, see Ducy and Karsenty, 2000). This may be the result of functional redundancy among members of this family (there are now more than 30), and further experiments will be required to elucidate unequivocally the role of BMP family members in osteoblast differentiation. However, TGFβ has been shown to have biphasic effects on osteoblast development *in vitro*, inhibiting early stage progenitors while stimulating matrix production by more mature cells in the lineage, including osteoblasts (see Chapter 49). These diverse effects may help account for the complex effects seen when TGFβ is overexpressed via the OCN promoter in transgenic mice; these mice have low bone mass, with increased resorption, but increased osteocyte numbers and hypomineralized matrix (Erlebacher and Derynck, 1996). Further studies from Derynck's group showed that when a dominant-negative TGFβ type II receptor was expressed in osteoblasts, osteocyte number, bone mass, and bone remodeling were all influenced in a manner suggesting that TGFβ increases the steady-state rate of osteoblastic differentiation from osteoprogenitor cells to terminally differentiated osteocytes (Erlebacher *et al.,* 1998) while also

regulating bone remodeling, structure, and biomechanical properties (Filvaroff *et al.,* 1999).

Review of all regulatory factors is beyond the scope of this chapter, and indeed, we have not touched on a growing number of reports on matrix and matrix–integrin effects on osteoblast development and/or activity *in vitro* and *in vivo* (see, e.g., Zimmerman *et al.,* 2000). However, given its inclusion in the majority of CFU-F and CFU-O assays *in vitro* reported in this chapter, it is worth considering glucocorticoids (most often dexamethasone in *in vitro* assays) in more detail. Glucocorticoid effects *in vivo* and *in vitro* are complex and often opposite, i.e., stimulating osteoblast differentiation *in vitro* (reviewed in Aubin and Liu, 1996) while resulting in glucocorticoid-induced osteoporosis *in vivo* (Weinstein *et al.,* 1998). An emerging picture of glucocorticoid-induced stimulation of osteoprogenitor cell recruitment, self-renewal, and differentiation (Aubin and Liu, 1996) opposed by the glucocorticoid-induced inhibition of several molecules synthesized by the mature osteoblast (Lukert and Kream, 1996) and a glucocorticoid-induced increase in osteoblast apoptosis (Weinstein *et al.,* 1998) may account for some of the discrepancy.

An area of considerable interest is the stimulatory activity of glucocorticoids on osteoprogenitors (see also Aubin, 1998). One mechanism by which dexamethasone or other glucocorticoids may act is through autocrine or paracrine regulatory feedback loops in which the production of other factors is modulated, including growth factors and cytokines that themselves regulate the differentiation pathway. For example, in rat calvaria cultures, glucocorticoids downregulate the endogenous production of LIF, which is known to be inhibitory to bone nodule formation when cells are treated at a late progenitor/preosteoblast stage (Malaval *et al.,* 1998), and upregulate BMP-6, which is stimulatory possibly through LMP-1, a LIM domain protein (Boden *et al.,* 1998). These are but two of a growing list of examples of dexamethasone regulation of endogenously produced factors with apparently autocrine or paracrine activities on osteoblast lineage cells.

Many factors of interest have effects on gene expression in mature osteoblasts that may correlate with effects on the differentiation process and may be opposite for different osteoblast genes, with glucocorticoids being a case in point. The molecular mechanisms mediating these complex effects are generally poorly understood; however, the ability to form particular transcription factor complexes, localization and levels of endogenous expression of cytokine/hormone/growth factor receptors, and expression of cognate or other regulatory ligands within specific subgroups of osteogenic cells as they progress from a less to a more differentiated state may all play roles. As mentioned earlier, growing evidence shows that the probabilities for self-renewal versus differentiation of hemopoietic stem cells is regulated, at least in part, by the maintenance of required/critical signaling ligands (soluble or matrix or cell-bound) above a threshold level. While there are few explicit data or experiments examining these issues in MSCs or osteoprogenitor populations, it seems likely that similar threshold controls may apply.

Stem Cell/Osteoprogenitor Cell Changes in Disease and Aging

As described previously, the formation of colonies (CFU-F, CFU-O, etc) may give some index of the stem/progenitor cell status of an individual or a tissue site. This colony-counting method was used, together with expression of ALP as an osteogenic marker, in a large study of 99 patients, with an age range from 14 to 97 years who were osteoarthritic, osteoporotic, or showed no evidence of metabolic bone disease (Oreffo *et al.,* 1998a,b). No evidence was found that CFU-F decreased significantly with age or disease in either the total populations or in males or females, but colony size decreased in control, osteoporotic, and osteoarthritic patients with age. Other reports document a decline in total CFU-F in patient groups, but these involve lower sample populations. However, the fact that discrepant results in CFU-F, CFU-O, and CFU-ALP size and number have also been reported in studies done on populations isolated from mice and rats (Bergman *et al.,* 1996; Brockbank *et al.,* 1983; Egrise *et al.,* 1996; Gazit *et al.,* 1998; Schmidt *et al.,* 1987) suggests that many of the issues affecting colony assays *in vitro* remain to be elucidated. Nevertheless, the new results on human cells are interesting because, in osteoporotics, not only was colony size reduced but the number of ALP-positive colonies was also reduced compared to controls. A similar conclusion regarding osteoprogenitor cell number was made by Nishida *et al.* (1999) by studying human CFU-Fs harvested from iliac bone marrow of 49 females aged 4 to 88 years. Numbers of CFU-ALP fell markedly after 10 years of age with a gradual decline with increasing age. This decline is also seen in bone progenitors isolated from human vertebrae (D'Ippolito *et al.,* 1999).

The responsiveness of CFU-F to systemic or locally released osteotropic growth factors has also been reported to decrease with age as suggested earlier (Pfeilschifter *et al.,* 1993). For example, the stimulatory effect of TGFβ on colony number and cells per colony in human osteoprogenitor cells derived from 98 iliac crest biopsies declined significantly with donor age (Erdmann *et al.,* 1999). In BALB/c mice, Gazit, *et al.* (1998) suggested that changes in the osteoprogenitor cell/CFU-F compartment occur with aging because of a reduction in the amount and/or activity of TGFβ1. Ligand concentration-dependent ERα induction and loss of receptor regulation and diminution of ligand–receptor signal transduction with increasing donor age have also been reported (Ankrom *et al.,* 1998). In other studies, PGE_2 was found to exert stimulatory and inhibitory effects on osteoblast differentiation and bone nodule formation through the EP1/IP3 and EP2/EP4-cAMP pathways, respectively, in cells from young rats. However, the EP1/IP3 pathway was reported to be inactive in cells isolated from

aged rats (Fujieda *et al.,* 1999). Thus, the known loss of bone with aging or menopause may be due to a reduced responsiveness of osteoprogenitor cells to biological factors resulting in an alteration in their subsequent differentiation potentials or to local changes in these factors. This has fundamental and strategic implications regarding therapeutic intervention to prevent bone loss and to increase bone mass in postmenopausal women and in aging populations. It also opens up possibilities for experimental studies to test whether the necessary growth factors can be supplied to the deficient site by the transfer of marrow stroma from one bone tissue site to another or by genetically engineered autologous cell therapy.

Relatively little attention has been paid to the interesting issue of telomerase presence and activity and telomere length in osteoblast lineage cells at different developmental ages or differentiation stages. However, in one study, telomere length was compared in cultured human trabecular osteoblasts undergoing cellular aging and in peripheral blood leukocytes (PBL) obtained from women in three different groups [young (aged 20–26 years, $n = 15$), elderly (aged 48–85 years, $n = 15$), and osteoporotic (aged 52–81 years, $n = 14$)] (Kveiborg *et al.,* 1999). Telomere shortening was observed with population doubling increases similar to what has been reported in human fibroblasts, but data from osteoporotic patients and age-matched controls did not support the notion of the occurrence of a generalized premature cellular aging in osteoporotic patients.

Tissue Engineering and Stem Cell Therapy for Skeletal Diseases

There has been enormous interest in the potential of stem cells for therapy in many degenerative disorders of metabolic, environmental, and genetic origins. In particular, the potential for using embryonic stem cells to generate multiple cell types for use in tissue regeneration and repair is receiving much attention (Colman and Kind, 2000). Therapeutic possibilities for use of these and other postnatal, tissue-derived stem cells are apparent for many tissues and organs, not only skeletal tissues (Bach *et al.,* 2000; McCarthy, 2000; Service, 2000). The explosion of interest is documented by the rapid rise in research publications in this area, and MSCs have been noted to have potentials far beyond skeletal reconstruction and augmentation of skeletal mass.

The field of tissue engineering, which combines biomaterials and cell and developmental biology, is a rapidly expanding, new research area of increasing importance. Its main focus is the synthesis of tissues or artificial constructs based on living cells and cell matrix (Caplan, 2000; Reddi, 2000). It is expected that practically all tissues will be capable of being repaired by tissue-engineering principles. Basic requirements are thought to include a scaffold conducive to cell attachment and maintenance of cell function, together with a rich source of progenitor cells. In the latter respect, bone is a special case and there is a vast potential for regeneration from cells with stem cell characteristics (Triffitt, 1996). About 60% of single CFU-F colony-derived marrow stromal cell strains from human donors have been shown to form bone in a model system *in vivo*, although strains differ from each other in osteogenic capacity (Kuznetsov *et al.,* 1997b). It is likely that a lower proportion of these colonies have stem cell characteristics as found by Friedenstein (1990) in other species and predicted based on the observed heterogeneity of individual CFU-F in various assays [e.g., expression of ALP (Herbertson and Aubin, 1997)] or multilineage potential (Pittenger *et al.,* 1999)].

Consistent with data from earlier studies on clonal cell lines (summarized in Aubin, 1998), the development of osteoblasts, chondroblasts, adipoblasts, myoblasts, and fibroblasts results from marrow stromal colonies derived from single cells (Bianco *et al.,* 1999; Krebsbach *et al.,* 1999; Park *et al.,* 1999; Pittenger *et al.,* 1999, 2000; Robey, 2000; Triffitt *et al.,* 1998). They may thus theoretically be useful for the regeneration of all tissues that this variety of cells comprise: bone, cartilage, fat, muscle, and tendons and ligaments. Indeed, as summarized earlier, the recent discoveries of transcription factors such as Cbfa1 and "master genes", which govern tissue development, offer potentials for further tissue engineering involving genetic manipulation that would open additional avenues for skeletal therapy (Nuttall and Gimble, 2000). Fibroblasts with proliferative activity are also present in blood and are inducible with respect to bone formation (Luria *et al.,* 1971), but their origins and relationships to repopulation of skeletal sites are unknown. The hypothesis that osteoprogenitors may circulate systemically and be recruited to bone-forming sites in specific conditions will undoubtedly be tested by numerous laboratories in the near future.

As life expectancy increases, so does the incidence of skeletal diseases such as osteoporosis and osteoarthritis and the resultant requirements for new and more adequate methods of replacing skeletal mass and refurbishing bone and joint structures (Oreffo and Triffitt, 1999). In addition, a number of other genetic and metabolic conditions affecting the skeletal tissues of younger individuals requires even more effective replacement of missing or damaged tissues. Rare genetic conditions such as osteogenesis imperfecta (OI) produce life-long crippling in some patients, and treatment of the skeletal defect is dependent on strengthening and correction by mechanical orthopedic procedures. Any future advances in strengthening the skeleton by other methods in this condition would provide significant improvements in prognosis.

Somatic Cell and Gene Therapy

Relevant to tissue reconstruction is the field of genetic engineering, which as a principal step in gene therapy would be the introduction of a cloned functional specific human DNA into the cells of a patient with a genetic dis-

ease that affects mainly a particular tissue or organ. Such a situation might be OI, for example, where the skeleton is affected with a severity from gross to mild. In certain patients with OI, null allelic mutations result in a 50% reduction in collagen and a mild phenotype (Prockop, 1997). Bone marrow fibroblastic cells could be removed from an affected patient, amplified in culture, genetically manipulated to replace the null allele, and reinserted into the affected individual. Even in severely affected individuals, any improvements in long bone quality would be beneficial to the patient. To this end, even cell culture-expanded allogeneic but tissue-matched normal marrow may be sufficient to effect adequate recovery at particular sites. Unfortunately, no evidence of benefit to OI patients of allogeneic stromal fibroblasts has been demonstrated conclusively to date (Horwitz *et al.,* 1999). Other experimental approaches should be tested thoroughly by investigations of replacement by human marked cells systemically or in intramedullary sites in immunodeficient animals initially before any application to possible human therapy.

Retroviral-mediated gene transfer is not only potentially of value for the correction of defective genes but also for the study of progenitor cell fate. This has been shown to be a reproducible method for infecting large numbers of primitive osteoprogenitors at high efficiency. The Moloney-derived retrovirus containing both LacZ and NeoR genes has been used to transduce human and murine bone marrow stromal cells and the stromal cell phenotype and function not observed to be significantly altered after retroviral-mediated transfer of these marker genes (Bulabois *et al.,* 1998).

With a triple transfection method, a reporter gene (*lacZ*) and a selective marker gene (neo^r) have been transfected retrovirally into the genomes of human bone marrow fibroblasts, and stable persistence of these markers has been shown for at least 8 months in culture (Oreffo, 2001). Such work enables a combination of *in vivo* animal experimentation with human cell populations, as well as with cells from experimental animals, that are marked routinely by these and other marker genes to assess in detail their differentiation into tissues and their extended interactions *in vivo* with other cell systems. It also allows more detailed experimentation on the fate of such marked osteoprogenitor cells from human and experimental animal origins.

Generally, studies on marrow transplants in humans and mice for hematological reconstitutions have concluded that the only donor cells surviving are hemopoietic, with the stromal fibroblasts being of host origin. The concept that marrow fibroblasts never repopulate in radiochimeras or home to the marrow site is rejected by recent experiments, however. A number of reports indicate that donor marrow fibroblasts persist in a variety of tissues following their systemic infusion when assessed by sensitive methods of detection. For example, Pereira *et al*. (1995) showed that 1–5 months after intravenous transplantation into irradiated mice of mouse bone marrow stromal cells enriched by adherence to plastic, expanded in culture, and marked with the human COL1A1 minigene, there was a widespread tissue distribution of connective tissue cells containing and expressing the gene. Among tissues, such as brain, spleen, marrow, and lung, bone also expressed the minigene as detected by PCR. In a very different approach (Nilsson *et al.,* 1999), whole unfractionated uncultured male mouse bone marrow was transplanted intravenously into nonablated female mice. Significant numbers of donor cells were detected by fluorescence *in situ* hybridization (FISH) in whole femoral sections, as both osteocytes encapsulated by mineralized matrix and residing within the bone lacunae and as flattened bone-lining cells in the periosteum, suggesting that donor cells may participate in normal biological turnover (Prockop, 1998). Data support the hypothesis proposed by Horwitz *et al*. (1999) that whole marrow contains enough of these cells to be able to replace sufficient osteoblasts to be clinically useful in diseases such as OI, where marrow ablation is standard preparative treatment. A cloned pluripotent cell line from mouse bone marrow stroma labeled with a genetic marker was traced by fluorescence activated cell sorting (FACS) analysis upon systemic injection into syngeneic mice (Dahir *et al.,* 2000). This manipulation did not affect obviously subsequent differentiation of the cells, the osteogenic characteristics of the cell line were retained in diffusion chamber implantations, and the genetically marked cells were shown to repopulate the marrow in syngeneic animals. It is concluded that differences in views of the transplantability of stromal fibroblastic cells may be explained by the different cell populations and the amounts administered. It could also be dependent on the method of administration and whether and how the medullary site is prepared and these possibilities should be considered.

The potential for therapy in other stromal systems may also be considered feasible as Ferrari *et al*. (1998) have demonstrated that genetically marked bone marrow-derived myogenic progenitors transplanted into immunodeficient mice migrated into areas of induced muscle damage, were able to undergo myogenic differentiation, and contributed, albeit minimally, to muscle regeneration. Genetically modified, marrow-derived myogenic progenitors could thus potentially target therapeutic genes to muscle tissue and provide alternative strategies for the treatment of muscular dystrophies.

A number of species were used to investigate the efficiency of transduction by a variety of viral vectors by Mosca *et al*. (2000). Optimal transduction for human, baboon, canine, and rat MSCs was a effected with amphotropic vectors. However, sheep, goat, and pig MSCs were transduced most effectively by xenotropic retroviral vectors, with rabbit cells requiring gibbon ape-enveloped vectors for best transduction. These authors used a myeloablative canine transplantation model with gene-marked canine mesenchymal stem cells to determine the physiological fate of infused cells. Most marked cells were found in the bone marrow samples.

For xenogeneic implantation of human cells, more strains of immunocompromised mice are now readily available and

their use has revolutionized the possibilities for investigations of human cells in a variety of physiological and regeneration schemes. Severe combined immunodeficient (SCID) mice and a variety of related strains (e.g., SCID/NOD) have been used extensively in studies on development in many xenogeneic and allogeneic cell systems and the persistence and tissue distribution patterns of injected cells determined. Marrow cells and, in a few cases, skeletal cells (Boynton *et al.,* 1996) have been shown to persist and differentiate in these systems. In the hemopoietic field the use of marrow stromal cells has been suggested as a vehicle for cell-mediated gene transfer to replace defective hemopoietic growth factors and deliver the gene product *in situ* to the marrow microenvironment. This also has relevance to the skeletal field particularly as studies (Oreffo, *et al.,* 1998a,b) strongly suggest that local tissue environmental factors rather than decreased stem cell number are basic contributory causes of age and osteoporosis defects in human skeletal quality. In the future, specific gene therapy may be relevant and necessary in certain cases to correct these defects. Model studies on direct cell implantations in animals, however, require assessments of origins of the implanted cells by species-specific antibody methods or by *in situ* hybridization with species-specific nucleic acid probes unless the implanted cells are marked genetically with a distinctive, easily detected marker. The acceptance of these xenografted cells opens new avenues for investigation of how human cells react in neophysiological situations and is an important area of future research, which will extend possibilities for clinical application of basic work on osteogenesis. Requirements for the future include the necessity to show functional relevance of the cells localized in the tissue after injection locally or systemically, and the participation in significant tissue reconstruction. The reticuloendothelial system has long been known to accumulate nonphysiological moieties or "undesirable material" present in the systemic circulation, and the leaky sinusoids of marrow are known to be prime sites for such interactions (Ham, 1969). Even if the "homing" of living cells to marrow in this context is a "nonphysiological" event, some benefit to skeletal reconstruction directly or by expression of growth factors may still be possible.

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CHAPTER 5

Transcriptional Control of Osteoblast Differentiation and Function

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The study of most cellular differentiation processes, such as myogenesis, hematopoiesis, neurogenesis, and adipogenesis, has demonstrated the importance of transcription factors in controlling cell-specific differentiation and gene expression (Arnold and Winter, 1998; Engel and Murre, 1999; Bang and Goulding, 1996; Wu *et al.,* 1999). Compared to other cell lineages, the study of the transcriptional control of osteoblast differentiation has progressed slowly for several reasons. At first, unlike other cell types, osteoblasts in culture do not undergo obvious morphological changes during differentiation that could be used to monitor gene expression. The only morphological feature that distinguishes an osteoblast from a fibroblast lies outside the cell. It is the accumulation of a mineralized matrix, by a process that is still poorly understood and that requires several weeks in culture (Aubin, 1998). Moreover, for a long time, *Osteocalcin* was the only known osteoblast-specific gene whose promoter could serve as a tool to identify osteoblast-specific transcription factors. *Osteocalcin* is expressed late during osteoblast differentiation, thus making it difficult to identify transcription factors acting at earlier stages. The apparent lack of osteoblast-specific markers and morphological features explains why the study of the transcriptional control of osteoblast differentiation has been rather difficult in the past. However, after the identification of Cbfa1 as a lineage-specific transcriptional activator of osteoblast differentiation and with the availability of human and mouse genetics as an experimental tool, we are now beginning to get more insights into this important area of skeletal biology.

Cbfa1: A Master Control Gene of Osteoblast Differentiation and Function

To date, only one transcription factor has been identified that is specifically expressed in cells of the osteoblast lineage, Cbfa1 (core binding factor 1). *Cbfa1* was originally cloned in 1993 as the α subunit of the polyomavirus enhancer binding protein 2 (Ogawa *et al.,* 1993). It represents one of three mammalian homologues (*Cbfa1-3*) of the *Drosophila* transcription factor runt that is required for embryonic segmentation and neurogenesis (Kagoshima *et al.,* 1993). The name given to this gene has changed several times since it was isolated. It was first called *Pebp2a1*, then *Aml3*, and later *Cbfa1* or *Runx2*. For the sake of clarity, we will refer to it as *Cbfa1* in this chapter. Initially, *Cbfa1* was thought to be important for T-cell-specific gene expression as it was found to be expressed in thymus and T-cell lines, albeit at very low levels, but not in B-cell lines (Ogawa *et al.,* 1993; Satake *et al.,* 1995). However, 4 years after the initial cloning of *Cbfa1*, its crucial importance as a transcriptional activator of osteoblast differentiation was demonstrated by several investigators at the same time using different experimental approaches (Ducy *et al.,* 1997; Komori *et al.,* 1997; Otto *et al.,* 1997; Mundlos *et al.,* 1997).

One approach was aiming at the identification of osteoblast-specific transcription factors using the *Osteocalcin* gene as a tool. The analysis of a proximal promoter

fragment of one of the two mouse *Osteocalcin* genes led to the definition of two osteoblast-specific *cis*-acting elements, termed OSE1 and OSE2 (Ducy *et al.,* 1997). Sequence inspection of OSE2 revealed homology to the DNA-binding site of runt family transcription factors, and subsequent analysis demonstrated that the factor binding to OSE2 is related immunologically to Cbfa transcription factors (Geoffroy *et al.,* 1995; Merriman *et al.,* 1995). Eventually, the screening of a mouse primary osteoblast cDNA library revealed that only one of the three mammalian *Cbfa* genes, *Cbfa1*, is expressed in cells of the osteoblast lineage, but not in any other cell types or tissues (Ducy *et al.,* 1997). As such, Cbfa1 is the earliest and most specific marker of osteogenesis identified to date.

During mouse development, *Cbfa1* is first expressed in the lateral plate mesoderm at 10.5 dpc, and later in cells of the mesenchymal condensations. Until 12.5 dpc these cells that prefigure the future skeleton represent common precursors of osteoblasts and chondrocytes. At 14.5 dpc osteoblasts first appear and maintain the expression of *Cbfa1*, whereas in chondrocytes, *Cbfa1* expression is decreased significantly and restricted to prehypertrophic and hypertrophic chondrocytes. After birth, *Cbfa1* expression is strictly restricted to osteoblasts. Taken together, this spatial and temporal expression pattern suggested a critical role for Cbfa1 as a regulator of osteoblast differentiation and function. Such a role was demonstrated by molecular biology and genetic experiments.

First, in addition to the *Osteocalcin* promoter, functional OSE2-like elements were identified in the promoter regions of all genes that are expressed at relatively high levels in osteoblasts such as *α1(I) collagen*, *Osteopontin*, and *Bone sialoprotein* (Ducy *et al.,* 1997). Second, and more importantly, the forced expression of *Cbfa1* in nonosteoblastic cell lines such as C3H10T1/2 mesenchymal cells, primary skin fibroblasts, and even myoblasts induced osteoblast-specific gene expression in these cells, demonstrating that Cbfa1 acts as a transcriptional activator of osteoblast differentiation *in vitro* (Ducy *et al.,* 1997). The ultimate demonstration that Cbfa1 is an indispensable transcriptional activator of osteoblast differentiation came from genetic studies in mice and humans. At the same time, two groups deleted the *Cbfa1* gene from the mouse genome, both expecting an immunological phenotype based on the assumption that *Cbfa1* was a T-cell-specific transcription factor (Komori *et al.,* 1997; Otto *et al.,* 1997). *Cbfa1*-deficient mice die shortly after birth without any detectable immunological defects. Instead, the cause of death is the lack of both endochondral and intramembranous bone formation. The complete absence of bone in *Cbfa1*-deficient mice is a consequence of the maturational arrest in osteoblast differentiation. This was confirmed by *in situ* hybridization analysis showing the absence of osteoblastic markers such as *Osteocalcin* and *Osteopontin* (Komori *et al.,* 1997). Thus, although the skeleton of *Cbfa1*-deficient mice is of normal size and shape, it is entirely cartilaginous. This latter result indicates that *Cbfa1* expression is necessary for osteoblast differentiation.

A more detailed analysis of *Cbfa1*-deficient mice has revealed that some skeletal elements, such as humerus and femur, lack hypertrophic chondrocytes (Inada *et al.,* 1999; Kim *et al.,* 1999), suggesting that Cbfa1 is also required for hypertrophic chondrocyte differentiation, at least in some skeletal elements (Inada *et al.,* 1999; Kim *et al.,* 1999). Such a function was further substantiated by the generation of *Cbfa1*-deficient mice expressing *Cbfa1* in nonhypertrophic chondrocytes under the control of the *α1(II)-collagen* promoter (Takeda *et al.,* 2001). The continuous expression of *Cbfa1* in nonhypertrophic chondrocytes in transgenic *Cbfa1*+/+ mice induces chondrocyte hypertrophy and endochondral ossification in locations where it normally never occurs. This result, consistent with the osteoblast differentiation ability of Cbfa1 indicates that *Cbfa1* expression is sufficient to induce endochondral bone formation ectopically. To test whether Cbfa1 is also a hypertrophic differentation factor, in addition to being an osteoblast differentation factor, these transgenic mice were crossed with *Cbfa1*-deficient mice. The presence of the *α1(II)-Cbfa1* transgene in a *Cbfa1*-deficient background restores chondrocyte hypertrophy and vascular invasion, but does not induce osteoblast differentiation. This latter observation identifies Cbfa1 as a hypertrophic chondrocyte differentiation factor and provides a genetic argument for a common regulation of osteoblast and chondrocyte differentiation mediated, at least in part, by Cbfa1.

The critical importance of Cbfa1 during osteoblast differentiation is demonstrated further by the fact that mice heterozygous for *Cbfa1* display defects in osteoblast, but not in chondrocyte differentiation. *Cbfa1*+/− mice are characterized by hypoplastic clavicles and a delay in the suture of fontanelles, indicating that the dosage of Cbfa1 is critical for osteoblast differentiation in bone structures formed by intramembranous ossification (Otto *et al.,* 1997). The phenotype of *Cbfa1*+/− mice is identical to a classical mouse mutation called *Cleidocranial dysplasia* (*Ccd*) (Selby and Selby, 1978). *Cbfa1* maps to a region on mouse chromosome 17 that is partially deleted in *Ccd* mice (Otto *et al.,* 1997), and two groups at the same time identified mutations of the human *CBFA1* gene in the genome of patients affected with CCD (Lee *et al.,* 1997; Mundlos *et al.,* 1997). Taken together, these lines of evidence demonstrate that Cbfa1 is necessary for osteoblast differentiation *in vivo*, although it remains to be determined if it is by itself sufficient for osteoblast differentiation.

The role of Cbfa1 in osteoblast biology is not limited to osteoblast differentiation. *Cbfa1* expression in osteoblasts is maintained after birth, and the expression of *Osteocalcin*, for instance, a gene only expressed in fully differentiated osteoblasts, is controlled by Cbfa1. This suggested a function for Cbfa1 beyond cellular differentiation. Such a function was confirmed by the generation of transgenic mice expressing a dominant-negative variant of *Cbfa1* (ΔCbfa1) under the control of an *Osteocalcin* promoter fragment (Ducy *et al.,* 1999). This promoter fragment is only active in fully differentiated osteoblasts and only after birth, resembling the specific temporal and spatial expression pat-

tern of the *Osteocalcin* gene. Accordingly, these transgenic mice are born alive because osteoblast differentiation is not affected. However, once the *Osteocalcin* promoter becomes fully active and ΔCbfa1 is expressed, these mice develop an osteoporosis-like phenotype characterized by a decreased bone formation rate, with a normal osteoblast number (Ducy *et al.,* 1999). This phenotype is explained by the fact that the expression of genes encoding bone extracellular matrix proteins, such as *type I collagen*, *Osteocalcin, Bone sialoprotein* and *Osteopontin* is decreased significantly. These findings demonstrate that Cbfa1 not only controls the differentiation of osteoblasts, but is also a regulator of their function, i.e., the production of the bone extracellular matrix.

After the discovery of Cbfa1 as a transcriptional activator of osteoblast differentiation and function, many studies have been performed in several laboratories that further underscored the importance of Cbfa1 in osteoblasts. For example, Cbfa1-binding sites could now be identified in a large number of genes that are expressed in osteoblasts such as *Collagenase 3, TGFβ-type I receptor*, and *RANKL/ODF* (Jimenez *et al.,* 1999; Ji *et al.,* 1998; Kitazawa *et al.,* 1999). Moreover, the identification of Cbfa1 has provided a handle to search for other factors playing a role in osteoblast differentiation. There are especially two equally important future research approaches. (1) the biochemical characterization of Cbfa1 in order to understand the molecular basis of its action and (2) the identification of molecules that regulate the spatial and temporal expression pattern and the level of expression of *Cbfa1*. Both issues are of paramount importance because a positive regulation of Cbfa1 expression or function is likely to be beneficial in bone loss diseases such as osteoporosis.

Like all transcription factors belonging to the Runt family, Cbfa1 contains a DNA-binding region of 128 amino acids, called the runt domain, followed C-terminally by a proline–serine–threonine-rich region, called the PST domain. The PST domain contributes to the transactivation function of Cbfa1 and contains a short sequence at the C terminus that mediates transcriptional repression through interactions with TLE2, a mammalian homologue of the *Drosophila* transcriptional repressor Groucho (Aronson *et al.,* 1997; Thirunavukkarasu *et al.,* 1998). In contrast to other Runt proteins, Cbfa1 has two unique domains located at the N terminus that are also involved in activating transcription. One of them, the so-called QA domain, which is rich in glutamine and alanine, prevents heterodimerization of Cbfa1 with Cbfβ, a known partner of other Runt family transcription factors (Wang *et al.,* 1993; Thirunavukkarasu *et al.,* 1998). Cbfa1 has been shown to be phosphorylated by PKA and MAPK *in vitro* (Selvamurugan *et al.,* 2000; Xiao *et al.,* 2000). Furthermore, physical interactions of Cbfa1 with Smad proteins and with the bHLH protein HES-1 have been shown to modulate Cbfa1 function *in vitro* (Hanai *et al.,* 1999; McLarren *et al.,* 2000). It is likely that the coming years will provide more detailed insights into the signaling pathways controlling Cbfa1 function in osteoblasts. Potentially, this area of research could lead to the identification of compounds that specifically regulate Cbfa1 activity and that may be useful in future therapeutic approaches to treat degenerative bone diseases.

Transcriptional Regulation of *Cbfa1* Expression and the Role of Homeodomain Proteins

The importance of understanding the molecular mechanisms controlling the rate of *Cbfa1* expression in osteoblasts is obvious, as haploinsufficiency at the *Cbfa1* locus leads to severe skeletal dysplasia. Thus, it is conceivable that a moderate increase in the level of Cbfa1 may lead to increased bone formation by activating either, osteoblast differentiation or function. However, given the size and the complex organization of the *Cbfa1* gene (Geoffroy *et al.,* 1998), progress in understanding the regulation of *Cbfa1* expression has been rather slow so far.

One important regulator of *Cbfa1* expression is Cbfa1 itself. Three OSE2-like elements are present in the mouse *Cbfa1* gene, one of them located in the proximal promoter, the others 3′ from the transcriptional start site (Ducy *et al.,* 1999). These elements are high-affinity-binding sites for Cbfa1 and activate transcription *in vitro* in a Cbfa1-dependent manner. The fact that Cbfa1 activates its own expression *in vivo* is clearly demonstrated by the finding that its expression is nearly abolished in transgenic mice expressing a dominant-negative variant of *Cbfa1* in osteoblasts (Ducy *et al.,* 1999). This suggests that once *Cbfa1* expression is turned on in osteoprogenitor cells, it is enhanced by an autoregulatory feedback loop and is maintained during the course of differentiation and thereafter. Thus, the nature of the molecules that are initially activating *Cbfa1* expression becomes even more important. Recently described mouse genetic experiments have given some initial answers to this question. They have shown that certain homeodomain transcription factors are involved in controlling *Cbfa1* expression in specific skeletal elements.

Msx1 and *Msx2* are two mammalian homologues of the *Drosophila* muscle segment homeobox gene *(msh)*. Both factors have been suggested to play roles in osteoblast differentiation, as binding sites were found in the *Osteocalcin* promoters of mouse and rat (Towler *et al.,* 1994; Hoffmann *et al.,* 1994). Msx2 especially gained further attention because it was found to act as a transcriptional repressor of osteoblast-specific gene expression *in vitro* (Towler *et al.,* 1994; Dodig *et al.,* 1999). Several genetic experiments have helped to understand the role of Msx2 as a regulator of osteoblast differentiation *in vivo*. Transgenic mice overexpressing *Msx2* under the control of its own promoter are characterized by enhanced calvarial bone growth resulting from an increased number of 5′-bromo-2′-deoxyuridine (BrdU)-positive osteoblastic cells at the osteogenic fronts (Liu *et al.,* 1999). Likewise, a gain-of-function mutation of *MSX2* in humans causes Boston-type craniosystosis (Jabs *et al.,* 1993). In contrast, *Msx2*-deficient mice display

a defective ossification of the skull resulting from decreased proliferation of osteoblast progenitor cells (Satokata *et al.,* 2000). A similar defect is observed in the skull of patients affected with enlarged parietal foramina, a disease caused by a loss-of-function mutation of *MSX2* (Wilke *et al.,* 2000). Taken together, these data demonstrate that Msx2 is an important regulator of craniofacial bone development and that it acts by maintaining osteoblast precursors in a proliferative stage through inhibition of their terminal differentiation. *Msx2*-deficient mice also display defects in endochondral bone formation. In the tibia and the femur of these mice the numbers of osteoblasts are reduced, thus leading to a decrease in trabecular and cortical thickness. The expression of *Osteocalcin* and *Cbfa1* is strongly reduced in *Msx2*-deficient mice. This suggests that Msx2 is required for osteoblast differentiation in endochondral bones and is a positive regulator of *Cbfa1* expression *in vivo*.

The fact that the expression patterns of *Msx1* and *Msx2* are partially overlapping during craniofacial development suggested a functional redundancy between these two related transcription factors. This has been demonstrated by the generation of mice lacking both *Msx1* and *Msx2*. In these double mutant mice, calvarial ossification is completely absent, leading to perinatal lethality (Satokata *et al.,* 2000). This finding underscores the critical importance of Msx proteins for craniofacial bone formation. However, their relationship to *Cbfa1* expression in both intramembranous and endochondral ossification needs to be investigated further.

Dlx5, a homologue of Distal-less (Dll) in *Drosophila,* is another homeodomain transcription factor that has been suggested to play a role in osteoblast differentiation based on its upregulation in mineralizing calvarial cultures (Ryoo *et al.,* 1997). Transfection of an osteoblastic cell line with a *Dlx5* expression vector results in an increased production of Osteocalcin and in accelerated maturation of a mineralized matrix (Miyama *et al.,* 1999). Importantly, *Cbfa1* expression is not affected in these experiments, suggesting that Dlx5 does not act upstream of Cbfa1 to promote osteoblast differentiation *in vitro* (Miyama *et al.,* 1999). *In vivo, Dlx5* is expressed early during skeletal development in the cranial neural crest and the developing limbs (Simeone *et al.,* 1994; Depew *et al.,* 1999). In adult mice, *Dlx5* expression is detectable in brain, long bones, and calvaria, but not in several other tissues (Ryoo *et al.,* 1997, Miyama *et al.,* 1999). *Dlx5*-deficient mice have been generated (Acampora *et al.,* 1999; Depew *et al.,* 1999). These mice die shortly after birth due to severe defects in craniofacial development. Most of the cranial bones and teeth in *Dlx5*-deficient mice are dysmorphic. It is not clear yet if these abnormalities are the result of patterning defects or if they are a direct consequence of impaired osteoblast differentiation. In contrast, no overt defects are observed in limbs and other appendages. However, upon closer examination Acampora *et al*. (1999) found a mild increase in Osteocalcin-positive periostal cells in femurs of newborn *Dlx5*-deficient mice. This indicates that in long bones, Dlx5 may act as a repressor of *Osteocalcin* expression and possibly osteoblast differentiation. Importantly, *Cbfa1* expression is not affected in *Dlx5*-deficient mice, suggesting that Dlx5 uses a Cbfa1-independent pathway to regulate osteoblast differentiation (Acampora *et al.,* 1999).

The severity of phenotype of *Dlx5*-deficient mice, especially in endochondral bones, may be blunted by a functional redundancy with *Dlx6,* another *Dll*-related gene that is coexpressed with *Dlx5* in developing skeletal structures (Simeone *et al.,* 1994). Thus, the generation of *Dlx5/Dlx6* double mutants may be required to fully uncover the role of Dlx proteins in osteoblast differentiation. One interesting aspect of the *in vitro* experiments is that Dlx5 induces *Osteocalcin* expression in the calvaria-derived cell line MC3T3-E1 (Miyama *et al.,* 1999), whereas it leads to decreased *Osteocalcin* expression in the long bone-derived osteosarcoma cell line ROS 17/2.8 (Ryoo *et al.,* 1997). These findings are in fact consistent with the phenotype of *Dlx5*-deficient mice. They suggest that the role of Dlx5 in osteoblast differentiation may differ in the two types of ossification. The same may be the case for Msx2 where the defects in calvaria and long bones could be caused by different mechanisms.

Another homeodomain protein controlling *Cbfa1* expression at an early stage of osteoblast differentiation is Bapx1, a homologue of the *Drosophila* transcription factor bagpipe. *Bapx1* is expressed early in development in the sclerotome of the somites and later in the cartilaginous condensations prefiguring the future skeleton (Tribioli *et al.,* 1997). *Bapx1*-deficient mice die at birth and display a severe dysplasia of the axial skeleton characterized by malformations or absence of specific skeletal elements in the vertebral column and craniofacial structures. In contrast, the appendicular skeleton is not affected (Tribioli and Lufkin, 1999; Lettice *et al.,* 1999). This phenotype is a consequence of impaired cartilage formation because several markers of chondrocyte differentiation, such as *α1(II) Collagen*, *Indian hedgehog*, and *Sox9* are downregulated in *Bapx1*-deficient mice (Tribioli and Lufkin, 1999). In wild-type mice, *Cbfa1* is coexpressed with these chondrocytic markers at 12.5 dpc in common mesenchymal precursor cells of chondrocytes and osteoblasts. Accordingly, in *Bapx1*-deficient mice, *Cbfa1* is downregulated in these precursor cells (Tribioli and Lufkin, 1999). This indicates that at a very early stage of osteoblast differentiation, Bapx1 acts upstream of Cbfa1 in some skeletal elements. It remains to be determined if Bapx1 regulates *Cbfa1* expression in these elements directly or if this regulation is mediated through other genes downstream of *Bapx1*.

Hoxa-2 is a homeodomain transcription factor that prevents *Cbfa1* expression specifically in skeletal elements of the second branchial arch. This is demonstrated by the fact that *Cbfa1* is upregulated in this region of *Hoxa*-2-deficient mice, resulting in ectopic bone formation (Kanzler *et al.,* 1998). Additionally, transgenic mice expressing *Hoxa-2* in craniofacial bones under the control of the *Msx2* promoter

lack several bones in the craniofacial area, indicating that *Hoxa-2* acts as an inhibitor of intramembranous bone formation (Kanzler *et al.,* 1998). Thus, Hoxa-2 inhibits ectopic bone formation in the second branchial arch by downregulating, directly or indirectly, the expression of *Cbfa1* in this region.

Taken together, these results suggest that in contrast to *Cbfa1,* a determining gene for osteoblast differentiation in all skeletal elements, transcription factors acting upstream of Cbfa1 may act only in specific areas of the skeleton. This is also the case for the growth factor Indian hedgehog required for *Cbfa1* expression and osteoblast differentiation only in bones formed by endochondral ossification (St-Jacques *et al.,* 1999). Although mouse genetic experiments have already revealed some genes that influence *Cbfa1* expression *in vivo,* in all cases molecular biology experiments are required to analyze how these effects are mediated. Additionally, the fact that Dlx5 has an influence on osteoblast differentiation without affecting *Cbfa1* expression suggests that parallel pathways may play a role in differentiation along the osteoblast lineage and may be specific for certain skeletal elements. Thus, the next section focuses on further transcription factors that possibly have important functions during osteoblast differentiation, although their connection to Cbfa1 is still unknown.

Are There Additional Osteoblast-Specific Transcription Factors in Addition to Cbfa1?

Given what we have learned from the study of other cell lineages, where multiple transciption factors are required for differentiation, it is likely that other transcription factors that are specifically expressed in cells of the osteoblast lineage are required to activate osteoblast differentiation throughout the skeleton or in parts of the skeleton. Molecular biology and biochemical experiments have provided evidence for the existence of at least two additional osteoblast-specific transcription factors that remain to be identified.

As was the case for the identification of Cbfa1, one of these factors was initially discovered through the analysis of a short promoter fragment of the mouse *Osteocalcin* gene (Ducy and Karsenty, 1995). On addition to OSE2, the binding site of Cbfa1, this promoter fragment includes another osteoblast-specific *cis*-acting element, termed OSE1, that is equally important as OSE2 for *Osteocalcin* promoter activity *in vitro* and in transgenic mice (Schinke and Karsenty, 1999). Multimers of OSE1 confer osteoblast-specific activity to a heterologous promoter *in vitro*, and an osteoblast-specific nuclear factor, provisionally termed Osf1, binds to the OSE1 oligonucleotide in electrophoretic mobility shift assays (Ducy and Karsenty, 1995; Schinke and Karsenty, 1999). The identification of Osf1 is complicated by the fact that the OSE1 core sequence does not share obvious similarities to binding sites of known transcription factors, suggesting that Osf1 may be a novel protein. Interestingly, Osf1-binding activity declines during the differentiation of mouse primary osteoblasts, being absent in fully mineralized cultures, suggesting that Osf1 is a stage-specific transcription factor that may act early during osteoblast differentiation. The fact that a functional OSE1 site is present in the *Cbfa1* promoter raises the hypothesis that Osf1 may act upstream of Cbfa1 (Schinke and Karsenty, 1999). However, the question of where Osf1 resides in a genetic cascade controlling osteoblast-specific gene expression will need to be addressed once a cDNA becomes available.

Another osteoblast-specific *cis*-acting element has been identified in the promoter of the rat and mouse *pro-α1(I) collagen* genes (Dodig *et al.,* 1996; Rossert *et al.,* 1996). *α1(I) collagen* is not specifically expressed by osteoblasts, but it has been shown that separate *cis*-acting elements control its expression in different cell types (Pavlin *et al.,* 1992; Rossert *et al.,* 1995). After initially identifying a 2.3-kb *α1(I) collagen* promoter fragment that directs bone-specific expression of a reporter gene in transgenic mice, Rossert *et al*. (1996) further narrowed down the critical region to a 117-bp promoter fragment of the mouse *α1(I) collagen* gene. Multimers of this 117-bp fragment cloned upstream of a minimal *α1(I) collagen* promoter led to osteoblast-specific expression of a *lacZ* reporter gene in transgenic mice. An osteoblast-specific factor binds to a sequence within this element in electrophoretic mobility shift assays. Although this sequence is similar to binding sites of homeodomain transcription factors, the molecular nature of the osteoblast-specific factor binding to it is still unknown. At the same time, Dodig *et al*. (1996) described the presence of the same element in the rat *α1(I) collagen* promoter. The authors found that Msx2 can bind to this element in electrophoretic mobility shift assays. However, overexpression of *Msx2* in an osteoblastic cell line led to a reduction of *α1(I) collagen* expression, and the expression pattern of *Msx2* during the course of osteoblast differentiation did not correlate with the observed binding activity. In conclusion, this element may serve as a binding site for an osteoblast-specific transcription factor, also called bone-inducing factor, that remains to be identified. This factor may act at later stages during osteoblast differentiation as the binding activity is only observed in differentiated osteoblast cultures (Dodig *et al.,* 1996).

The Function of AP-1 Family Transcription Factors during Osteoblast Differentiation

Activator protein 1 (AP-1) is a dimeric complex of Fos and Jun proteins that belongs to the bZIP transcription factor family. Known members of the AP-1 family are c-Fos, FosB, and the Fos-related antigens Fra-1 and Fra-2 that heterodimerize with c-Jun, JunB, or JunD (Karin *et al.,* 1997). Several lines of evidence suggest that members of the AP-1 family are involved in the regulation of osteoblast

differentiation. First, functional AP-1-binding sites have been found in the promoter regions of several genes expressed in osteoblasts, including *alkaline phosphatase*, *α1(I) collagen*, and *Osteocalcin* (McCabe *et al.,* 1996). Second, a number of extracellular signaling molecules, such as TGF*β* and PTH, have been shown to induce the expression of AP-1 components in osteoblastic cells (Clohisy *et al.,* 1992; Lee *et al.,* 1994). Third, various members of the AP-1 family have been shown to be expressed in osteoblast cultures and can be detected at sites of active bone formation *in vivo* (Dony and Gruss, 1987). *In vitro*, most of these factors are expressed preferentially in the proliferative stage before the onset of osteoblast differentiation. The exception is Fra-2, which is expressed at later stages (McCabe *et al.,* 1996). The best argument for a role of AP-1 transcription factors in osteoblast differentiation comes from *in vivo* studies where it has been shown that certain AP-1 family members can affect osteoblast proliferation or differentiation, although the underlying mechanisms are not fully understood yet.

One AP-1 family member affecting osteoblast proliferation is c-Fos. The overexpression of *c-Fos* in transgenic mice under the control of the MHC class I H2-K^b promoter leads to osteosarcomas in all mice examined (Grigoriadis *et al.,* 1993). Such a phenotype is not observed in mice overexpressing *FosB* or *cJun*, thus indicating that the impairment of osteoblast proliferation is a specific property of c-Fos. This finding is consistent with the high level of *c-Fos* expression observed in murine und human osteosarcomas (Schon *et al.,* 1986; Wu *et al.,* 1990) and suggests that c-Fos is one regulator of osteoblast proliferation *in vivo*. However, the phenotype of *c-Fos*-deficient mice argues against such a function. These mice display a severe osteopetrosis due to a decreased differentiation of bone-resorbing osteoclasts (Johnson *et al.,* 1992; Wang *et al.,* 1992). Importantly, there are no defects of bone formation in *c-Fos*-/- mice, indicating that osteoblast proliferation and differentiation are not impaired. There are two possibilities to explain this discrepancy between the gain-of-function and the loss-of-function experiment. First, there could be functional redundancies, and other transcription factors belonging to the AP-1 family could fulfill the function of c-Fos in osteoblasts of *c-Fos*-/- mice. Second, the overexpression of *c-Fos* in transgenic mice could perturb the function of yet another unidentified bZIP transcription factor that would be required to regulate osteoblast proliferation in the physiological situation.

Two other AP-1 family members, both lacking a transcriptional activation domain, have been shown to affect the differentiation of osteoblasts *in vivo*. The expression of *Fra-1* in transgenic mice under the control of the MHC class I H2-K^b promoter leads to a severe osteosclerosis due to an increased number of osteoblasts (Jochum *et al.,* 2000). This phenotype is explained by a cell autonomous increase in osteoblast differentiation. Interestingly, *Cbfa1* expression is not altered in primary osteoblast cultures derived from these transgenic mice, although their differentiation is accelerated *ex vivo*. This suggests that the effect of Fra-1 on osteoblast differentiation is independent of the Cbfa1 pathway. This is confirmed by the fact that the overexpression of *Fra-1* on a *Cbfa1*+/− background leads to osteosclerosis without rescuing the CCD phenotype of the *Cbfa1*+/− mice (Jochum *et al.,* 2000). Fra-1 appears to be dispensable for osteoblast differentiation *in vivo*, as newborn *Fra-1*-/- mice derived by injecting *Fra-1*-/- embryonic stem cells into tetraploid wild-type blastocysts show no defects in osteoblastogenesis (Jochum *et al.,* 2000). However, this question will be better addressed by an osteoblast-specific deletion of *Fra-1*, and also of *c-Fos*. Interestingly, transgenic mice expressing Δ*FosB*, a splice variant of *FosB* lacking an apparent transactivation domain, display an osteosclerotic phenotype similar to the one observed in mice overexpressing *Fra-1* (Sabatakos *et al.,* 2000). The only difference between these two mouse models is the fact that the cell autonomous increase of osteoblast differentiation in *ΔFosB* transgenic mice is at the expense of adipogenesis that is reduced significantly in these mice. Again, inducing osteoblast differentiation does not seem to be a physiologic function of ΔFosB because *FosB*-/- mice have normal bone formation (Gruda *et al.,* 1996).

The fact that both Fra-1 and ΔFosB lack a major transcriptional activation domain (Metz *et al.,* 1994) is puzzling and led to the suggestion of two possible mechanisms to explain the osteosclerosis in both transgenic mouse models (Karsenty, 2000). The first possibility is that both factors heterodimerize with an activator of osteoblast differentiation to increase its DNA binding or transactivation ability. In contrast, both factors could heterodimerize with an inhibitor of osteoblast differentiation and therefore act by releasing a brake on osteoblast differentiation that might exist in the physiological situation. Taken together, all these lines of evidence strongly suggest that bZIP transcription factors play a critical role in osteoblast proliferation and differentiation. Although the evidence is only based on overexpression studies so far, c-Fos, Fra-1, and ΔFosB provide excellent handles to elucidate the molecular mechanisms underlying osteoblast differentiation in the normal situation.

Perspectives

Our knowledge about the transcriptional control of osteoblast differentiation and function was almost nonexistent until recently. This has now changed substantially due to the discovery of Cbfa1. There is little doubt that *Cbfa1* is a major player during osteoblast differentiation, although this does not rule out the possibility that other transcription factors may also play important regulatory roles. This premise is supported experimentally by the fact that other osteoblast-specific DNA-binding activities have been described, but also by the severe bone phenotypes of mice overexpressing certain AP-1 transcription factors. The identification of further osteoblast-specific transcription

factors and the elucidation of the molecular mechanisms underlying the AP-1 action on osteoblasts will definitely give a more complete picture of the regulation of osteoblast differentiation in the future. Also, future studies should provide a link between the transcription factors and the extracellular signaling molecules that affect osteoblast differentiation. Additionally, once we know more about the molecules involved, it should be possible to uncover a genetic cascade controlling osteoblast differentiation and function, as it is already known for the bone-resorbing osteoclast (Karsenty, 1999). This would lead to a more complete understanding of bone remodeling, a balanced process mediated by osteoblasts and osteoclasts, and could provide several therapeutic targets for the treatment of degenerative bone diseases, mostly osteoporosis, where bone formation is decreased relative to bone resorption.

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CHAPTER 6

The Osteocyte

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Introduction

The osteocyte is the most abundant cell type of bone. There are approximately 10 times as many osteocytes as osteoblasts in adult human bone (Parfitt, 1977), and the number of osteoclasts is only a fraction of the number of osteoblasts. Our current knowledge of osteocytes, however, lags behind what we know of the properties and functions of both osteoblasts and osteoclasts. However, the striking structural design of bone predicts an important role for osteocytes.

Considering that osteocytes have a very particular location in bone, not on the bone surface but spaced regularly throughout the mineralized matrix, and considering their typical morphology of stellate cells, which are connected with each other via long, slender cell processes, a parallel with the nerve system springs to one's mind. Are the osteocytes the "nerve cells" of the mineralized bone matrix, and if so, what are the stimuli that "excite" the cells? The answers to these questions may very well come from studies in which biomechanical concepts and techniques are applied to bone cell biology. Both theoretical considerations and experimental results have strengthened the notion that osteocytes are the pivotal cells in the biomechanical regulation of bone mass and structure (Cowin *et al.,* 1991; Mullender and Huiskes, 1994, 1995; Klein-Nulend *et al.* 1995b). This idea poses many new questions that have to be answered. By which mechanism(s) are loading stimuli on bone translated into biochemical stimuli that regulate bone (re)modeling and what is the nature of these signaling molecules? How and where do the mechanical and hormonal regulatory systems of bone interact? Are osteocytes mere signaling cells or do they contribute actively to bone metabolic processes such as mineralization, a process that takes place around newly incorporated osteocytes at some distance of the bone matrix formation front?

The development of osteocyte isolation techniques, the use of highly sensitive (immuno)cytochemical and *in situ* hybridization procedures, and the usefulness of molecular biological methods even when only small numbers of cells are available have rapidly increased our knowledge about this least understood cell type of bone in the recent past and will certainly continue to do so in the future. This chapter compiles and analyzes the most recent findings and it is hoped that it contributes to the development of new ideas and thoughts about the role of osteocytes in the physiology of bone.

The Osteocytic Phenotype

The Osteocyte Syncytium

Mature osteocytes are stellate shaped or dendritic cells enclosed within the lacunocanalicular network of bone. The lacunae contain the cell bodies. From these cell bodies, long, slender cytoplasmic processes radiate in all directions, but with the highest density perpendicular to the bone surface (Fig. 1). They pass through the bone matrix via small canals, the canaliculi. Processes and their canaliculi may be branched. They appear generally not to cross the cement lines separating adjacent osteons. The more mature osteocytes are connected by these cell processes to neighboring osteocytes, the most recently incorporated osteocytes to neighboring osteocytes and to the cells lining the bone surface. Some of the processes oriented to the bone surface, however, appear not to connect with the lining

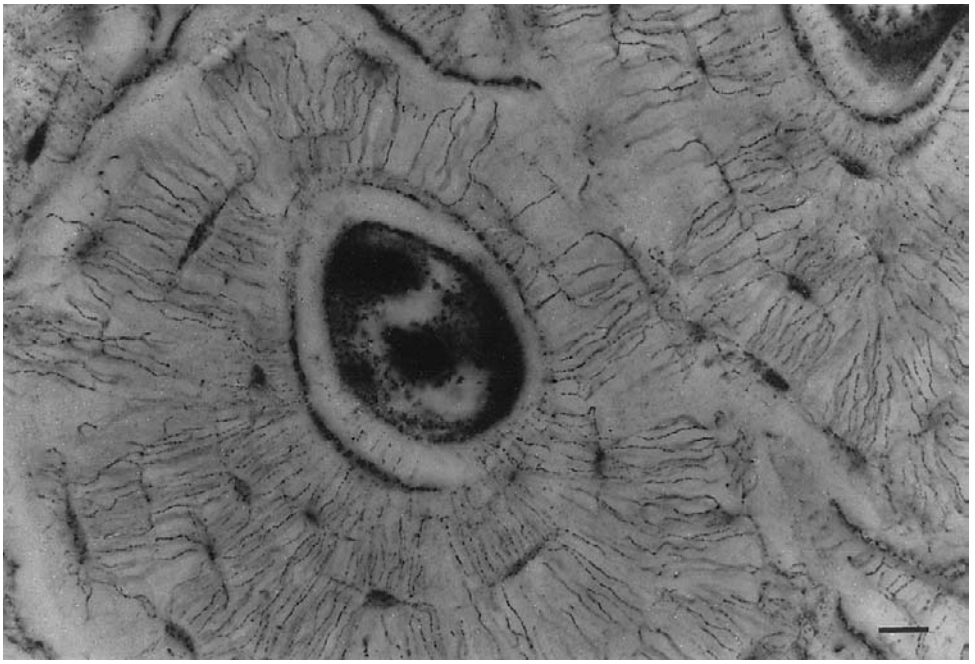

Figure 1 Osteon in mature human bone. Osteocytes are arranged in concentric circles around the central haversian channel. Note the many cell processes, radiating from the osteocyte cell bodies, in particular in the perpendicular directions. Schmorl staining. (Original magnification: ×390; bar: 25μm.)

cells, but pass through this cell layer, thereby establishing a direct contact between the osteocyte syncytium and the extraosseus space. This intriguing observation by Kamioka *et al*. (2001) suggests the existence of a signaling system between the osteocyte and the bone marrow compartment without intervention of the osteoblasts/lining cells.

The typical morphology of the osteocyte was originally thought to be enforced on differentiating osteoblasts during their incorporation in the bone matrix. Osteocytes have to remain in contact with other cells and ultimately with the bone surface to ensure the access of oxygen and nutrients. Culture experiments with isolated osteocytes have shown, however, that although the cells lose their stellate shape in suspension, they reexpress this morphology as soon as they settle on a support (Van der Plas and Nijweide, 1992) (Fig. 2). Apparently, the typical stellate morphology and the need to establish a cellular network are intrinsic characteristics of terminal osteocyte differentiation.

In bone, gap junctions are present between the tips of the cell processes of connecting osteocytes (Doty, 1981). Within each osteon or hemiosteon (on bone surfaces), therefore, osteocytes form a syncytium of gap junction-coupled cells. As the lacunae are connected via the canaliculi, the osteocyte syncytium represents two network systems: an intracellular one and an extracellular one. This feature is probably the key to understanding the function of the osteocyte.

Osteocyte Formation and Death

Osteogenic cells arise from multipotential mesenchymal stem cells (see Chapter 4). These stem cells have the capacity to also differentiate into other lineages, including those of chondroblasts, fibroblasts, adipocytes, and myoblasts (Aubin *et al.,* 1995; Chapter 4). By analogy with hemopoietic differentiation, each of these differentiation lineages is thought to originate from a different committed progenitor, which for the osteogenic lineage is called the osteoprogenitor. Osteodifferentiation progresses via a number of progenitor and precursor stages to the mature osteoblast. Osteoblasts may then differentiate to the ultimate differentiation stage, the osteocyte. The mechanism by which osteoblasts differentiate into osteocytes is, however, still unknown. Marotti (1996) has postulated that a newly formed osteocyte starts to produce an osteoblast inhibitory signal when its cytoplasmic processes connecting the cell with the osteoblast layer have reached their maximal length. The osteoid production of the most adjacent, most intimately connected osteoblast will be relatively more inhibited by that signal than that of its neighbors. The inactivated osteoblast then spreads over a larger bone surface area, thereby even reducing its linear appositional rate of matrix production further. A second consequence of the widening of the cell is that it may intercept more osteocytic processes carrying the inhibitory signal. This positive feedback mechanism results in the embedding of the cell in matrix produced by the neighboring osteoblasts. Ultimately, the cell will acquire the typical osteocyte morphology and the surrounding matrix will become calcified. The theory of Marotti is based entirely on morphological observations. There is no biochemical evidence about the nature or even the existence of the proposed inhibitory factor. Martin (2000) has, however, used the concept successfully in explaining

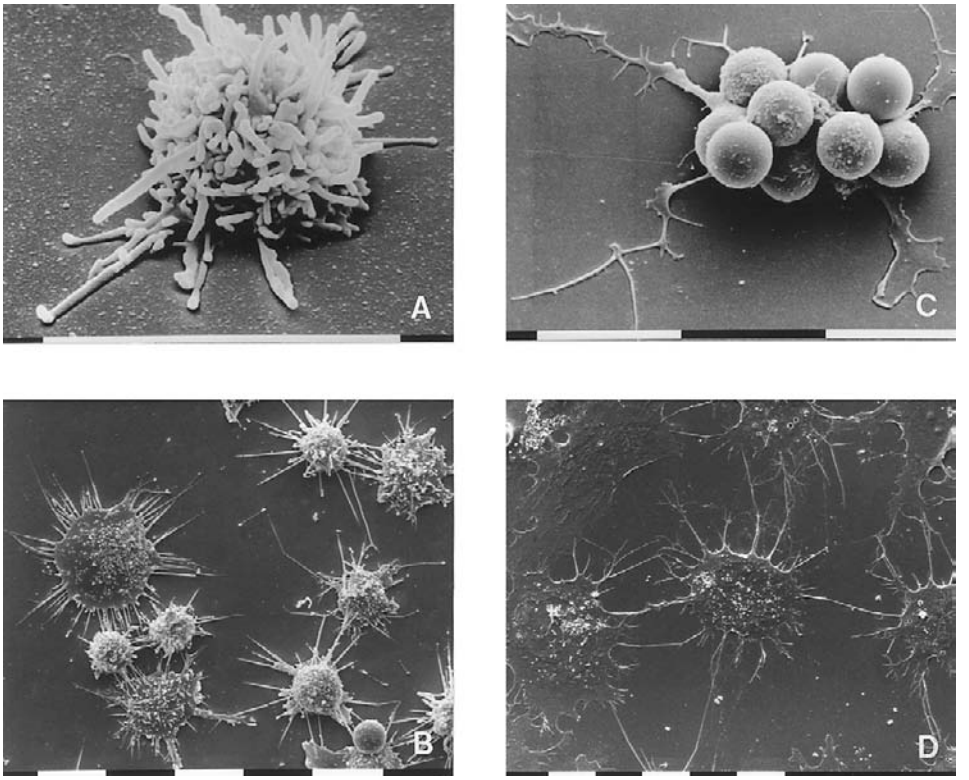

Figure 2 Isolated osteocytes in culture. Osteocytes were isolated by an immunodissection method using MAb OB7.3-coated magnetic beads. After isolation the cells were seeded on a glass support, cultured for 5 min (A), 30 min (B), or 24 hr (C and D) and studied with a scanning electron microscope. Immediately after attachment, osteocytes form cytoplasmic extrusions in all directions (A). During subsequent culture the cell processes perpendicular on the support disappear, while the processes in the plane of the support elongate (B) and ultimately form smooth connections between neighboring cells (D). In A, B, and D the immunobeads were removed from the cells before seeding, in C the beads were left on the cells. (Original magnification: (A) $\times$7200, (B) $\times$1400, (C) $\times$2900, and (D) $\times$940; bar: 10μm).

mathematically the changing rates of matrix formation during bone remodeling.

As described earlier, osteocytes derive from active osteoblasts. Most bone surfaces, particularly in mature bone, are, however, occupied by inactive (in terms of matrix production) bone-lining cells. How then is bone formation and osteocyte differentiation on an inactive bone surface started? Imai *et al*. (1998) found evidence that osteocytes may stimulate osteoblast differentiation and the accompanying matrix and osteocyte formation by expressing osteoblast stimulating factor-1 (OSF-1) (Tezuka *et al.,* 1990). OSF-1 or heparin-binding, growth-associated molecule (HB-GAM) (Rauvala, 1989) accumulates on bone surfaces near by the osteocytes that produce it (Imai *et al.,* 1998) and induces osteoblast formation by coupling to *N*-syndecan, a receptor for OSF-1 (Raulo *et al.,* 1994), present on osteoblast precursor cells. According to Imai *et al*. (1998), the expression of OSF-1 in osteocytes may be activated by local damage to bone or local mechanical stress. The issue of stress-mediated induction of bone formation and the role of osteocytes are discussed in more detail in the second part of this chapter.

The life span of osteocytes is probably largely determined by bone turnover, when osteoclasts resorb bone and "liberate" osteocytes. Osteocytes may have a half-life of decades if the particular bone they reside in has a slow turnover rate. The fate of living osteocytes that are liberated by osteoclast action is presently unknown. There is little evidence that osteocytes may reverse their differentiation back into the osteoblastic state (Van der Plas *et al.,* 1994). Some of them, only half released by osteoclastic activity, may be reembedded during new bone formation that follows the resorption process (Suzuki *et al.,* 2000). These osteocytes are then the cells that cross the cement lines between individual osteons, sometimes seen in cross sections of osteonal bone. Most of the osteocytes, however, will probably die by apoptosis and become phagocytosed. Phagocytosis of osteocytes by osteoclasts as part of the bone resorption process has been documented in several reports (Bronckers *et al.,* 1996; Elmardi *et al.,* 1990). Apoptosis of osteocytes in their lacunae is attracting growing attention because of its expected consequence of decreased bone mechanoregulation, which may lead to osteoporosis. Apoptotic changes in osteocytes were shown to be associated with high bone turnover (Noble *et al.,* 1997). However, fatigue-related microdamage in bone may cause decreased osteocyte accessibility for nutrients and oxygen

inducing osteocyte apoptosis and subsequent bone remodeling (Burger and Klein-Nulend, 1999; Verborgt *et al.,* 2000). Also, loss of estrogen (Tomkinson *et al.,* 1998) and chronic glucocorticoid treatment (Weinstein *et al.,* 1998) were demonstrated to induce osteocyte apoptosis, which may, at least in part, explain the bone-deleterious effects of these conditions.

Osteocyte Isolation

Analysis of osteocyte properties and functions has long been hampered by the fact that they are embedded in a mineralized matrix. Although sensitive methods are now available, such as immunocytochemistry and *in situ* hybridization, by which osteocytes can be studied in the tissue in some detail, osteocyte isolation and culture offer a major step forward. This approach became possible by the development of osteocyte-specific antibodies (Fig. 3) directed to antigenic sites on the outside of the cytoplasmic membrane (Bruder and Caplan, 1990; Nijweide and Mulder, 1986). Using an immunodissection method, Van der Plas and Nijweide (1992) subsequently succeeded in the isolation and purification of chicken osteocytes from mixed bone cell populations isolated from fetal bones by enzymatic digestion. Isolated osteocytes appeared to behave *in vitro* like they do *in vivo* in that they reacquired their stellate morphology and, when seeded sparsely, formed a network of cells coupled to one another by long, slender, often branched cell processes (Fig. 2). The cells retained this morphology in culture throughout the time studied (5–7 days) and even reexpressed it when passaged for a second time (Van der Plas and Nijweide, 1992). As *in vivo* isolated osteocytes were postmitotic (Van der Plas *et al.,* 1994). When seeded on dentin slices, they did not resorb or dissolve the dentin to any extent when observed with a scanning electron microscope. These results therefore do not support the earlier hypothesis of osteocytic osteolysis as a function for osteocytes (Bélanger, 1969).

Mikuni-Takagaki *et al*. (1995) have isolated seven cell fractions from rat calvariae by sequential digestion. They claimed that the last fraction consisted of osteocytic cells. The cells displayed dendritic cell processes, were negative for alkaline phosphatase, had high extracellular activities of casein kinase II and ecto-5'nucleotidase, and produced large amounts of osteocalcin. After a few days of little change in cell number, the cells of fraction VII proliferated, however, equally fast as those of fraction III, the osteoblastic cells, in culture. Because osteocyte-specific antibodies are not yet available for the rat system, the final identification of the cells in fraction VII has to await the development of additional identification methods.

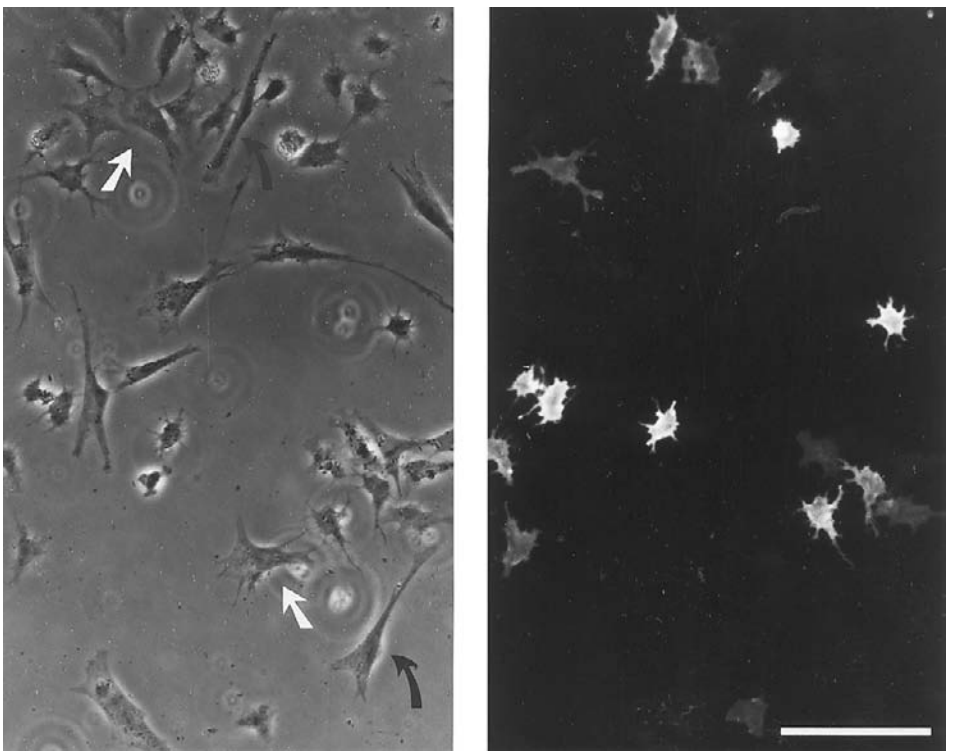

Figure 3 MAb OB7.3 immunostaining of osteocytes. Cells were isolated from periosteum-free 18-day-old chicken calvariae by collagenase digestion, seeded, and cultured for 24 hr. Subsequently the osteocytes in the mixed population were specifically stained with MAb OB7.3 in combination with biotinylated horse–antimouse IgG and streptavidin-Cy3. (Left) Phase contrast. (Right) Immunofluorescence. Black arrows, fibroblast-like cells; white arrows, osteoblast-like cells. (Original magnification: ×300; bar: 100 μm.)

Osteocytic Cell Lines

Because the number of osteocytes that can be isolated each time (Van der Plas and Nijweide, 1992) is limited, several groups have tried to establish osteocytic cell lines. Basically, an osteocytic cell line is a *contradictio in terminis*. Osteocytes are postmitotic. However, a cell line of proliferating precursor cells that would differentiate to osteocytes under specific circumstances, could prove to be very valuable in the study of osteocyte properties and functions. HOB-01-C1 (Bodine *et al.,*1996) may meet these requirements. It is a temperature-sensitive cell line, prepared from immortalized, cloned human adult bone cells. It proliferates at 34°C but stops dividing at 39°C. HOB-01-C1 cells display putative osteocytic markers, such as cellular processes, low alkaline phosphatase activity, high osteocalcin production, and the expression of CD44.

MLO-Y4 (Kato *et al.,* 1997) is another osteocyte-like cell line in that the cells, when seeded at low density, display complex dendritic processes. They produce high amounts of osteocalcin and osteopontin, express CD44, and have low alkaline phosphatase activity. MLO-Y4 cells are, however, strongly proliferative.

The problem of defining these cell lines as really osteocytic lies in the fact that the markers just mentioned are each on their own not osteocyte specific (see later) although the combination of their markers may resemble that of the osteocyte phenotype.

Osteocyte Markers

In bone, osteocytes are fully defined by their location within the bone matrix and their typical stellate morphology. Related to this stellate morphology, osteocytes have a typical cytoskeletal organization. The prominent actin bundles in the osteocytic processes, together with the abundant presence of the actin-bundling protein fimbrin, are exemplary for osteocytes and are retained after isolation (Tanaka-Kamioka *et al.,* 1998). In addition, osteocytes are generally found to express osteocalcin, osteonectin, and osteopontin, but show little alkaline phosphatase activity, particularly the more mature cells (Aarden *et al*. 1996b). As stated previously, these metabolic markers have, however, little discriminating value in mixtures of isolated cells. A promising newly found protein in this class of markers is OF45 (Petersen *et al.,* 2000), a RGD-containing matrix protein particularly expressed by bone-embedded osteocytes. Its degree of mRNA expression appears to correlate with the progressing differentiation of osteoblastic cells *in vitro*. However, whether an antibody generated with the protein can specifically recognize osteocytes in bone cell mixtures is not yet clear. The function of the protein is also not known.

At present the best markers for isolated osteocytes are their typical morphology, which they reacquire in culture (Mikuni-Takagaki *et al.,* 1995; Van der Plas and Nijweide, 1992) in combination with their reaction with monoclonal antibodies, which have been proven to be osteocyte specific in tissue sections. Examples are the monoclonal antibodies MAb OB7.3 (Nijweide and Mulder, 1986) (Fig. 3), MAb OB37.11 (Nijweide *et al.,* 1988), and MAb SB5 (Bruder and Caplan, 1990). All three are specific for avian osteocytes and do not cross-react with mammalian cells. The antigenic sites of OB7.3 and OB37.11 are not identical (Aarden *et al.,* 1996a). Whether SB5 reacts with either or reacts with another, different site is not known. The identities of the three antigens involved have not been reported, although that of OB7.3 has been elucidated (Westbroek *et al.,* submitted). E11 is a monoclonal antibody that reacts specifically with highly mature osteoblasts and with osteocytes in tissue sections of rat bone (Wetterwald *et al.,* 1996). In primary rat osteoblast and ROS 17/2.8 cultures the antibody recognizes a subset of cells (Schulze *et al.,* 1999; Wetterwald *et al.,* 1996). The antigen of the antibody is OTS-8, a transmembrane protein that interacts with the membrane-bound glycoprotein CD44 (Ohizumia *et al.,* 2000). Because CD44 is involved in cell attachment and cell movement and because the E11 antigen is only present in young, recently-embedded osteocytes and is limited in osteoblasts to their basal side (Wetterwald *et al.,* 1996), it is attractive to hypothesize that the CD44–OTS-8 complex is associated with the formation of dendritic processes during osteocyte formation. Osteocytes were indeed found to be strongly immunoreactive to CD44, whereas, again, in osteoblasts attached to the bone surface, CD44 immunoreactivity was restricted to the cytoplasmic processes on the basal side (Nakamura and Ozawa, 1996).

Matrix Synthesis

The subcellular morphology of osteocytes and the fact that they are encased in mineralized matrix do not suggest that osteocytes partake to a large extent in matrix production. Osteocytes, especially the more mature, have relatively few organelles necessary for matrix production and secretion. Nevertheless, a limited secretion of specific matrix proteins may be essential for osteocyte function and survival. Several arguments are in favor of such limited matrix production. First, as the mineralization front lags behind the osteoid formation front in areas of new bone formation, osteocytes may be involved in the maturation and mineralization of the osteoid matrix by secreting specific matrix molecules. It is, however, also possible that osteocytes enable the osteoid matrix to be mineralized by phosphorylating certain matrix constituents, as was suggested by Mikuni-Takagaki *et al*. (1995). However, osteocytes have to inhibit mineralization of the matrix directly surrounding them to ensure the diffusion of oxygen, nutrients, and waste products through the lacunocanalicular system. Osteocalcin, which is expressed to a relative high extent by osteocytes, may play an important role here (Aarden *et al.,* 1996b; Ducy *et al.,* 1996; Mikuni-Takagaki *et al.,* 1995). Finally, if osteocytes are the mechanosensor cells of bone (see later), the attachment of osteocytes to matrix molecules is likely of major importance for the transduction of

stress signals into cellular signals. Production and secretion of specific matrix molecules offer a possibility for the cells to regulate their own adhesion and, thereby, sensitivity for stress signals.

Currently available information about osteocyte capacity to produce certain matrix molecules is based almost entirely on immunocytochemical and *in situ* hybridization studies using sections of bone and isolated osteocytes. Osteocytes have been found positive for osteocalcin and osteonectin (Aarden *et al.,* 1996b), molecules that are probably involved in the regulation of calcification. Osteopontin, fibronectin, and collagen type I (Aarden *et al.,* 1996b) have also been demonstrated in and immediately around (isolated) osteocytes. These proteins may be involved in osteocyte attachment to the bone matrix (see later).

In addition to collagenous and noncollagenous proteins, the bone matrix contains proteoglycans. These macromolecules consist of a core protein to which one or more glycosaminoglycan (GAG) side chains are covalently bound. Early electron microscopical studies (Jande, 1971) already showed that the osteocyte body, as well as its cell processes, is surrounded by a thin layer of unmineralized matrix containing collagen fibrils and proteoglycans. The proteoglycans were shown to consist of chondroitin 4-sulfate, dermatan sulfate, and keratan sulfate with immunocytochemical methods (Maeno *et al.,* 1992; Smith *et al.,* 1997; Takagi *et al.,* 1997). These observations are supported by the findings of Sauren *et al*. (1992), who demonstrated an increased presence of proteoglycans in the pericellular matrix by staining with the cationic dye cuprolinic blue. Of special interest is the reported presence of hyaluronan in osteocyte lacunae (Noonan *et al.,* 1996). CD44, which is highly expressed on the osteocyte membrane, is a hyaluronan-binding protein. CD44 binds, however, also to collagen, fibronectin, and osteopontin (Nakamura and Ozawa, 1996; Yamazaki *et al.,* 1999).

The Osteocyte Cytoskeleton and Cell–Matrix Adhesion

As mentioned earlier, the cell–matrix adhesion of osteocytes is likely of importance for the translation of biomechanical signals produced by loading of bone into chemical signals. Study of the adhesion of osteocytes to extracellular matrix molecules became feasible with the development of osteocyte isolation and culture methods (Van der Plas and Nijweide, 1992). These studies found little difference between the adhesive properties of osteocytes and osteoblasts, although the pattern of adhesion plaques (osteocytes, many small focal contacts, osteoblasts, larger adhesion plaques) was quite different (Aarden *et al.,* 1996a). Both cell types adhered equally well to collagen type I, osteopontin, vitronectin, fibronectin, and thrombospondin. Integrin receptors are involved, as is shown by the inhibiting effects of small peptides containing a RGD sequence on the adhesion to some of these proteins. Adhesion to all aforementioned matrix molecules was blocked by an antibody reacting with the β_1-integrin subunit (Aarden *et al.,* 1996a). The identity of the α units involved is yet unknown.

Deformation of the bone matrix upon loading may cause a physical "twisting" of integrins at sites where osteocytes adhere to the matrix. Integrins are coupled to the cytoskeleton via molecules such as vinculin, talin, and α-actinin. In osteocytes, especially in the osteocytic cell processes, the actin-bundling protein fimbrin appears to play a prominent role (Tanaka-Kamioka *et al.,* 1998). Mechanical twisting of the cell membrane via integrin-bound beads has been demonstrated to induce cytoskeletal rearrangements in cultured endothelial cells (Wang and Ingber, 1994). The integrin–cytoskeleton complex may therefore play a role as an intracellular signal transducer for stress signals. In addition to the integrins, the nonintegrin adhesion receptor CD44 may attribute to the attachment of osteocytes to the surrounding matrix. CD44 is present abundantly on the osteocyte surface (Hughes *et al.,* 1994; Nakamura and Ozawa, 1995) and is also linked to the cytoskeleton.

Hormone Receptors in Osteocytes

Parathyroid hormone (PTH) receptors have been demonstrated on rat osteocytes *in situ* (Fermor and Skerry, 1995) and on isolated chicken osteocytes (Van der Plas *et al.,* 1994). Administered *in vitro*, PTH was reported to increase cAMP levels in isolated chicken osteocytes (Van der Plas *et al.,* 1994; Miyauchi *et al.,* 2000) and, administered *in vivo*, to increase fos protein (Takeda *et al.,* 1999) and the mRNAs of c-fos, c-jun, and Il-6 in rat osteocytes (Liang *et al.,* 1999). Therefore, although the original theory of osteocytic osteolysis and its regulation by calciotropic hormones such as PTH (Bélanger, 1969) has been abandoned, the presence of PTH receptors on osteocytes and their short-term responses to PTH suggest a role for PTH in osteocyte function. As it is now generally accepted that osteocytes are involved in the transduction of mechanical signals into chemical signals regulating bone (re)modeling, PTH might modulate the osteocytic response to mechanical strain. Injection of PTH in rats was shown to augment the osteogenic response of bone to mechanical stimulation *in vivo*, whereas thyroparathyroidectony abrogated the mechanical responsiveness of bone (Chow *et al.,* 1998). However, such an approach cannot separate an effect at the level of osteocyte mechanosensing from one at the level of osteoprogenitor recruitment. One mechanism by which PTH may act on osteocytes is suggested by the reports of Schiller *et al*. (1992) and Donahue *et al*. (1995). These authors found that PTH increases connexin-43 gene expression and gap junctional communication in osteoblastic cells. In osteocytes, where cell-to-cell communication is so important, a similar effect might lead to more efficient communication within the osteocyte syncytium. Activation of receptors for 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], which were also shown to be present in osteocytes immunocytochemically (Boivin *et al.,* 1987) and by *in situ* hybridization (Davideau *et al.,* 1996), may have similar effects.

Another important hormone involved in bone metabolism is estrogen. As is demonstrated in many studies, the decrease of blood estrogen levels is accompanied by a loss of bone mass. One explanation for this phenomenon is that estrogen regulates the set point for the mechanical responsiveness of bone (Frost, 1992), i.e, that lowering the ambient estrogen level increases the level of strain in bone necessary for the bone to respond with increased bone formation. If osteocytes are the main mechanosensors of bone, it is reasonable to suppose that osteocytes are the site of set point regulation by estrogen. Estrogen receptors (ERα) were demonstrated in osteocytes with immunocytochemistry and *in situ* hybridization (Braidman *et al.,* 1995; Hoyland *et al.,* 1999) in tissue sections. In addition, Westbroek *et al*. (2000b) found higher levels of ERα in isolated osteocytes than in osteoblast and osteoblast-precursor populations. At this time, no experimental evidence for the estrogen regulation of osteocyte mechanosensitivity is available, but Joldersma *et al*. (2001) reported an increased responsiveness to fluid shear stress by human bone cells as a result of estrogen treatment.

Next to receptors for PTH, $1,25(OH)_2D_3$, and estrogen, the androgen receptor (Abu *et al.,* 1997), the glucocorticoid receptor α (Abu *et al.,* 2000; Silvestrini *et al.,* 1999), and various prostaglandin receptors (Lean *et al.,* 1995; Sabbieti *et al.,* 1999) have been described in osteocytes. The latter may be important for communication within the osteocyte network during mechanotransduction (see later).

Osteocyte Function

Blood–Calcium Homeostasis

The organization of osteocytes as a syncytium of gap junction-coupled cells in each osteon represents such an unique structure that one expects it to have an important function in the metabolism and maintenance of bone. The syncytium offers two advantages that may be exploited by the tissue.

1. A tremendous cell–bone surface contact area, about two orders of magnitude larger than the contact area the osteoblasts and lining cells have (Johnson, 1966).
2. An extensive intracellular and an extracellular communication system between sites within the bone and the bone surface.

The first consideration has led Bélanger (1969) and others to propose the hypothesis that osteocytes are capable of local bone remodeling or osteocytic osteolysis. According to this hypothesis, osteocytes are coresponsible for blood–calcium homeostasis. Later studies (Boyde, 1980; Marotti *et al.,* 1990) supplied alternative explanations for the observations that appeared to support the osteocytic osteolysis theory. The possibility remains, however, that osteocytes are involved in the facilitation of calcium diffusion in and out of the bone (Bonucci, 1990). Although the bulk of calcium transport in and out of the bone is apparently taken care of by osteoblasts and osteoclasts (Boyde, 1980; Marotti *et al.,* 1990), osteocytes may have a function in the fine regulation of blood–calcium homeostasis. The major emphasis of present day thinking is, however, on the role of the osteocyte syncytium as a three-dimensional sensor and communication system in bone.

Functional Adaptation, Wolff's Law

Functional adaptation is the term used to describe the ability of organisms to increase their capacity to accomplish a specific function with increased demand and to decrease this capacity with lesser demand. In the 19th century, the anatomist Julius Wolff proposed that mechanical stress is responsible for determining the architecture of bone and that bone tissue is able to adapt its mass and three-dimensional structure to the prevailing mechanical usage to obtain a higher efficiency of load bearing (Wolff, 1892). For the past century, Wolff's law has become widely accepted. Adaptation will improve an individual animal's survival chance because bone is not only hard but also heavy. Too much of it is probably as bad as too little, leading either to uneconomic energy consumption during movement (for too high bone mass) or to an enhanced fracture risk (for too low bone mass). This readily explains the usefulness of mechanical adaptation as an evolutionary driver, even if we do not understand how it is performed.

Osteocytes as Mechanosensory Cells

In principle, all cells of bone may be involved in mechanosensing, as eukaryotic cells in general are sensitive to mechanical stress (Oster, 1989). However, several features argue in favor of osteocytes as the mechanosensory cells *par excellence* of bone as discussed earlier in this chapter. In virtually all types of bone, osteocytes are dispersed throughout the mineralized matrix and are connected with their neighbor osteocytes via long, slender cell processes that run in slightly wider canaliculi of unmineralized matrix. The cell processes contact each other via gap junctions (Doty, 1981; Donahue *et al.,* 1995), thereby allowing direct cell-to-cell coupling. The superficial osteocytes are connected with the lining cells covering most bone surfaces, as well as the osteoblasts that cover (much-less abundant) surfaces where new bone is formed. From a cell biological viewpoint therefore, bone tissue is a three-dimensional network of cells, most of which are surrounded by a very narrow sheath of unmineralized matrix, followed by a much wider layer of mineralized matrix. The sheath of unmineralized matrix is penetrated easily by macromolecules such as albumin and peroxidase (McKee *et al.,* 1993; Tanaka and Sakano, 1985). Therefore, there is an intracellular as well as an extracellular route for the rapid passage of ions and signal molecules. This allows for several manners of cellular signaling from osteocytes lying

deep within the bone tissue to surface-lining cells and vice versa (Cowin *et al.,* 1995).

Experimental studies indicate that osteocytes are indeed sensitive to stress applied to intact bone tissue. *In vivo* experiments using the functionally isolated turkey ulna have shown that immediately following a 6-min period of intermittent (1 Hz) loading, the number of osteocytes expressing glucose-6-phosphate dehydrogenase (G6PD) activity was increased in relation to local strain magnitude (Skerry *et al.,* 1989). The tissue strain magnitude varied between 0.05 and 0.2% (500–2000 microstrain) in line with *in vivo* peak strains in bone during vigorous exercise. Other models, including strained cores of adult dog cancellous bone, embryonic chicken tibiotarsi, rat caudal vertebrae, and rat tibiae, as well as experimental tooth movement in rats, have demonstrated that osteocytes in intact bone change their enzyme activity and RNA synthesis rapidly after mechanical loading (El Haj *et al.,* 1990; Dallas *et al.,* 1993; Lean *et al.,* 1995; Forwood *et al.,* 1998; Terai *et al.,* 1999). These studies show that intermittent loading produces rapid changes of metabolic activity in osteocytes and suggest that osteocytes may indeed function as mechanosensors in bone. Computer simulation studies of bone remodeling, assuming this to be a self-organizational control process, predict a role for osteocytes, rather than lining cells and osteoblasts, as stress sensors of bone (Mullender and Huiskes, 1995, 1997; Huiskes *et al.,* 2000). A regulating role of strain-sensitive osteocytes in basic multicellular unit (BMU) coupling has been postulated by Smit and Burger (2000). Using finite-element analysis, the subsequent activation of osteoclasts and osteoblasts during coupled bone remodeling was shown to relate to opposite strain distributions in the surrounding bone tissue. In front of the cutting cone of a forming secondary osteon, an area of *decreased* bone strain was demonstrated, whereas a layer of *increased* strain occurs around the closing cone (Smit and Burger, 2000). Osteoclasts therefore attack an area of bone where the osteocytes are underloaded, whereas osteoblasts are recruited in a bone area where the osteocytes are overloaded. Hemiosteonic remodeling of trabecular bone showed a similar strain pattern (Smit and Burger, 2000). Thus, bone remodeling regulated by strain-sensitive osteocytes can explain the maintenance of osteonic and trabecular architecture as an optimal mechanical structure, as well as adaptation to alternative external loads (Huiskes *et al.,* 2000; Smit and Burger, 2000).

If osteocytes are the mechanosensors of bone, how do they sense mechanical loading? This key question is, unfortunately, still open because it has not yet been established unequivocally how the loading of intact bone is transduced into a signal for the osteocytes. The application of force to bone during movement results in several potential cell stimuli. These include changes in hydrostatic pressure, direct cell strain, fluid flow, and electric fields resulting from electrokinetic effects accompanying fluid flow (Pienkowski and Pollack, 1983). Evidence has been increasing steadily for the flow of canalicular interstitial fluid as the likely stress-derived factor that informs the osteocytes about the level of bone loading (Cowin *et al.,* 1991, 1995; Cowin, 1999; Weinbaum *et al.,* 1994; Klein-Nulend *et al.,* 1995b; Knothe-Tate 2000; Burger and Klein-Nulend, 1999; You *et al.,* 2000). In this view, canaliculi are the bone porosity of interest, and the osteocytes the mechanosensor cells.

Canalicular Fluid Flow and Osteocyte Mechanosensing

In healthy, adequately adapted bone, strains as a result of physiological loads (e.g., resulting from normal locomotion) are quite small. Quantitative studies of the strain in bones of performing animals (e.g., galloping horses, fast-flying birds, even a running human volunteer) found a maximal strain not higher than 0.2–0.3% (Rubin, 1984; Burr *et al.,* 1996). This poses a problem in interpreting the results of *in vitro* studies of strained bone cells, where much higher deformations, in the order of 1–10%, were needed to obtain a cellular response (for a review, Burger and Veldhuijzen, 1993). In these studies, isolated bone cells were usually grown on a flexible substratum, which is then strained by stretching or bending. For instance, unidirectional cell stretching of 0.7% was required to activate prostaglandin E_2 production in primary bone cell cultures (Murray and Rushton, 1990). However, in intact bone, a 0.15% bending strain was already sufficient to activate prostaglandin-dependent adaptive bone formation *in vivo* (Turner *et al.,* 1994; Forwood, 1996). If we assume that bone organ strain is somehow involved in bone cell mechanosensing, then bone tissue seems to possess a lever system whereby small matrix strains are transduced into a larger signal that is detected easily by osteocytes. The canalicular flow hypothesis proposes such a lever system. The flow of extracellular tissue fluid through the lacunocanalicular network as a result of bone tissue strains was made plausible by the theoretical study of Piekarski and Munro (1977) and has been shown experimentally by Knothe-Tate and colleagues (1998, 2000). This strain-derived extracellular fluid flow may help keep osteocytes healthy, particularly the deeper ones, by facilitating the exchange of nutrients and waste products between the Haversian channel and the osteocyte network of an osteon (Kufahl and Saha, 1990). However, a second function of this strain-derived interstitial fluid flow could be the transmission of "mechanical information" (Fig. 4). The magnitude of interstitial fluid flow through the lacunocanalicular network is directly related to the amount of strain of the bone organ (Cowin *et al.,* 1991). Because of the narrow diameter of the canaliculi, bulk bone strains of about 0.1% will produce a fluid shear stress in the canaliculi of roughly 1 Pa (Weinbaum *et al.,* 1994), enough to produce a rapid response in endothelial cells (Frangos *et al.,* 1985; Kamiya and Ando, 1996).

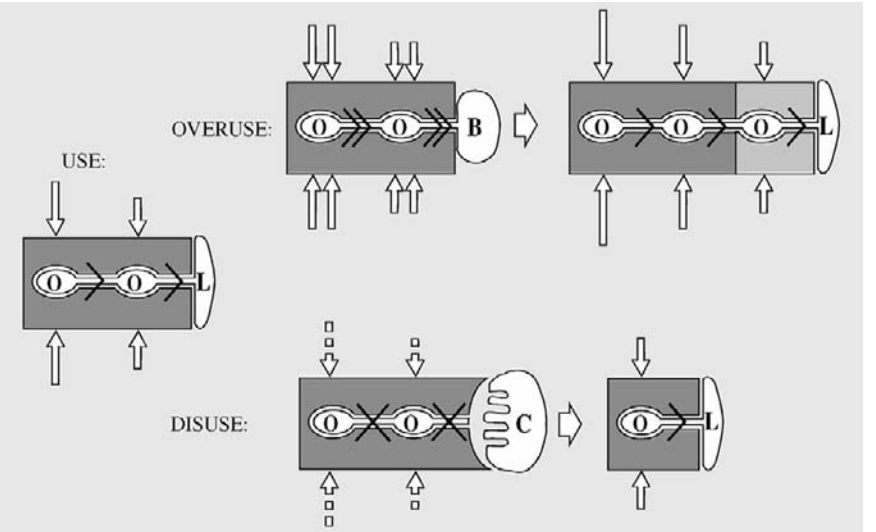

Figure 4 Schematic representation of how the osteocyte network may regulate bone modeling. In the steady state (USE), normal mechanical use ensures a basal level of fluid flow through the lacunocanalicular porosity, indicated by an arrowhead through the canaliculi. This basal flow keeps the osteocytes viable and also ensures basal osteocyte activation and signaling, thereby suppressing osteoblastic activity as well as osteoclastic attack. During (local) overuse (OVERUSE), osteocytes are overactivated by enhanced fluid flow (indicated by double arrowheads), leading to the release of osteoblast-recruiting signals. Subsequent osteoblastic bone formation reduces the overuse until normal mechanical use is reestablished, thereby reestablishing the steady state of basal fluid flow. During (local) disuse (DISUSE), osteocytes are inactivated by lack of fluid flow (indicated by crosses through canaliculi). Inactivation leads to a release of osteoclast-recruiting signals, to a lack of osteoclast-suppressing signals, or both. Subsequent osteoclastic bone resorption reestablishes normal mechanical use (or loading) and basal fluid flow. O, osteocyte; L, lining cell; B, osteoblast; C, osteoclast; dark-gray area, mineralized bone matrix; light-gray area, newly formed bone matrix; white arrows represent direction and magnitude of loading. Adapted from Burger and Klein-Nulend (1999).

Experimental studies *in vitro* have demonstrated that osteocytes are indeed quite sensitive to the fluid shear stress of such a magnitude compared to osteoblasts and osteoprogenitor cells (Klein-Nulend *et al.,* 1995a, b; Ajubi *et al.,* 1996; Westbroek *et al.,* 2000a). These results suggest that the combination of the cellular three-dimensional network of osteocytes and the accompanying porous network of lacunae and canaliculi acts as the mechano sensory organ of bone. The flow of interstitial fluid through the bone canaliculi will have two effects: a mechanical one derived from the fluid shear stress and an electrokinetic one derived from streaming potentials (Pollack *et al.,* 1984; Salzstein and Pollack, 1987). Either of the two, or both in combination, might activate the osteocyte. For instance, streaming potentials might modulate the movement of ions such as calcium across the cell membrane (Hung *et al.,* 1995, 1996), whereas shear stress will pull at the macromolecular attachments between the cell and its surrounding matrix (Wang and Ingber, 1994). Both ion fluxes and cellular attachment are powerful modulators of cell behavior and therefore good conveyors of physical information (Sachs, 1989; Ingber, 1991).

Response of Osteocytes to Fluid Flow *in Vitro*

The technique of immunodissection, as discussed earlier in this chapter, made it possible to test the canalicular flow hypothesis by comparing the responsiveness of osteocytes, osteoblasts, and osteoprogenitor cells to fluid flow. The strength of the immunodissection technique is that three separate cell populations with a high ((90%) degree of homogeneity are prepared, representing (1) osteocytes with the typical "spider-like" osteocyte morphology and little matrix synthesis, (2) osteoblasts with a high synthetic activity of bone matrix-specific proteins, and (3) (from the periosteum) osteoprogenitor cells with a fibroblast-like morphology and very high proliferative capacity (Nijweide *et al.,* 1986). Because the cells are used within 2 days after isolation from the bone tissue, they may well represent the three differentiation steps of osteoprogenitor cell, osteoblast, and osteocyte. In contrast, mixed cell cultures derived from bone that are generally used to represent "osteoblastic" cells likely contain cells in various stages of differentiation. Therefore, changes in mechanosensitivity related to progressive cell differentiation cannot be studied in such cultures.

Using these immunoseparated cell populations, osteocytes were found to respond far stronger to fluid flow than osteoblasts and these stronger than osteoprogenitor cells (Klein-Nulend *et al.,* 1995a,b; Ajubi *et al.,* 1996; Westbroek *et al.,* 2000a). Pulsating fluid flow (PFF) with a mean shear stress of 0.5 Pa (5 dynes/cm^2) with a cyclic variation of plus or minus 0.2 Pa at 5 Hz stimulated the release of nitric oxide (NO) and prostaglandin E_2 and I_2 (PGE_2 and PGI_2) rapidly from osteocytes within minutes (Klein-Nulend *et al.,* 1995a; Ajubi *et al.,* 1996). Osteoblasts showed less response, and osteoprogenitor cells (periosteal fibroblasts) still less. Intermittent hydrostatic compression (IHC) of 13,000 Pa peak pressure at 0.3 Hz (1 sec compression followed by 2 sec relaxation) needed more than 1 hr application before prostaglandin production was increased, again more in osteocytes than in osteoblasts, suggesting that mechanical stimulation via fluid flow is more effective than hydrostatic compression (Klein-Nulend *et al.,* 1995b). A 1-hr treatment with PFF also induced a sustained release of PGE_2 from the osteocytes in the hour following PFF treatment (Klein-Nulend *et al.,* 1995b). This sustained PGE_2 release, continuing after PFF treatment had been stopped, could be ascribed to the induction of prostaglandin G/H synthase-2 (or cyclo-oxygenase 2, COX-2) expression (Westbroek *et al.,* 2000a). Again, osteocytes were much more responsive than osteoblasts and osteoprogenitor cells, as only a 15-min treatment with PFF increased COX-2 mRNA expression by three-fold in osteocytes but not in the other two cell populations (Westbroek *et al.,* 2000). Upregulation of COX-2 but not COX-1 by PFF had been shown earlier in a mixed population of mouse calvarial cells (Klein-Nulend *et al.,* 1997) and was also demonstrated in primary bone cells from elderly women (Joldersma *et al.,* 2000), whereas the expression of COX-1 and -2 in osteocytes and osteoblasts in intact rat bone has been documented (Forwood *et al.,* 1998). These *in vitro* experiments on immunoseparated cells suggest that as bone cells mature, they increase their capacity to produce prostaglandins in response to fluid flow (Burger and Klein-Nulend, 1999). First, their immediate production of PGE_2, PGI_2, and probably $PGF_2\alpha$ (Klein-Nulend *et al.,* 1997) in response to flow increases as they develop from osteoprogenitor cell, via the osteoblastic stage into osteocytes. Second, their capacity to increase expression of COX-2 in response to flow, and thereby to continue to produce PGE_2 even after the shear stress has stopped (Westbroek *et al.,* 2000a), increases as they reach terminal differentiation. Because induction of COX-2 is a crucial step in the induction of bone formation by mechanical loading *in vivo* (Forwood, 1996), these results provide direct experimental support for the concept that osteocytes, the long-living terminal differentiation stage of osteoblasts, function as the "professional" mechanosensors in bone tissue.

Pulsating fluid flow also rapidly induced the release of NO in osteocytes but not osteoprogenitor cells (Klein-Nulend *et al.,* 1995a). Rapid release of NO was also found when whole rat bone rudiments were mechanically strained in organ culture (Pitsillides *et al.,* 1995) and in human bone cells submitted to fluid flow (Sterck *et al.,* 1998). In line with these *in vitro* observations, inhibition of NO production inhibited mechanically induced bone formation in animal studies (Turner *et al.,* 1996; Fox *et al.,* 1996). NO is a ubiquitous messenger molecule for intercellular communication, involved in many tissue reactions where cells must collaborate and communicate with each other (Koprowski and Maeda, 1995). An interesting example is the adaptation of blood vessels to changes in blood flow. In blood vessels, enhanced blood flow, e.g., during exercise, leads to widening of the vessel to ensure a constant blood pressure. This response depends on the endothelial cells, which sense the increased blood flow, and produce intercellular messengers such as NO and prostaglandins. In response to these messengers, the smooth muscle cells around the vessel relax to allow the vessel to increase in diameter (Kamiya and Ando, 1996). The capacity of endothelial cells to produce NO in response to fluid flow is related to a specific enzyme, endothelial NO synthase or ecNOS. Interestingly, this enzyme was found in rat bone lining cells and osteocytes (Helfrich *et al.,* 1997; Zaman *et al.,* 1999) and in cultured bone cells derived from human bone (Klein-Nulend *et al.,* 1998). Treatment with pulsatile fluid flow increased the level of ecNOS RNA transcripts in the bone cell cultures (Klein-Nulend *et al.,* 1998), a response also described in endothelial cells (Busse and Fleming, 1998; Uematsu *et al.,* 1995). Enhanced production of prostaglandins is also a well-described response of endothelial cells to fluid flow (Busse and Fleming, 1998; Kamiya and Ando, 1996). It seems, therefore, that endothelial cells and osteocytes possess a similar sensor system for fluid flow and that both cell types are "professional" sensors of fluid flow. This is an indication that osteocytes sense bone strains via the (canalicular) fluid flow resulting from bone strains.

Mechanotransduction starts by the conversion of physical loading-derived stimuli into cellular signals. Several studies suggest that the attachment complex between intracellular actin cytoskeleton and extracellular matrix macromolecules, via integrins and CD44 receptors in the cell membrane, provides the site of mechanotransduction (Wang *et al.,* 1993; Watson, 1991; Ajubi *et al.,* 1996, 1999; Pavalko *et al.,* 1998). An important early response is the influx of calcium ions through mechanosensitive ion channels in the plasma membrane and the release of calcium from internal stores (Hung *et al.,* 1995, 1996; Ajubi *et al.,* 1999; Chen *et al.,* 2000; You *et al.,* 2000). The signal transduction pathway then involves protein kinase C and phospholipase A_2 to activate arachidonic acid production and PGE_2 release (Ajubi *et al.,* 1999). However, many other steps in the mechanosignaling cascade are still unknown in osteocytes as well as other mechanosensory cells.

Cell Stretch versus Fluid Flow

To mimic the effect of physiological bone loading in monolayer cell cultures, several authors have used cell stretching via deformation of the cell culture substratum (Murray and

Rushton, 1990; Pitsillides *et al.,* 1995; Zaman *et al.,* 1999; Neidlinger-Wilke *et al.,* 1995; Kaspar *et al.,* 2000; Mikuni-Takagaki *et al.,* 1996; Kawata and Mikuni-Takagaki, 1998; for a review of the older literature, see Burger and Veldhuijzen, 1993). Stretchloading by hypoosmotic cell swelling was also used (Miyauchi *et al.,* 2000). The results are generally in agreement with the studies using fluid flow, including the high sensitivity of osteocytes to strain. The advantage of cell straining via the cell culture substratum is that this technique allows to precisely determine the amount of cell strain that is applied, provided that the cell does not modulate its attachment to the substratum during stretching. Cell strain in *vitro* can therefore be adjusted to bone tissue strain *in vivo* during exercise. Using fluid flow *in vitro* means that only the fluid shear *stress* can be well determined, but not the ensuing cell strain (deformation). Attempts to measure cell deformation as a result of fluid flow showed that osteocyte deformation is below the detection limit using phase-contrast microscopy (S. C. Cowin, C. M. Semeins, J. Klein-Nulend, and Burger, E. H. unpublished results). The canalicular fluid shear stress *in vivo*, as predicted by Weinbaum *et al*. (1994) on the basis of a theoretical model validated with respect to the streaming potentials (Cowin *et al.,* 1995), is of the order of 1 Pa (0.8–3 N/m^2). *In vitro,* fluid shear stress between 0.4 and 1.2 Pa dose-dependently stimulated the release of NO and PGE_2 from primary bone cells (Bakker *et al.,* 2001) in good agreement with this figure, but more direct determination of canalicular shear stress is lacking. However, two independent studies concluded that the actual physical cell stimulus during substratum-mediated cell stretching is the *flow* of culture medium over the cell surface (Owan *et al.,* 1997; You *et al.,* 2000). When this medium fluid flow was prevented, 10% cell stretch was needed to induce the increase of cell calcium levels (You *et al.,* 2000). You and co-workers (2000) suggested that bone cell mechanotransduction may involve two distinct pathways relating to two different events. One is the mechanical adaptation of intact bone that occurs throughout life mediated by canalicular loading-induced fluid flow derived from small bone strains of the order of 0.1%. The other relates to fracture healing, when large cell deformations of the order of 10% can be expected. Interestingly, such large cell deformations are also applied during distraction osteogenesis and in orthodontic tooth displacement, where rapid activation of osteoprogenitor cells occurs (Ikegame *et al.,* 2001). It seems therefore that osteoprogenitor cells are activated to express their osteoblastic phenotype when they experience large strains in biological processes related to rapid bone healing. However, the more subtle effects of physiological bone loading seem to be mediated by fluid flow in the osteocyte network in intact bone. Because the flow *in vitro* resulting from substrate stretching or bending is difficult to determine and is probably complicated in pattern, it follows that for the study of physiological strains, the use of a system designed to apply flow is to be preferred. This approach allows at least the determination of the magnitude and pattern of the applied fluid shear stress (Bakker *et al.,* 2001).

Summary and Conclusions

Although we are still at the beginning of understanding the role of osteocytes in bone physiology, important progress has been made. Morphological studies of bone have illustrated the intricate process of osteocyte differentiation and formation of the complex osteocyte network. Studies with isolated osteocytes have shown that the formation of this network is an intrinsic property of osteocytes. Future experiments will have to elucidate the mechanism by which the osteocyte syncytium is formed.

According to the current predominant view, this three-dimensional osteocyte network provides the site for information acquirement underlying Wolff's law of adaptive bone remodeling, in other words, the site where bone is informed about local osteopenia or bone redundancy in relation to its usage. Although deformation of the cells themselves as a result of loading-induced matrix strain cannot yet be excluded as the signaling mechanism, the modulation of canalicular fluid flow resulting from compression/relaxation of the bone matrix seems a more sensitive mechanism of mechanosignaling in bone. This hypothesis does justice to the anatomy of the lacunocanalicular porosity and osteocyte syncytium. The osteocyte, cell body, and processes are surrounded by a thin sheath of unmineralized matrix, which allows a loading-derived flow of interstitial fluid flow over the osteocyte surface, as is demonstrated by the loading–facilitation of macromolecule diffusion. In addition, comparative studies of osteoprogenitor cells, osteoblasts, and osteocytes have shown that bone cells increase their sensitivity for shear stress as they mature, with the highest sensitivity being shown by the terminally differentiated cell stage, the osteocyte. This high sensitivity may be related to the composition of the cytoskeleton of the osteocyte, which in some ways differs from that of the osteoblast. Because the interaction of the cytoskeleton with the surrounding matrix is considered to be one of the possible sites of shear stress transduction, the composition of the matrix seems to be of major importance. Several studies have reported on the specialized matrix of the osteocyte sheath and the presence of receptors on the osteocyte that may interact with molecules in this matrix.

The hypothesis of the osteocyte's three-dimensional cellular network as the mechanosensory organ of bone has spawned several cell-based concepts that explain adaptive bone remodeling at the level of cells, osteons and trabeculae. These models are generally in agreement with Frost's mechanostat hypothesis, but go a step further in explaining the actual cell behavior during adaptive remodeling. As such they are a major step forward in our understanding of bone physiology.

The nature of the signaling molecules produced in the shear-strained osteocyte that modulate the recruitment or activity of the effector cells of loading-related bone remodeling, osteoblasts and osteoclasts, has been a subject of study in many investigations. Generally, authors agree on the importance of NO and prostaglandins. The enzymes

that produce these molecules are thought to be activated directly by changes in the intracellular calcium and the cytoskeletal organization and/or indirectly by the induction of gene expression. Clearly, most of the steps in the signaling cascades that are involved still have to be elucidated.

In sum, the field of molecular and, possibly, electrical signaling during adaptive bone remodeling is still wide open and provides an exciting area of future research. Further analysis of the specific properties of osteocytes, including identification of the antigens of known and newly developed osteocyte-specific antibodies, may help in the understanding of the role of osteocytes in bone physiology.

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CHAPTER 7

Cells of Bone

Osteoclast Generation

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Introduction

Osteoclasts, the multinucleated giant cells that resorb bone, develop from hemopoietic cells of the monocyte–macrophage lineage. We have developed a mouse coculture system of osteoblasts/stromal cells and hemopoietic cells in which osteoclasts are formed in response to bone-resorbing factors such as $1\alpha,25$-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$], parathyroid hormone (PTH), prostaglandin E_2 (PGE_2), and interleukin 11 (IL-11). A series of experiments using this coculture system have established the concept that osteoblasts/stromal cells are crucially involved in osteoclast development. Cell-to-cell contact between osteoblasts/stromal cells and osteoclast progenitors was necessary for the induction of osteoclast differentiation in the coculture system. Studies on macrophage colony-stimulating factor (M-CSF, also called CSF-1)-deficient *op/op* mice have shown that M-CSF produced by osteoblasts/stromal cells is an essential factor for inducing osteoclast differentiation from monocyte–macrophage lineage cells. Subsequently, in 1998, another essential factor for osteoclastogenesis, receptor activator of nuclear factor κB ligand (RANKL) was cloned molecularly. RANKL [also known as osteoclast differentiation factor (ODF)/osteoprotegerin ligand (OPGL)/TNF-related activation-induced cytokine (TRANCE)] is a new member of the tumor necrosis factor (TNF)-ligand family, which is expressed as a membrane-associated protein in osteoblasts/stromal cells in response to many bone-resorbing factors. Osteoclast precursors that possess RANK, a TNF receptor family member, recognize RANKL through cell–cell interaction with osteoblasts/stromal cells and differentiate into osteoclasts in the presence of M-CSF. Mature osteoclasts also express RANK, and RANKL induces their bone-resorbing activity. Osteoprotegerin [OPG, also called osteoclastogenesis inhibitory factor (OCIF)] mainly produced by osteoblasts/stromal cells is a soluble decoy receptor for RANKL. OPG has been shown to function as an inhibitory factor for osteoclastogenesis *in vivo* and *in vitro*. Thus, the rapid advances in osteoclast biology have elucidated the precise mechanism by which osteoblasts/stromal cells regulate osteoclast differentiation and function. Activation of nuclear factor κB (NF-κB) and c-Jun N-terminal protein kinase (JNK) through RANK-mediated signals appears to be involved in the differentiation and activation of osteoclasts. Findings also indicate that TNFα directly induces differentiation of osteoclasts by a mechanism independent of the RANKL–RANK interaction. This chapter describes the current knowledge of the regulatory mechanisms of osteoclast differentiation induced by osteotropic hormones and cytokines.

Role of Osteoblasts/Stromal Cells in Osteoclast Differentiation and Function

Osteoblasts/Stromal Cells Regulate Osteoclast Differentiation

Development of osteoclasts proceeds within a local microenvironment of bone. This process can be reproduced *ex vivo* in a coculture of mouse calvarial osteoblasts and hemopoietic cells (Chambers *et al.,* 1993; Suda *et al.,* 1992; Takahashi *et al.,* 1988). Multinucleated cells formed in the coculture exhibit major characteristics of osteoclasts, including tartrate-resistant acid phosphatase (TRAP) activity, expression of calcitonin receptors, c-Src (p60c-src),

vitronectin receptors ($\alpha v\beta 3$), and the ability to form resorption pits on bone and dentine slices (Suda *et al.,* 1992). Some mouse stromal cell lines, such as MC3T3-G2/PA6 and ST2, are able to support osteoclastogenesis when cultured with mouse spleen cells (Udagawa *et al.,* 1989). In this coculture, osteoclasts were formed in response to various osteotropic factors, including $1\alpha,25(OH)_2D_3$, PTH, PGE_2, and IL-11. Cell-to-cell contact between osteoblasts/stromal cells and osteoclast progenitors is required to induce osteoclastogenesis. Subsequent experiments have established that the target cells of osteotropic factors for inducing osteoclast formation *in vitro* are osteoblasts/stromal cells (Table I).

IL-6 exerts its activity via a cell surface receptor that consists of two components: a ligand-binding IL-6 receptor (IL-6R) and a nonligand-binding but signal-transducing protein gp130 (Taga and Kishimoto, 1997). The genetically engineered soluble IL-6R (sIL-6R), which lacks both transmembrane and cytoplasmic domains, has been shown to mediate IL-6 signals through gp130 in response to IL-6. Neither recombinant IL-6 nor sIL-6R alone induced osteoclast formation in the coculture, but osteoclasts were formed in response to IL-6 in the presence of sIL-6R (Tamura *et al.,* 1993). This suggests that a signal(s) mediated by gp130 is involved in osteoclast development. Using transgenic mice constitutively expressing human IL-6R, it was shown that the expression of human IL-6R in osteoblasts was indispensable for inducing osteoclast recruitment (Udagawa *et al.,* 1995) (Table I). When osteoblasts obtained from human IL-6R transgenic mice were cocultured with normal spleen cells, osteoclast formation was induced in response to human IL-6 without the addition of human sIL-6R. Indeed, cytokines such as IL-11, oncostatin M, and leukemia inhibitory factor (LIF), which transduce their signals through gp130 in osteoblasts/stromal cells, induced osteoclast formation in the coculture. These results established for the first time the concept that bone-resorbing cytokines using gp130 as a common signal transducer act directly on osteoblasts/stromal cells but not on osteoclast progenitors to induce osteoclast formation.

Requirement of PTH/PTHrP receptors (PTHR1) in the osteoblast was confirmed using cocultures of osteoblasts and spleen cells obtained from PTHR1 knockout mice (Liu *et al.,* 1998). Osteoblasts obtained from PTHR1(−/−) mice failed to support osteoclast development in cocultures with normal spleen cells in response to PTH (Table I). Osteoclasts were formed in response to PTH in cocultures of spleen cells obtained from PTHR1(−/−) mice and normal calvarial osteoblasts. This suggests that the expression of PTHR1 in osteoblasts/stromal cells is critical for PTH-induced osteoclast formation *in vitro*.

PGE_2 exerts its effects through PGE receptors (EPs) that consist of four subtypes (EP1, EP2, EP3, and EP4) (Breyer and Breyer, 2000). Intracellular signaling differs among the receptor subtypes: EP1 is coupled to Ca^{2+} mobilization and EP3 inhibits adenylate cyclase activity, whereas both EP2 and EP4 stimulate adenylate cyclase activity. It was reported that 11-deoxy-PGE1 (an EP4 and EP2 agonist) stimulated osteoclast formation more effectively than butaprost (an EP2 agonist) and other EP agonists in the coculture of primary osteoblasts and bone marrow cells, suggesting that EP4 is the main receptor responsible for PGE_2-induced osteoclast formation (Sakuma *et al.,* 2000). Furthermore, the PGE_2-induced osteoclast formation was not observed in the coculture of osteoblasts from EP4(−/−) mice and spleen cells from wild-type mice, whereas osteoclasts were formed in the coculture of wild-type osteoblasts and EP4(−/−) spleen cells (Sakuma *et al.,* 2000) (Table I). These results indicate that PGE_2 enhances osteoclast formation through the EP4 subtype on osteoblasts. Li *et al.* (2000b) used cells from mice in which the EP2 receptor had been disrupted to test the role of EP2 in osteoclast formation. The response to PGE_2 for osteoclast formation was also reduced in cultures of bone marrow cells obtained from EP2(−/−) mice. In mouse calvarial organ cultures, the EP4 agonist stimulated bone resorption markedly, but its maximal stimulation was less

Table I Osteoclast Formation in Cocultures with hIL-6R Transgenic Mice or Mice Carrying the Disrupted Genes of VDR, PTHR1, or EP4

Osteotropic factor	Coculture system[a]		Osteoclast formation	Reference
	Osteoblasts	Hemopoietic cells		
hIL-6	wt	hIL-6R tg	−	Udagawa *et al.* (1995)
	hIL-6R tg	wt	+	
PTH	PTHR1(−/−)	wt	−	Liu *et al*. (1998)
	wt	PTHR1(−/−)	+	
PGE_2	EP4(−/−)	wt	−	Sakuma *et al.* (2000)
	wt	EP4(−/−)	+	
$1\alpha,25(OH)_2D_3$	VDR(−/−)	wt	−	Takeda *et al.* (1999)
	wt	VDR(−/−)	+	

[a] Osteoblasts or hemopoietic cells obtained from human IL-6R (hIL-6R) transgenic (tg) mice, VDR knockout [VDR(−/−)] mice, PTHR1 knockout [PTHR1(−/−)] mice, or EP4 knockout [EP4(−/−)] mice were cocultured with their counterpart (osteoblasts or hemopoietic cells) obtained from wild-type (wt) mice.

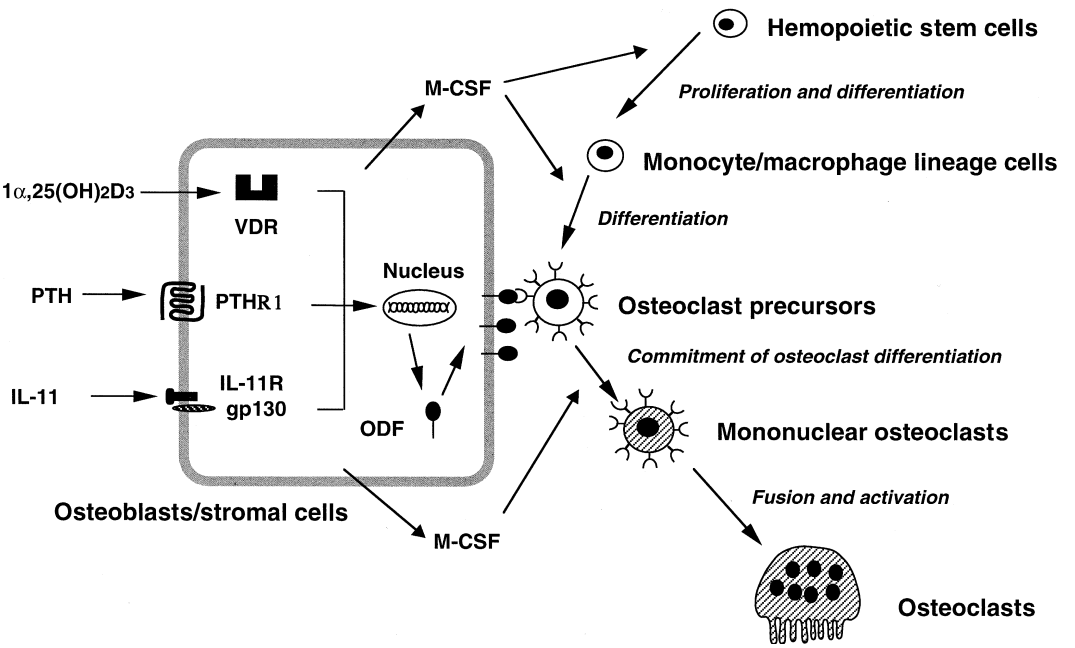


Figure 1 Hypothetical concept of osteoclast differentiation and a proposal for osteoclast differentiation factor (ODF). Osteotropic factors such as $1\alpha,25(OH)_2D_3$, PTH, and IL-11 stimulate osteoclast formation in mouse cocultures of osteoblasts/stromal cells and hemopoietic cells. The target cells of these osteotropic factors are osteoblasts/stromal cells. Three independent signaling pathways mediated by $1\alpha,25(OH)_2D_3$–VDR, PTH–PTHR1, and IL-11–IL-11R/gp130 interactions induce ODF as a membrane-associated factor in osteoblasts/stromal cells in a similar manner. Osteoclast progenitors of the monocyte–macrophage lineage recognize ODF through cell–cell interaction with osteoblasts/stromal cells and differentiate into osteoclasts. M-CSF produced by osteoblasts/stromal cells is a prerequisite for both proliferation and differentiation of osteoclast progenitors. This hypothetical concept has been proven molecularly by the discovery of the RANKL–RANK interaction.

than that induced by PGE_2 (Suzawa *et al.,* 2000). The EP2 agonist also stimulated bone resorption, but only slightly. EP1 and EP3 agonists showed no effect on bone resorption. These findings suggest that PGE_2 stimulates bone resorption by a mechanism involving cAMP production in osteoblasts/stromal cells, mediated mainly by EP4 and partially by EP2.

The other known pathway used for osteoclast formation is that stimulated by $1\alpha,25(OH)_2D_2$. Using $1\alpha,25(OH)_2D_3$ receptor (VDR) knockout mice, Takeda *et al*. (1999) clearly showed that the target cells of $1\alpha,25(OH)_2D_3$ for inducing osteoclasts in the coculture were also osteoblasts/stromal cells but not osteoclast progenitors (Table I). Spleen cells from VDR(−/−) mice differentiated into osteoclasts when cultured with normal osteoblasts in response to $1\alpha,25(OH)_2D_3$. In contrast, osteoblasts obtained from VDR(−/−) mice failed to support osteoclast development in coculture with wild-type spleen cells in response to $1\alpha,25(OH)_2D_3$. These results suggest that the signals mediated by VDR are also transduced into osteoblasts/stromal cells to induce osteoclast formation in the coculture.

Thus, the signals induced by almost all the bone-resorbing factors are transduced into osteoblasts/stromal cells to recruit osteoclasts in the coculture. Therefore, we proposed that osteoblasts/stromal cells express ODF, which is hypothesized to be a membrane-bound factor in promoting the differentiation of osteoclast progenitors into osteoclasts through a mechanism involving cell-to-cell contact (Suda *et al.,* 1992) (Fig. 1).

M-CSF Produced by Osteoblasts/Stromal Cells Is an Essential Factor for Osteoclastogenesis

Experiments with the *op/op* mouse model have established the role for M-CSF in osteoclast formation. Yoshida *et al.* (1990) demonstrated that there is an extra thymidine insertion at base pair 262 in the coding region of the M-CSF gene in *op/op* mice. This insertion generated a stop codon (TGA) 21 bp downstream, suggesting that the M-CSF gene of *op/op* mice cannot code for the functionally active M-CSF protein. In fact, administration of recombinant human M-CSF restored the impaired bone resorption of *op/op* mice *in vivo* (Felix *et al.,* 1990; Kodama *et al.,* 1991). Calvarial osteoblasts obtained from *op/op* mice could not support osteoclast formation in cocultures with normal spleen cells, even in the presence of $1\alpha,25(OH)_2D_3$ (Suda *et al.,* 1997a). The addition of M-CSF to the coculture with *op/op* osteoblastic cells induced osteoclast formation from normal spleen cells in response to $1\alpha,25(OH)_2D_3$. In contrast, spleen cells obtained from *op/op* mice were able to differentiate into osteoclasts when cocultured with normal osteoblasts. It was shown that M-CSF is involved in both proliferation of osteoclast progenitors and differentiation into osteoclasts (Felix *et al.,* 1994; Tanaka *et*

al., 1993). Begg *et al.* (1993) investigated age-related changes in osteoclast activity in *op*/*op* mice. Femurs of newborn *op*/*op* mice were infiltrated heavily with bone, and the marrow hemopoiesis was reduced significantly. However, the femoral marrow cavity of *op*/*op* mice enlarged progressively with the concomitant appearance of TRAP-positive osteoclasts, and by 22 weeks of age the marrow hemopoiesis was comparable to that of controls. Niida *et al*. (1999) reported that a single injection in *op*/*op* mice with recombinant human vascular endothelial growth factor (VEGF) induced osteoclast recruitment. These results suggest that factors other than M-CSF, including VEGF, can substitute for M-CSF to induce osteoclast formation under special occasions.

Osteoblasts/Stromal Cells Regulate Osteoclast Function

One of the major technical difficulties associated with the analysis of mature osteoclasts is their strong adherence to plastic dishes. We have developed a collagen-gel culture using mouse bone marrow cells and osteoblasts/stromal cells to obtain a cell preparation containing functionally active osteoclasts (Akatsu *et al.,* 1992; Suda *et al.,* 1997a). The purity of osteoclasts in this preparation was only 2–3%, contaminated with numerous osteoblasts. However, this crude osteoclast preparation proved to be a useful source to establish a reliable resorption pit assay on dentine slices. Therefore, osteoclasts were purified via centrifugation of the crude osteoclast preparation in a 30% Percoll solution (Jimi *et al.,* 1996b). Interestingly, these highly purified osteoclasts (purity: 50–70%) cultured for 24 h on dentine slices failed to form resorption pits. Resorptive capability of the purified osteoclasts was restored when osteoblasts/stromal cells were added to the purified osteoclast preparation. Similarly, Wesolowski *et al.* (1995) obtained highly purified mononuclear and binuclear prefusion osteoclasts using echistatin from mouse cocultures of bone marrow cells and osteoblastic MB 1.8 cells. These enriched prefusion osteoclasts failed to form resorption pits on bone slices, but their bone-resorbing activity was induced when both MB 1.8 cells and $1\alpha,25(OH)_2D_3$ were added to the prefusion osteoclast cultures. These results suggest that osteoblasts/stromal cells play an essential role not only in the stimulation of osteoclast formation, but also in the activation of mature osteoclasts to resorb bone, which is also a cell-to-cell contact-dependent process (Suda *et al.,* 1997b).

Discovery of the RANKL–RANK Interaction for Osteoclastogenesis

Discovery of OPG

OPG was cloned as a new member of the TNF receptor superfamily in an expressed sequence tag cDNA project (Simonet *et al.,* 1997). Interestingly, OPG lacked a transmembrane domain and presented as a secreted form. Hepatic expression of OPG in transgenic mice resulted in severe osteopetrosis. Osteoclastogenesis inhibitory factor (OCIF), which inhibited osteoclast formation in the coculture of osteoblasts and spleen cells, was isolated as a heparin-binding protein from the conditioned medium of human fibroblast cultures (Tsuda *et al.,* 1997). The cDNA sequence of OCIF was identical to that of OPG (Yasuda *et al.,* 1998a). Tan *et al.* (1997) also identified a new member of the TNF receptor family called TNF receptor-like molecule 1 (TR1) from a search of an expressed sequence tag database. TR1 was also found to be identical to OPG/OCIF.

OPG contains four cysteine-rich domains and two death domain homologous regions (Fig. 2). The death domain homologous regions share structural features with "death domains" of TNF type I receptor (p55) and Fas, both of which mediate apoptotic signals. Analysis of the domain-deletion mutants of OPG revealed that the cysteine-rich domains, but not the death domain homologous regions, are essential for inducing biological activity *in vitro*. When the transmembrane domain of Fas was inserted between the cysteine-rich domains and the death domain homologous regions, and the mutant protein was then expressed in the human kidney cell line 293-EBNA, apoptosis was induced in the transfected cells (Yamaguchi *et al.,* 1998). The biological significance of the death domain homologous regions in the OPG molecule, however, remains largely unknown at present.

OPG strongly inhibited osteoclast formation induced by $1\alpha,25(OH)_2D_3$, PTH, PGE_2, or IL-11 in the cocultures. Analyses of transgenic mice overexpressing OPG and animals injected with OPG have demonstrated that this factor increases bone mass by suppressing bone resorption (Simonet *et al.,* 1997; Yasuda *et al.,* 1998a). Administration of OPG to rats decreased the serum calcium concentration rapidly (Yamamoto *et al.,* 1998). The physiological role of OPG was investigated further in OPG-deficient mice (Bucay *et al.,* 1998; Mizuno *et al.,* 1998). These mutant mice were viable and fertile, but adolescent and adult OPG(−/−) mice exhibited a decrease in bone mineral density (BMD) characterized by severe trabecular and cortical bone porosity, marked thinning of parietal bones of the skull, and a high incidence of fractures. Interestingly, osteoblasts derived from OPG(−/−) mice strongly supported osteoclast formation in the coculture even in the absence of any bone-resorbing agents (Udagawa *et al.,* 2000). Bone-resorbing activity in organ cultures of fetal long bones derived from OPG(−/−) mice was also strikingly higher in the absence of bone-resorbing factors when compared to that of wild-type mice. Osteoblasts prepared from OPG(−/−) mice and wild-type mice expressed comparable levels of RANKL mRNA. These results indicate that OPG produced by osteoblasts/stromal cells functions as an important negative regulator in osteoclast differentiation and activation *in vivo* and *in vitro*.

Discovery of the RANKL–RANK Interaction

The mouse bone marrow-derived stromal cell line ST2 supports osteoclast formation in the coculture with mouse

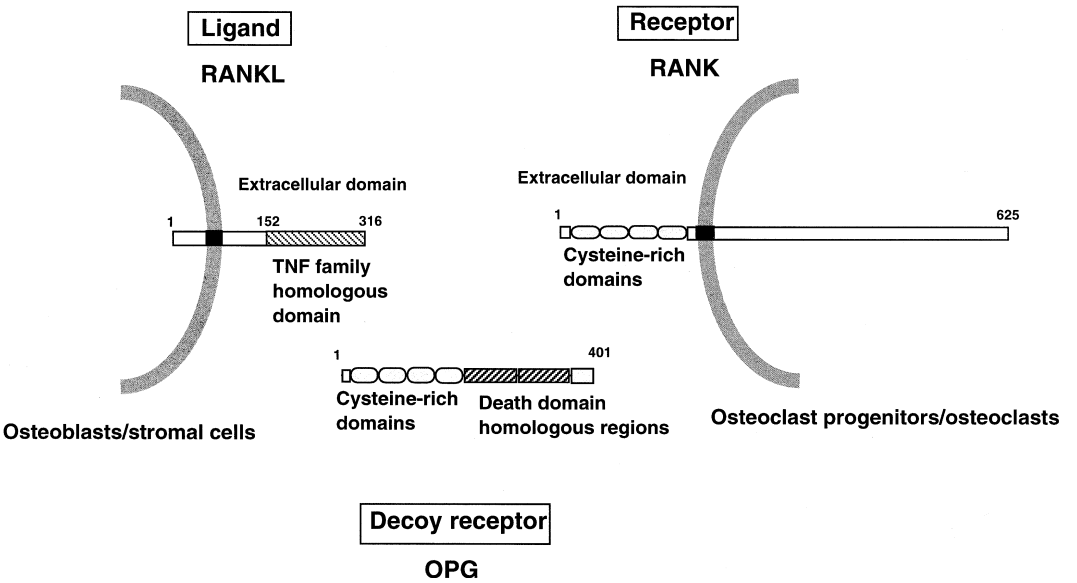


Figure 2 Diagrammatic representation of the ligand, receptor, and decoy receptor of the new TNF receptor–ligand family members essentially involved in osteoclastogenesis. Mouse RANKL is a type II transmembrane protein composed of 316 amino acid residues. The TNF homologous domain exists in Asp_{152}-Asp_{316}. RANK is a type I transmembrane protein of 625 amino acid residues. Four cysteine-rich domains exist in the extracellular region of the RANK protein. OPG, a soluble decoy receptor for RANKL, is composed of 401 amino acid residues without a transmembrane domain. OPG also contains four cysteine-rich domains and two death domain homologous regions. The cysteine-rich domains but not the death domain homologous regions of OPG are essential for inhibiting osteoclast differentiation and function.

spleen cells in the presence of $1\alpha,25(OH)_2D_3$ and dexamethasone (Udagawa *et al.,* 1989). OPG bound to a single class of high-affinity binding sites appearing on ST2 cells treated with $1\alpha,25(OH)_2D_3$ and dexamethasone (Yasuda *et al.,* 1998a). Using OPG as a probe, Yasuda *et al.* (1998b) cloned a cDNA with an open reading frame encoding 316 amino acid residues from an expression library of ST2 cells. The OPG-binding molecule was a type II transmembrane protein of the TNF ligand family (Fig. 2). Because the OPG-binding molecule satisfied major criteria of ODF, this molecule was renamed ODF. Lacey *et al.* (1998) also succeeded in the molecular cloning of the ligand for OPG (OPGL) from an expression library of the murine myelomonocytic cell line 32D. Molecular cloning of ODF/OPGL demonstrated that it was identical to TRANCE (Wong *et al.,* 1997b) and RANKL (Anderson *et al.,* 1997), which had been identified independently by other research groups in the immunology field.

TRANCE was cloned during a search for apoptosis-regulatory genes in murine T cell hybridomas (Wong *et al.,* 1997b). A recombinant soluble form of TRANCE induced activation of JNK in T lymphocytes and inhibited apoptosis of mouse and human dendritic cells. A new member of the TNF receptor family, termed "RANK," was cloned from a cDNA library of human dendritic cells (Anderson *et al.,* 1997). The mouse homologue was also isolated from a fetal mouse liver cDNA library. The mouse RANK cDNA encodes a type I transmembrane protein of 625 amino acid residues with four cycteine-rich domains in the extracellular region (Fig. 2). RANKL was cloned from a cDNA library of murine thymoma EL40.5 cells and was found to be identical to TRANCE. A soluble form of RANKL augmented the capability of dendritic cells to stimulate T cell proliferation in a mixed lymphocyte reaction and increased the survival of RANK-positive T-cells (Wong *et al.,* 1997a). The N-terminal region of RANK has a similar structure to that of OPG, a decoy receptor for RANKL (Fig. 2).

Polyclonal antibodies against the extracellular domains of RANK (anti-RANK Ab) have been shown to induce osteoclast formation in spleen cell cultures in the presence of M-CSF (Hsu *et al.,* 1999; Nakagawa *et al.,* 1998). This suggests that the clustering of RANK is required for the RANK-mediated signaling of osteoclastogenesis. In contrast, the anti-RANK antibody, which lacks the Fc fragment, (the Fab fragment), completely blocked the RANKL-mediated osteoclastogenesis (Nakagawa *et al.,* 1998). A soluble form of RANK, an extracellular domain of RANK, not only inhibited RANKL-mediated osteoclast formation, but also prevented the survival, multinucleation, and pit-forming activity of prefusion osteoclasts treated with RANKL (Jimi *et al.,* 1999a). Transgenic mice expressing a soluble RANK-Fc fusion protein showed osteopetrosis, similar to OPG transgenic mice (Hsu *et al.,* 1999). Taken together, these results suggest that RANK acts as the sole signaling receptor for RANKL in inducing differentiation and subsequent activation of osteoclasts (Fig. 2).

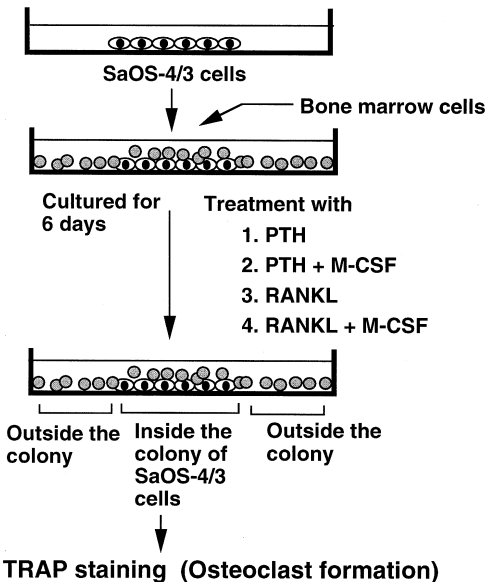
Thus, ODF, OPGL, TRANCE, and RANKL are different names for the same protein, which is important for the development and function of T cells, dendritic cells and osteoclasts. The terms "RANKL," "RANK," and "OPG" are

used in this chapter in accordance with the guideline of the American Society for Bone and Mineral Research President's Committee on Nomenclature (2000).

Role of RANKL in Osteoclast Differentiation and Function

When COS-7 cells transfected with a RANKL expression vector were fixed with paraformaldehyde and cocultured with mouse spleen cells in the presence of M-CSF, osteoclasts were formed on the fixed COS-7 cells (Yasuda *et al.,* 1998b). A genetically engineered soluble form of RANKL, together with M-CSF, induced osteoclast formation from spleen cells in the absence of osteoblasts/stromal cells, which was abolished completely by the simultaneous addition of OPG. Treatment of calvarial osteoblasts with $1\alpha,25(OH)_2D_3$, PTH, PGE_2, or IL-11 upregulated the expression of RANKL mRNA. Human osteoclasts were also formed in cultures of human peripheral blood mononuclear cells in the presence of RANKL and human M-CSF (Matsuzaki *et al.,* 1998). This suggests that the mechanism of human osteoclast formation is essentially the same as that of mouse osteoclast formation. Lum *et al*. (1999) reported that like TNFα, RANKL is synthesized as a membrane-anchored precursor and is detached from the plasma membrane to generate the soluble form of RANKL by a metalloprotease–disintegrin TNFα convertase (TACE). Soluble RANKL demonstrated a potent activity in the induction of dendritic cell survival and osteoclastogenesis. These findings suggest that the ectodomain of RANKL is released from the cells by TACE or a related metalloprotease-disintegrin and that this release is an important component of the function of RANKL in bone and immune homeostasis.

We carefully examined the mechanism of action of RANKL and M-CSF expressed by osteoblasts/stromal cells that support osteoclast formation (Itoh *et al.,* 2000b) (Fig. 3). SaOS-4/3, a subclone of the human osteosarcoma cell line SaOS-2, was established by transfecting the human PTHR1 cDNA. SaOS-4/3 cells supported human and mouse osteoclast formation in response to PTH in cocultures with human peripheral blood mononuclear cells and mouse bone marrow cells, respectively (Matsuzaki *et al.,* 1999). Osteoclast formation supported by SaOS-4/3 cells was completely inhibited by adding either OPG or antibodies against human M-CSF. This suggests that RANKL and M-CSF are both essential factors for inducing osteoclast formation in the coculture with SaOS-4/3 cells. To elucidate the functional form of both RANKL and M-CSF, SaOS-4/3 cells were spot cultured for 2 hr in the center of a culture well and then mouse bone marrow cells were plated uniformly over the well (Fig. 3). When the spot coculture was treated for 6 days with PTH together with or without M-CSF, osteoclast formation was induced exclusively inside the colony of SaOS-4/3 cells irrespective of the exogenous addition of M-CSF. Similarly, when the spot coculture was treated with RANKL, osteoclasts were formed only inside the colony of SaOS-4/3 cells, suggesting that M-CSF acts as a membrane- or matrix-associated form in the coculture. However, the concomitant treatment with RANKL and M-CSF induced osteoclast formation both inside and outside the colony of SaOS-4/3 (Fig. 3). Similar results were obtained in the spot coculture with OPG(−/−) mouse-derived osteoblasts



Treatment with	Osteoclast formation	
	Inside the colony of SaOS-4/3 cells	Outside the colony of SaOS-4/3 cells
1. PTH	+	—
2. PTH + M-CSF	+	—
3. RANKL	+	—
4. RANKL + M-CSF	+	+

Figure 3 Both M-CSF and RANKL act as membrane- or matrix-associated forms in osteoclast formation. SaOS-4/3 cells expressing recombinant human PTHR1 were spot cultured for 2 hr in the center of a culture well; subsequently, mouse bone marrow cells were plated uniformly over the well. Spot cocultures were treated for 6 days with PTH, PTH plus M-CSF, RANKL, or RANKL plus M-CSF. Cells were then fixed and stained for TRAP. The location of TRAP-positive osteoclasts in the culture well was observed under a microscope. Osteoclasts formed outside the colony of SaOS-4/3 cells were observed only when the spot cocultures were treated with both RANKL and M-CSF.

(Udagawa *et al.,* 2000). These results suggest that membrane- or matrix-associated forms of both M-CSF and RANKL are essentially involved in osteoclast formation supported by osteoblasts/stromal cells. Such a mechanism of action of RANKL and M-CSF on osteoclast progenitors may explain the reason why osteoclasts are localized in bone, despite the relatively wide distribution of RANKL (Kartsogiannis *et al*., 1999) and M-CSF (Felix *et al.,* 1994; Wood *et al.,* 1997).

Survival, fusion, and pit-forming activity of osteoclasts are also induced by RANKL (Fig. 4). Treatment of prefusion osteoclasts with OPG suppressed their survival, fusion, and pit-forming activity induced by RANKL (Jimi *et al.,* 1999a). RANKL increased bone resorption by isolated rat authentic osteoclasts (Burgess *et al.,* 1999; Fuller *et al*., 1998). Bone-resorbing factors, such as $1\alpha,25(OH)_2D_3$, PTH, and IL-11, enhanced pit formation by purified osteoclasts only in the presence of osteoblasts (Udagawa *et al.,* 1999). Treatment of prelabeled bone with $1\alpha,25(OH)_2D_3$, PGE_2, and PTH enhanced the release of ^{45}Ca from the bone, which was completely inhibited by the addition of OPG or anti-RANKL antibody (Tsukii *et al.,* 1998). These results suggest that osteoblasts/stromal cells are essentially involved in both differentiation and activation of osteoclasts through the expression of RANKL as a membrane-associated factor (Fig. 4).

RANKL- and RANK-Deficient Mice

The physiological role of RANKL was investigated by generating RANKL-deficient mice (Kong *et al.,* 1999b) (Table II). RANKL(−/−) mice exhibited typical osteopetrosis with total occlusion of bone marrow space within endosteal bone. RANKL(−/−) mice lacked osteoclasts but had normal osteoclast progenitors that can differentiate into functionally active osteoclasts when cocultured with normal osteoblasts/stromal cells. Osteoblasts obtained from RANKL(−/−) mice failed to support osteoclast formation in the coculture with wild-type bone marrow cells even in the presence of $1\alpha,25(OH)_2D_3$ and PGE_2. RANKL(−/−) mice exhibited defects in the early differentiation of T and B lymphocytes. In addition, RANKL(−/−) mice showed normal splenic structure and Peyer's patches, but lacked all lymph nodes. These results suggest that RANKL is an absolute requirement not only for osteoclast development, but it plays an important role in lymphocyte development and lymph node organogenesis.

The physiological role of RANK was also investigated by generating RANK-deficient mice (Dougall *et al.,* 1999) (Table II). The phenotypes of RANK(−/−) mice were essentially the same as those of RANKL(−/−) mice, except for some differences. Like RANKL-deficient mice, RANK(−/−) mice were characterized by severe osteopetrosis resulting from an apparent block in osteoclast differentiation. RANK expression was not required for the commitment, differentiation, and functional maturation of macrophages and dendritic cells from their myeloid precursors, but provided a necessary and specific signal for the differentiation of myeloid-derived osteoclasts. RANK(−/−) mice also exhibited a marked deficiency of B cells in the spleen. RANK(−/−) mice retained mucosal-associated lymphoid tissues, including Peyer's patches, but completely lacked all the other peripheral lymph nodes. These results demonstrate that RANK provides critical signals necessary for lymph node organogenesis and osteoclast differentiation.

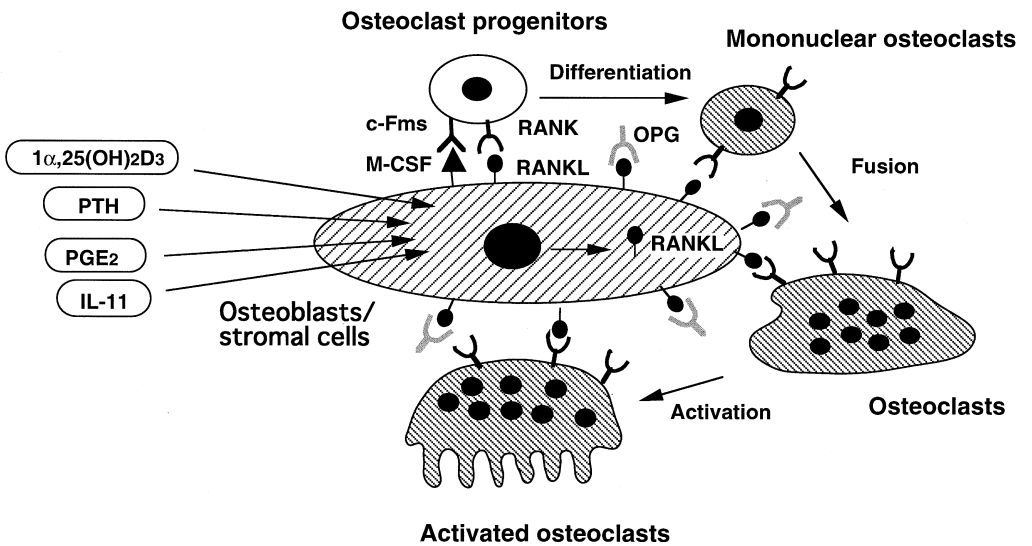


Figure 4 Schematic representation of osteoclast differentiation and function regulated by RANKL and M-CSF. Osteoclast progenitors and mature osteoclasts express RANK, the receptor for RANKL. Osteotropic factors such as $1\alpha,25(OH)_2D_3$, PTH, and IL-11 stimulate expression of RANKL in osteoblasts/stromal cells. Membrane- or matrix-associated forms of both M-CSF and RANKL expressed by osteoblasts/stromal cells are responsible for the induction of osteoclast differentiation in the coculture. RANKL also directly stimulates fusion and activation of osteoclasts. Mainly osteoblasts/stromal cells produce OPG, a soluble decoy receptor of RANKL. OPG strongly inhibits the entire differentiation, fusion, and activation processes of osteoclasts induced by RANKL.

Table II Comparison of Characteristics between RANKL-Deficient Mice and RANK-Deficient Mice

Characteristic	RANKL(−/−) mice	RANK(−/−) mice
Osteopetrosis	Severe	Severe
Osteoclasts in bone	Absence	Absence
Tooth eruption	Impaired	Impaired
Function of macrophages	Normal	Normal
Function of dendritic cells	Normal	Normal
B-cell development	Slightly impaired	Slightly impaired
T-cell development	Slightly impaired	Not impaired
Lymph node formation	Impaired	Impaired
Extramedullary hemopoiesis	Increased	Increased
Bone marrow transplantation	Not curable	Curable

Li *et al.* (2000a) further showed that RANK(−/−) mice lacked osteoclasts and had a profound defect in bone resorption and remodeling and in the development of the cartilaginous growth plates of endochondral bone. Osteopetrosis observed in these mutant mice was rescued by the transplantation of bone marrow from rag1 (recombinase activating gene 1)(−/−) mice, indicating that RANK(−/−) mice have an intrinsic defect in osteoclast lineage cells.

Osteoclastogenesis in RANK(−/−) mice was rescued by the transferring the RANK cDNA back into hematopoietic precursors. These data indicate that RANK is the intrinsic cell surface determinant that mediates RANKL effects on bone resorption.

Activating Mutations of RANK Found in Humans

Familial expansile osteolysis is a rare autosomal dominant disorder of bone characterized by focal areas of increased bone remodeling. The osteolytic lesions, which develop usually in the long bones during early adulthood, show increased osteoblast and osteoclast activity. Hughes *et al.* (2000) reported that the gene responsible for familial expansile osteolysis and familial Paget's disease of bone was mapped to the gene encoding RANK. Two mutations of heterozygous insertion were detected in the first exon of RANK in affected members of four families with familial expansile osteolysis or familial Paget's disease of bone. One mutation was a duplication of 18 bases and the other a duplication of 27 bases, both of which affected the signal peptide region (extracellular domain) of the RANK molecule. Expression of recombinant forms of the mutant RANK proteins revealed perturbations in the expression levels and lack of normal cleavage of the signal peptide. Both mutations caused an increase in RANK-mediated NF-κB signaling *in vitro*, consistent with the presence of an activating mutation. These results further confirm that RANK is involved in osteoclast differentiation and activation in humans as well.

Regulation of RANKL and OPG Expression

Osteoblasts/Stromal Cells

Treatment of calvarial osteoblasts with osteotropic factors such as $1\alpha,25(OH)_2D_3$, PTH, PGE_2 or IL-11, which stimulate osteoclast formation, up regulated the expression of RANKL mRNA. In many cases, expression of OPG mRNA is suppressed by those osteotropic factors. O'Brien *et al.* (1999) reported that the expression of dominant-negative STAT3 or dominant-negative gp130 suppressed RANKL expression in a stromal/osteoblastic cell line (UAMS-32) and osteoclast formation supporting activity stimulated by IL-6 together with soluble IL-6 receptor, oncostatin M, or IL-11 but not by PTH or $1\alpha,25(OH)_2D_3$. This suggests that the gp130/STAT3 signaling pathway induces RANKL expression in osteoblasts. The involvement of PGE receptor subtypes, EP1, EP2, EP3, and EP4, in PGE_2-induced bone resorption was examined using specific agonists for the respective EPs. Both the EP2 agonist and the EP4 agonist induced cAMP production and expression of RANKL mRNA in osteoblastic cells (Suzawa *et al.,* 2000). These results suggest that at least three signals are independently involved in RANKL expression by osteoblasts/stromal cells: VDR-mediated signals by $1\alpha,25(OH)_2D_3$; cAMP/protein kinase A (PKA)-mediated signals by PTH or PGE_2; and gp130-mediated signals by IL-11, IL-6, oncostatin M, and LIF (Fig. 5). Inverted TATA and CAAT boxes, a putative Cbfa1/Osf2/AML3 binding domain, and the repeated half-sites for VDR and the glucocorticoid receptor binding domain are found in the 5'-flanking basic promoter region of the mouse RANKL gene (Kitazawa *et al.,* 1999). Promoter analysis of the RANKL gene may elucidate the precise mechanism of the regulation of RANKL gene expression.

IL-1 stimulates osteoclast formation in the coculture of mouse primary osteoblasts and bone marrow cells, which is completely inhibited by the concomitant addition of indomethacin (Akatsu *et al.,* 1991). A positve correlation was observed between the number of osteoclasts induced by IL-1 and the amount of PGE_2 released into the culture

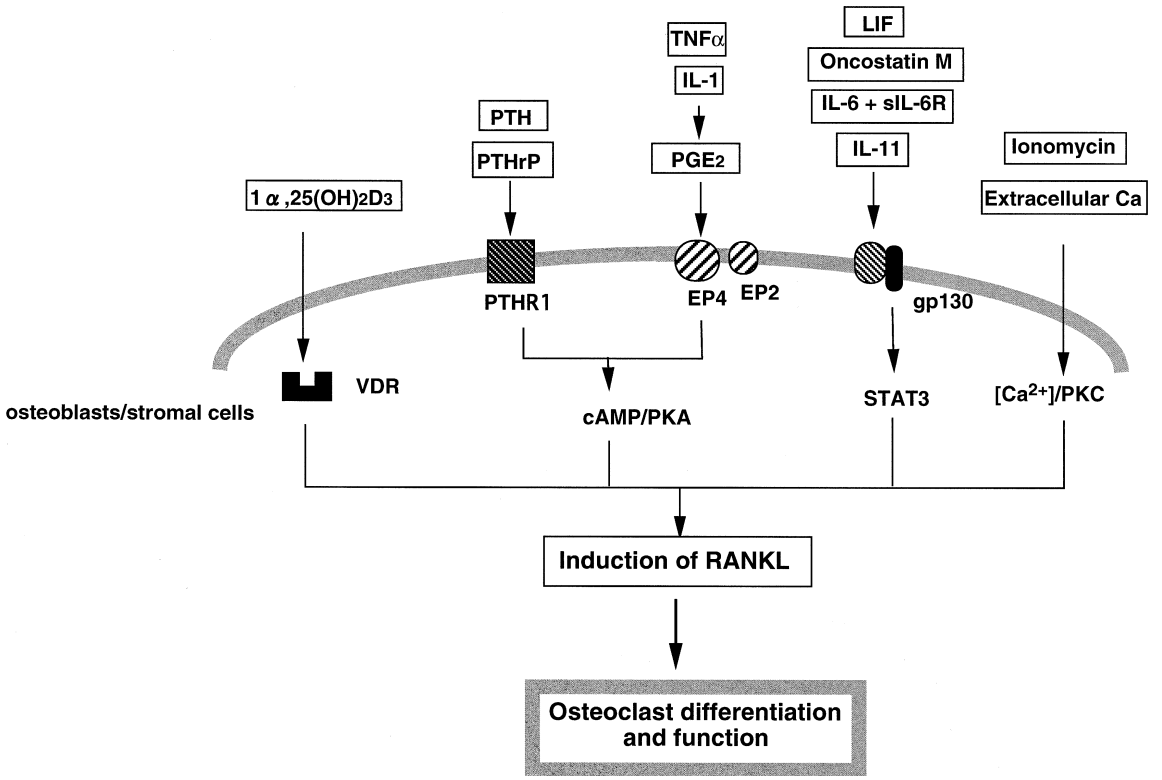


Figure 5 Signaling pathways for the induction of RANKL in osteoblasts/stromal cells. Three independent signals have been proposed to induce RANKL expression in osteoblasts/stromal cells: VDR-mediated signals by $1\alpha,25(OH)_2D_3$, cAMP/PKA-mediated signals by PTH and PGE_2, and gp130-mediated signals by IL-11, IL-6, oncostatin M, and LIF. IL-1 and TNFα also stimulate RANKL expression in osteoblasts/stromal cells through the up regulation of PGE_2 production. The calcium/PKC signal in osteoblasts/stromal cells, which is induced by iomomycin or high calcium concentrations of the culture medium, is now proposed to be the fourth signal involved in the induction of RANKL mRNA expression. RANKL expression induced by these four signals in osteoblasts/stromal cells in turn stimulates osteoclast differentiation and function.

medium. Sakuma *et al.* (2000) reported that osteoclasts were barely induced by IL-1 and TNFα in the coculture of primary osteoblasts and bone marrow cells prepared from EP4(−/−) mice, suggesting the crucial involvement of PGs and the EP4 subtype in osteoclast formation by IL-1 and TNFα (Fig. 5). In contrast, osteoclast formation induced by $1\alpha,25(OH)_2D_3$ was not impaired in the coculture of EP4(−/−) mouse-derived cells. These results suggest that PGE_2 is involved in the mechanism of IL-1- and TNFα-mediated osteoclast formation *in vitro* (Fig. 5).

Compounds, which elevate intracellular calcium, such as ionomycin, cyclopiazonic acid, and thapsigargin, also induced osteoclast formation in mouse cocultures of bone marrow cells and primary osteoblasts (Takami *et al.,* 1997). Similarly, high calcium concentrations of the culture medium induced osteoclast formation in the cocultures. Treatment of primary osteoblasts with these compounds or high medium calcium stimulated the expression of both RANKL and OPG mRNAs (Takami *et al.,* 2000). Phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC), also stimulated osteoclast formation in these cocultures and the expression of RANKL and OPG mRNAs in primary osteoblasts. PKC inhibitors, such as calphostin and staurosporin, suppressed ionomycin- and PMA-induced osteoclast formation in the coculture as well as the expression of RANKL and OPG mRNAs in primary osteoblasts. OPG strongly inhibited osteoclast formation induced by calcium-elevating compounds and PMA in the cocultures, suggesting that RANKL expression in osteoblasts is a rate-limiting step for osteoclast formation induced by calcium-elevating compounds. Forskolin , an activator of cAMP/protein kinase A (PKA) signals, also enhanced RANKL mRNA expression but, inversely, suppressed OPG mRNA expression in primary osteoblasts. Thus, calcium/PKC signals stimulate the expression of OPG mRNA whereas cAMP/PKA signals inhibit it, although both signals induce RANKL expression in osteoblasts/stromal cells in a similar manner. Therefore, the calcium/PKC signal is proposed to be the fourth signal pathway involved in the induction of RANKL mRNA expression, which in turn stimulates osteoclast formation (Fig. 5).

During embryonic bone development, osteoclasts appear just after bone mineralization takes place. Implantation of bone morphogenetic proteins (BMPs) into muscle or subcutaneous tissues induces ectopic bone formation at the site of the implantation. In this case, osteoclasts also appear just after bone tissue mineralization is initiated by BMPs. These results suggest that the physiological expression of RANKL in osteoblasts occurs in response to an endogeneous factor(s)

present in mineralized tissues. The calcium/PKC signaling system is one of the candidates that induce osteoclast formation in calcified bone. Further studies are necessary to elucidate the involvement of calcium/PKC signals in the regulation of osteoclast formation.

T LYMPHOCYTES

The RANKL–RANK interaction has been shown to regulate lymph node organogenesis, lymphocyte development, and interactions between T cells and dendritic cells in the immune system. RANKL expression in T cells is induced by antigen receptor engagement. Kong *et al.* (1999a) reported that activated T cells directly triggered osteoclastogenesis through RANKL expression. Using specific inhibitors, it was shown that the induction of RANKL by T cells depends on PKC, phosphoinositide-3 kinase, and calcineurin-mediated signaling pathways. RANKL was detected on the surface of activated T cells. Activated T cells also secreted soluble RANKL into culture medium. Both membrane-bound and soluble RANKL supported osteoclast development *in vitro*. Systemic activation of T cells *in vivo* also induced a RANKL-mediated increase in osteoclastogenesis and bone loss. In a T-cell-dependent model of rat adjuvant arthritis characterized by severe joint inflammation, treatment with OPG at the onset of the disease prevented bone and cartilage destruction but not inflammation. These results suggest that both systemic and local T-cell activation can lead to RANKL production and subsequent bone loss. Horwood *et al.* (1999) also reported that human peripheral blood-derived T cells, prepared with anti-CD3 antibody-coated magnetic beads, supported osteoclast differentiation from mouse spleen cells in the presence of Con A together with IL-1 or transforming growth factor-β (TGF-β) in the coculture. The expression of RANKL mRNA was stimulated in peripheral blood-derived T cells treated with the same factors. In synovial tissue sections with lymphoid infiltrates from patients with rheumatoid arthritis, the expression of RANKL was demonstrated in CD3-positive T cells. The ability of activated T lymphocytes to support osteoclast formation may provide a mechanism for the potentiation of osteoclast formation and bone destruction in diseases such as rheumatoid arthritis and periodontitis.

Teng *et al.* (2000) transplanted human peripheral blood lymphocytes from periodontitis patients into NOD/SCID mice. Human $CD4^{(+)}$ T cells, but not $CD8^{(+)}$ T cells or B cells, were identified as essential mediators of alveolar bone destruction in the transplanted mice. Stimulation of $CD4^{(+)}$ T cells by *Actinobacillus actinomycetemcomitans,* a well-known gram-negative anaerobic microorganism that causes human periodontitis, induced production of RANKL. *In vivo* inhibition of RANKL function with OPG diminished alveolar bone destruction and reduced the number of periodontal osteoclasts after microbial challenge. These data suggest that alveolar bone destruction observed in periodontal infections is mediated by the microorganism-triggered induction of RANKL expression on $CD4^{(+)}$ T cells.

Signal Transduction Mechanism of RANK

TRAFs as Signaling Molecules of RANK

Studies have indicated that the cytoplasmic tail of RANK interacts with TNF receptor-associated factor 1(TRAF1), TRAF2, TRAF3, TRAF5, and TRAF6 (Darnay *et al.,* 1998, 1999; Galibert *et al.,* 1998; Kim *et al.,* 1999; Wong *et al.,* 1998). Mapping of the structural requirements for TRAF/RANK interaction revealed that selective TRAF-binding sites are clustered in two distinct domains of the RANK cytoplasmic tail. In particular, TRAF6 interacts with the membrane-proximal domain of the cytoplasmic tail distinct from binding sites for TRAFs 1, 2, 3, and 5. When the proximal TRAF6 interaction domain was deleted, RANK-mediated NF-κB activation was completely inhibited and JNK activation partially inhibited (Galibert *et al.,* 1998). An N-terminal truncation mutant of TRAF6 (dominant-negative TRAF6) also inhibited RANKL-induced NF-κB activation (Darnay *et al.,* 1999). These results suggest that TRAF6 transduces a signal involved in RANK-mediated differentiation and activation of osteoclasts (Fig. 6).

Lomaga *et al.* (1999) have reported that TRAF6(−/−) mice are osteopetrotic with defects in bone resorption and tooth eruption due to impaired osteoclast function. A similar number of TRAP-positive osteoclasts were observed in bone tissues in wild-type and TRAF6(−/−) mice, but TRAP-positive osteoclasts in TRAF6(−/−) mice failed to form ruffled borders. Using *in vitro* assays, it was demonstrated that TRAF6 is crucial not only for IL-1 and CD40 signalings but also for lipopolysaccharide (LPS) signaling. Naito *et al.* (1999) reported independently that TRAF6(−/−) mice exhibited severe osteopetrosis. However, unlike the report by Lomaga *et al.* (1999), TRAF6(−/−) mice produced by Naito *et al*. (1999) were defective in osteoclast formation as well. *in vitro* experiments revealed that osteoclast precursors derived from TRAF6(−/−) mice are unable to differentiate into functional osteoclasts in response to RANKL and M-CSF. The cause of the difference between the two TRAF6(−/−) mice is not known at present, perhaps it was due to different experimental conditions, but TRAF6 is proposed to be an essential component of the RANK-mediated signaling pathway in bone metabolism and immune/inflammatory systems *in vivo* (Table III).

Takayanagi *et al.* (2000) reported that T-cell production of interferon-γ (IFN-γ) strongly suppresses osteoclastogenesis by interfering with the RANKL–RANK signaling pathway. IFNγ-induced rapid degradation of TRAF6 in osteoclast precursors, which resulted in strong inhibition of the RANKL-induced activation of the transcription factor NF-κB and JNK. These results suggest that there is cross-communication between the TNF and IFN families of cytokines, through which IFNγ provides a negative link between T-cell activation and bone resorption.

Soriano *et al.* (1991) were the first to report that the targeted disruption of the gene encoding c-Src (a member of the tyrosine kinase family) induced an osteopetrotic disorder.

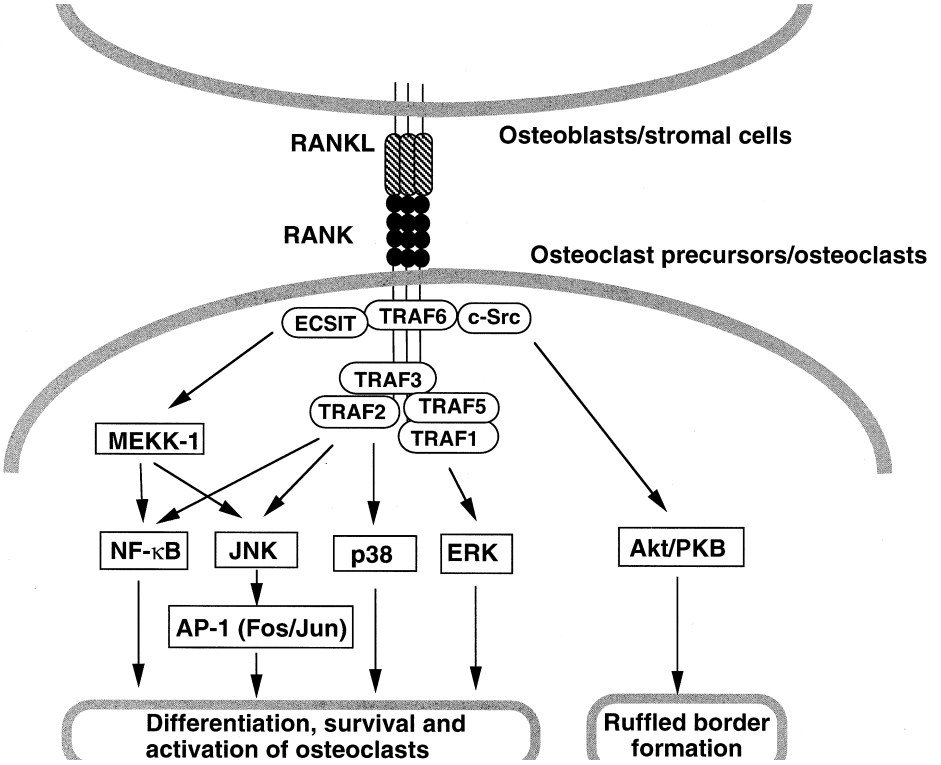


Figure 6 Signal transduction induced by the RANKL–RANK interaction in the target cell. The cytoplasmic tail of RANK interacts with TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6. The RANKL–RANK interaction induces activation of NF-κB, JNK, p38 MAP kinase, and ERK in osteoclast precursors, as well as in mature osteoclasts. In addition, RANKL activates Akt/PKB through a signaling complex involving c-Src and TRAF6. RANK-induced Akt/PKB signals appear to be involved in ruffled border formation. The adapter protein, ECSIT, may be important for TRAF6-mediated osteoclast differentiation and activation.

Local injection of PTH and IL-1 over the calvaria of c-Src-deficient mice increased the number of multinucleated cells with the morphological characteristics of osteoclasts in calvaria, but these multinucleated cells failed to develop ruffled borders (Boyce *et al.,* 1992). Using a mouse coculture system, it was shown that spleen cells obtained from c-Src-deficient mice differentiated into TRAP-positive multinucleated cells, but they did not form resorption pits on dentine slices (Lowe *et al.,* 1993). Transplantation of fetal liver cells into c-Src-deficient mice cured their osteopetrotic disorders. Indeed, osteoclasts have been shown to express high levels of c-Src (Horne *et al.,* 1992; Tanaka *et al.,* 1992). These findings suggest that c-Src expressed in osteoclasts plays a crucial role in ruffled border formation (Table III).

RANKL has been shown to activate the anti apoptotic serine/threonine kinase Akt/PKB (protein kinase B) through a signaling complex involving c-Src and TRAF6 (Wong *et al.,* 1999) (Fig. 6). A deficiency in c-Src or the addition of inhibitors of the Src family kinases blocked RANKL-mediated Akt/PKB activation in osteoclasts. The RANKL–RANK interaction triggered simultaneous binding of c-Src and TRAF6 to the intracellular domain of RANK, resulting in the enhancement of c-Src kinase activity leading to tyrosine phosphorylation of downstream signaling molecules such as c-Cbl (Tanaka *et al.,* 1996) and $p130^{Cas}$ (Nakamura *et al.,* 1998). These results suggest a mechanism by which RANKL activates Src family kinases and Akt/PKB. The results also provide evidence for the presence of cross-communication between TRAF proteins and Src family kinases. Kopp *et al.* (1999) identified a novel intermediate in the signaling pathways that bridges TRAF6 to MEKK-1 [mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) kinase kinase-1]. This adapter protein, named ECSIT (evolutionarily conserved signaling intermediate in Toll pathways), was shown to be specific for the Toll/IL-1 pathways and is a regulator of MEKK-1 processing. Expression of wild-type ECSIT accelerated the processing of MEKK-1 and NF-κB activation, whereas a dominant-negative fragment of ECSIT blocked both MEKK-1 processing and activation of NF-κB. These results indicate that ECSIT plays an important role for TRAF6-meditated osteoclast differentiation and function (Fig. 6).

RANK-Mediated Signals

Franzoso *et al.* (1997) and Iotsova *et al*. (1997) independently generated mice deficient in both p50 and p52 subunits

Table III Characteristics of Mice Carrying Disrupted Genes Involved in Osteoclast Differentiation and Function

Gene disrupted	Phenotype	State of bone resorption	Defective cells	References
c-src	Osteopetrosis	Osteoclasts are present, but fail to form ruffled borders	Osteoclast progenitors	Soriano *et al.* (1991), Boyce *et al.* (1992), Lowe *et al.* (1993)
c-fos	Osteopetrosis	Osteoclasts are absent	Osteoclast progenitors	Wang *et al.* (1992), Grigoriadis *et al.* (1994)
p50/p52 (NF-κB)	Osteopetrosis	Osteoclasts are absent	Osteoblasts progenitors	Franzoso *et al.* (1997), Iotsova *et al.* (1997)
rankl	Osteopetrosis	Osteoclasts are absent	Osteoblasts	Kong *et al.* (1999)
rank	Osteopetrosis	Osteoclasts are absent	Osteoclast progenitors	Dougall *et al.* (1999), Li *et al.* (2000)
opg	Osteoporosis	Osteoclastic bone resorption is enhanced	Osteoblasts	Mizuno *et al.* (1998), Bucay *et al.* (1998)
traf6 (1)	Osteopetrosis	Osteoclasts are present, but fail to form ruffled borders	Not determined	Lomaga *et al.* (1999)
traf6 (2)	Osteopetrosis	The number of osteoclasts are decreased markedly	Osteoclast progenitors	Naito *et al.* (1999)

of NF-κB (Table III). The double knockout mice developed severe osteopetrosis due to a defect in osteoclast differentiation. The osteopetrotic phenotype was rescued by bone marrow transplantation, indicating that the osteoclast progenitors are inactive in the double knockout mice. RANKL has been shown to activate NF-κB in the target cells, including osteoclast precursors and mature osteoclasts. These results suggest that RANKL-induced activation of NF-κB in osteoclast progenitors plays a crucial role in their differentiation into osteoclasts (Fig. 6).

Purified osteoclasts died spontaneously via apoptosis, whereas IL-1 promoted the survival of osteoclasts by preventing their apoptosis. Jimi *et al*. (1996a) reported that the pretreatment of purified osteoclasts with proteasome inhibitors suppressed the IL-1-induced activation of NF-κB and prevented the survival of osteoclasts supported by IL-1. When osteoclasts were pretreated with the antisense oligodeoxynucleotides to the p65 and p50 subunits of NF-κB, the expression of respective mRNAs by osteoclasts was suppressed, together with the concomitant inhibition of IL-1-induced survival of osteoclasts. These results indicate that IL-1 promotes the survival of osteoclasts through the activation of NF-κB. Miyazaki *et al.* (2000) also examined the role of mitogen-activated protein kinase and NF-κB pathways in osteoclast survival and activation, using adenovirus vectors carrying various mutants of signaling molecules. Inhibition of ERK activity by dominant-negative Ras overexpression induced the apoptosis of osteoclasts rapidly, whereas ERK activation by the introduction of constitutively active MEK (MAPK/ERK kinase) prolonged their survival remarkably. Neither inhibition nor activation of ERK affected the pit-forming activity of osteoclasts. In contrast, inhibition of the NF-κB pathway with dominant-negative IκB kinase suppressed the pit-forming activity of osteoclasts. NF-κB activation by constitutively active IκB kinase expression up regulated the pit-forming activity of osteoclasts without affecting their survival. IL-1 strongly induced both ERK and NF-κB activation. Matsumoto *et al.* (2000) found that treatment of bone marrow cells with an inhibitor of p38 MAP kinase (SB203580) suppressed osteoclast differentiation via inhibition of the RANKL-mediated signaling pathway. RAW264, a transformed mouse myeloid cell line, has been shown to differentiate into osteoclasts in response to RANKL (Hsu *et al.,* 1999). Expression of the dominant negative form of p38 MAP kinase in RAW264 cells inhibited their RANKL-induced differentiation into osteoclasts. These results indicate that activation of the p38 MAP kinase pathway also plays an important role in RANKL-induced osteoclast differentiation.

Mice lacking c-Fos have been shown to develop osteopetrosis due to an early differentiation block in the osteoclast lineage (Grigoriadis *et al.,* 1994; Wang *et al.,* 1992) (Table III). The dimeric transcription factor activator protein-1 (AP-1) is composed of mainly Fos proteins (c-Fos, FosB, Fra-1, and Fra-2) and Jun proteins (c-Jun, JunB, and JunD). RANKL activated JNK in the target cells, including purified osteoclasts and osteoclast progenitors. These results suggest that AP-1 appears to be located downstream of RANK-mediated signals. Unlike c-Fos, Fra-1 lacks transactivation domains required for oncogenesis and cellular transformation. Using a retroviral gene transfer, Matsuo *et al.* (2000) showed that all four Fos proteins, but not Jun proteins, rescued the differentiation block of c-Fos-deficient spleen cells into osteoclasts *in vitro*. Structure–function analysis demonstrated that the major carboxy-terminal transactivation domains of c-Fos and FosB are dispensable and that Fra-1 has the highest rescue activity. Moreover, a transgene expressing Fra-1 rescued the osteopetrosis of c-Fos-mutant mice *in vivo*. RANKL induced

transcription of Fra-1 expression in a c-Fos-dependent manner. These results indicate the presence of a link between RANK signaling and the expression of AP-1 proteins in inducing osteoclast differentiation (Fig. 6).

Cross-Communication between RANKL and TGF-β Superfamily Members

Bone is a major storage site for the cytokines of the TGF-β superfamily, such as TGF-β and BMPs. Osteoclastic bone resorption releases these growth factors from bone matrix. Receptors for TGF-β superfamily members are a family of transmembrane serine/threonine kinases and are classified as type I and type II receptors according to their structural and functional characteristics (Miyazono, 2000). Formation of a type I–type II receptor complex is required for the ligand-induced signals. Previous studies have shown that the extracellular domain of type I receptors is sufficient to mediate stable binding to TGF-β superfamily members and subsequent formation of a heteromeric complex with the intact type II receptors. Sells Galvin *et al.* (1999) first reported that TGF-β enhanced osteoclast differentiation in cultures of mouse bone marrow cells stimulated with RANKL and M-CSF. These results support the previous findings (1) that transgenic mice expressing TGF-β2 developed osteoporosis due to enhanced osteoclast formation (Erlebacher and Derynck, 1996) and (2) that osteoclast formation was reduced in transgenic mice expressing a truncated TGF-β type II receptor in the cytoplasmic domain (Filvaroff *et al.,* 1999). Fuller *et al.* (2000) also reported that activin A potentiated RANKL-induced osteoclast formation. Moreover, osteoclast formation induced by RANKL was abolished completely by adding soluble activin receptor type IIA or soluble TGF-β receptor type II, suggesting that activin A and TGF-β are essential factors for osteoclastogenesis. We further found that BMP-2 enhanced the differentiation of osteoclasts and the survival of osteoclasts supported by RANKL (Itoh *et al.,* 2000a). A soluble form of BMP receptor IA, which inhibits the binding of BMP-2 to BMP receptor IA, blocked RANKL-induced osteoclast formation. Thus, BMP-2 is yet another important determinant of osteoclast formation. Although the molecular mechanism by which TGF-β superfamily members potentiate the RANK-mediated signals is not known, cytokines released from bone matrix accompanying osteoclastic bone resorption appear to play an important role in RANKL-induced osteoclast formation. Further studies will elucidate the molecular mechanism of the cross-communication between TGF-β superfamily members and RANKL in osteoclast differentiation and function.

RANK Is Not the Sole Factor Responsible for Osteoclast Differentiation and Function

IL-1 stimulates not only osteoclast differentiation, but also osteoclast function through the IL-1 type 1 receptor (Jimi *et al.,* 1999b). As described earlier, purified osteoclasts placed on dentine slices failed to form resorption pits. When IL-1 or RANKL was added to the purified osteoclast cultures, resorption pits were formed on dentine slices within 24 hr (Jimi *et al.,* 1999b). Osteoclasts express IL-1 type 1 receptors, and IL-1 activated NF-κB rapidly in purified osteoclasts. The pit-forming activity of osteoclasts induced by IL-1 was inhibited completely by adding IL-1 receptor antagonist (IL-1ra) but not by OPG (Jimi *et al.,* 1999a). This suggests that IL-1 directly stimulates osteoclast function through IL-1 type 1 receptors in mature osteoclasts (Fig. 7).

Since the discovery of the RANKL–RANK signaling system, RANKL has been regarded as the sole factor responsible for inducing osteoclast differentiation. Azuma *et al.* (2000) and Kobayashi *et al.,* (2000) independently found that TNFα stimulates osteoclast differentiation in the absence of the RANKL–RANK interaction (Fig. 7). When mouse bone marrow cells were cultured with M-CSF, M-CSF-dependent bone marrow macrophages appeared within 3 days. In addition, TRAP-positive osteoclasts were formed in response to not only RANKL but also mouse TNFα, when bone marrow macrophages were cultured further for another 3 days with either ligand in the presence of M-CSF. Osteoclast formation induced by TNFα was inhibited by the addition of respective antibodies against TNF receptor type I (TNFRI, p55) and TNF receptor type II (TNFRII, p75), but not by OPG. Osteoclasts induced by TNFα formed resorption pits on dentine slices only in the presence of IL-1. These results demonstrate that TNFα stimulates osteoclast differentiation in the presence of M-CSF through a mechanism independent of the RANKL–RANK system (Fig. 7). TNFRI and TNFRII use TRAF2 as a common signal transducer in the target cells, suggesting that TRAF2-mediated signals play important roles in osteoclast differentiation. It has been reported that when osteotropic factors such as $1\alpha,25(OH)_2D_3$, PTHrP, and IL-1 were administered into RANK(−/−) mice, neither TRAP-positive cell formation nor hypercalcemia was induced (Li *et al.,* 2000a). In contrast, administration of TNFα into RANK(−/−) mice induced TRAP-positive cells near the site of injection even though the number of TRAP-positive cells induced by TNFα was not large. This suggests that TNFα somewhat induces osteoclasts in the absence of RANK-mediated signals *in vivo*. These results further strongly delineate that the RANKL–RANK interaction is not the sole pathway for inducing osteoclast differentiation *in vitro* and *in vivo*. It is, therefore, proposed that TNFα, together with IL-1, plays an important role in bone resorption in metabolic bone diseases such as rheumatoid arthritis, periodontitis, and possibly osteoporosis. Lam *et al.* (2000) also reported that a small amount of RANKL strongly enhanced osteoclast differentiation in a pure population of murine precursors in the presence of TNFα. These results suggest that RANKL-induced signals cross-communicate with TNFα-induced ones in the target cells.

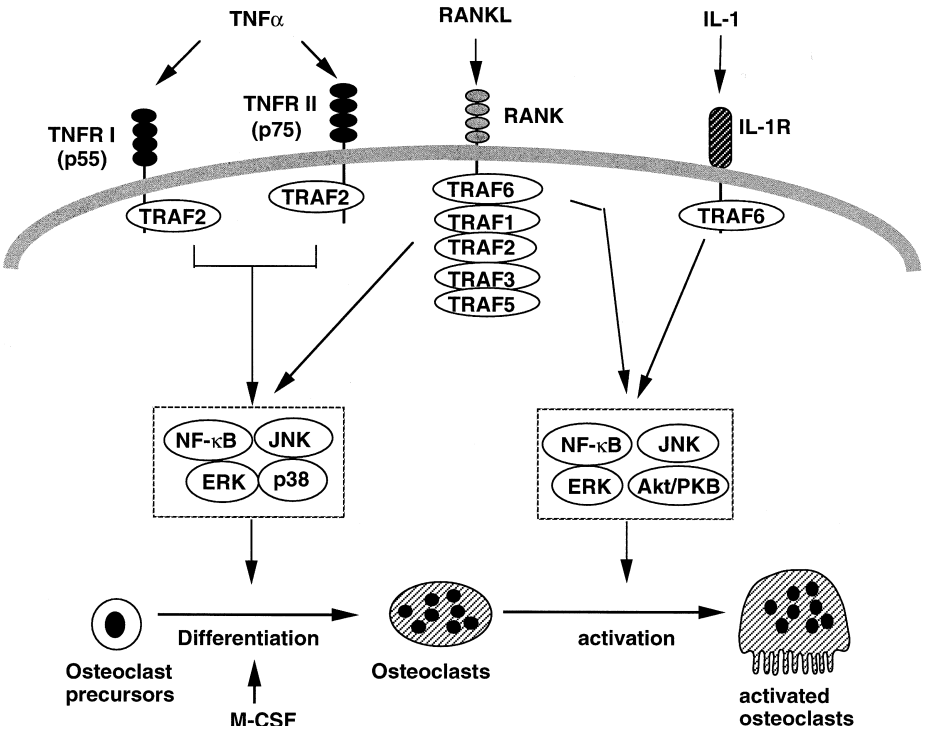


Figure 7 Schematic representation of ligand–receptor systems in osteoclast differentiation and function regulated by TNFα, RANKL, and IL-1. TNFα and RANKL stimulate osteoclast differentiation independently. Osteoclast differentiation induced by TNFα occurs via TNFRI (p55) and TNFRII (p75) expressed by osteoclast precursors. RANKL induces osteoclast differentiation through RANK-mediated signals. M-CSF is a common factor required by both TNFα- and RANKL-induced osteoclast differentiation. Activation of osteoclasts is induced by RANKL and IL-1 through RANK and IL-1 type I receptors, respectively. Common signaling cascades, such as NF-κB, JNK, p38 MAP kinase, and ERK activation, may be involved in the differentiation of osteoclasts induced by TNFα and RANKL. RANKL and IL-1 may activate osteoclast function though signals mediated by NF-κB, JNK, ERK, and Ark/PKB.

Conclusion

The discovery of the RANKL–RANK interaction now opens a wide new area in bone biology focused on the investigation of the molecular mechanism of osteoclast development and function. Osteoblasts/stromal cells, through the expression of RANKL and M-CSF, are involved throughout the osteoclast lifetime in all processes that govern their differentiation, survival, fusion, and activation. OPG produced by osteoblasts/stromal cells is an important negative regulator of osteoclast differentiation and function. Membrane- or matrix-associated forms of both M-CSF and RANKL expressed by osteoblasts/stromal cells appear to be essential for osteoclast formation. Both RANKL(−/−) mice and RANK(−/−) mice show similar features of osteopetrosis with a complete absence of osteoclasts in bone. Gain-of-function mutations of RANK have been found in patients suffering from familial expansile osteolysis and familial Paget's disease of bone. These findings confirm that the RANKL–RANK interaction is indispensable for osteoclastogenesis not only in mice but also in humans. The cytoplasmic tail of RANK interacts with the TRAF family members. TRAF2-mediated signals appear important for inducing osteoclast differentiation, and TRAF6-mediated signals are indispensable for osteoclast activation. Activation of NF-κB, JNK, and ERK, all induced by RANKL in osteoclast precursors and mature osteoclasts, may be involved in their differentiation and function. OPG strongly blocked all processes of osteoclastic bone resorption *in vivo,* suggesting that inhibiting either the RANKL–RANK interaction or RANK-mediated signals are ideal ways to prevent increased bone resorption in metabolic bone diseases such as rheumatoid arthritis, periodontitis, and osteoporosis. Studies have also shown that TNFα and IL-1 can substitute for RANKL in inducing osteoclast differentiation and function *in vitro*. These results suggest that signals other than RANK-induced ones may also play important roles in osteoclastic bone resorption under pathological conditions.

Under physiological conditions, osteoclast formation requires cell-to-cell contact with osteoblasts/stromal cells, which express RANKL as a membrane-bound factor in response to several bone-resorbing factors. In normal bone remodeling, osteoblastic bone formation occurs in a programmed precise and quantitative manner following osteoclastic bone resorption: bone formation is coupled to bone

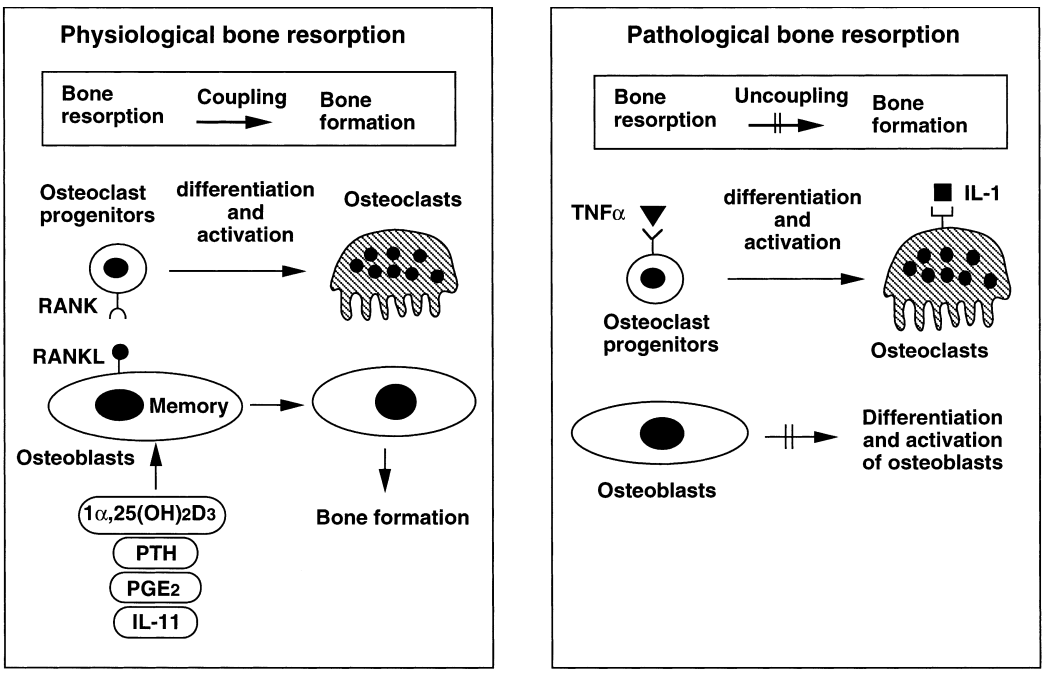


Figure 8 A hypothesis on the regulation of osteoblastic bone formation under physiological and pathological bone resorption. Under physiological conditions, osteoclast formation requires cell-to-cell contact with osteoblasts/stromal cells, which express RANKL as a membrane-associated factor in response to several bone-resorbing factors. In normal bone remodeling, osteoblastic bone formation occurs in a programmed precise and quantitative manner following osteoclastic bone resorption. It is so-called coupling between bone resorption and bone formation. In contrast, in pathological bone resorption, macrophages and/or T cells secrete inflammatory cytokines, such as TNFα and IL-1, as well as a soluble form of RANKL, which act directly on osteoclast progenitors and mature osteoclasts without cell-to-cell contact. This situation is characterized by uncoupling between bone resorption and bone formation. Cell-to-cell contact between osteoclast progenitors and osteoblasts may leave behind some memory for bone formation in osteoblasts.

resorption. In contrast, in pathological bone resorption, as in rheumatoid arthritis, macrophages and/or T cells secrete inflammatory cytokines such as TNFα and IL-1, which act directly on osteoclast progenitors and mature osteoclasts without cell-to-cell contact. This situation is characterized by uncoupling between bone resorption and bone formation. It is, therefore, noteworthy to consider that cell-to-cell contact between osteoclast progenitors and osteoblasts may leave behind in osteoblasts some memory for bone formation (Fig. 8). Further experiments are necessary to verify this hypothesis. Studies on the signal transduction of these TNF-ligand family members in osteoclast progenitors and in mature osteoclasts will provide novel approaches for the treatment of metabolic bone diseases.

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CHAPTER 8

Osteoclast Function

Biology and Mechanisms

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Introduction

There is a unanimous consensus among biologists that the main function of osteoclasts is to resorb mineralized bone, dentine, and calcified cartilage. Actually, according to our current knowledge, this seems to be the only function for those large and multinucleated cells that reveal several unique features. Resorption of mineralized tissues is obligatory for normal skeletal maturation, including bone growth and remodeling, as well as tooth eruption.

In evolution the appearance of osteoclasts opened a totally new strategy for skeletal development. However, it is very difficult to know exactly why natural selection during evolution has favored the development of osteoclasts in the first place. Was it because of the obvious advantages they offered for the flexible use of the skeleton? Perhaps the development of osteo (chondro) clast-like cells was favored by natural selection due to the advantages of the effective regulation of calcium homeostasis. The third possibility could be the need and pressure for the development of a safe environment for the hematopoetic tissue. No firm conclusions can be drawn from the current evidence and knowledge. Given the importance of calcium homeostasis and hematopoesis, one might speculate that resorptive cells were originally developed not at all for skeletal purposes, but to support those vital functions.

In the ontogenic development of most vertebrates, cartilage appears before bone. Based on this fact, many biologists, especially bone biologists, have concluded that cartilage is also more primitive than bone. This seems not to be the case, as it is likely that bone actually preceded cartilage during evolution. Thus it is not known yet for sure if functional osteoclasts were originally developed for the resorption of bone or calcified cartilage. It also remains to be clarified at what stage of the evolution resorbing osteoclasts appeared. Most probably this has taken place more than 300 millions years ago.

Is There More Than One Type of Bone-Resorbing Cell?

During skeletal growth, osteoclasts are needed for the resorption of calcified cartilage and modeling of growing bone. In adult bone, resorptive cells are responsible for remodeling. If necessary, they fulfill the requirements of calcium homeostasis via excessive resorption beyond normal remodeling. In addition to osteoclasts tumor cells, monocytes and macrophages have been suggested to have bone-resorbing capacity. However, later studies have not been able to confirm that tumor cells can resorb bone directly. Instead they can induce recruitment, as well as activity of osteoclasts, by secreting a large number of osteoclast regulating factors (Ralston, 1990). Bone resorption by macrophages has been demonstrated only *in vitro* and is probably due to the phagocytosis of bone particles rather than the more specialized mechanisms used by the osteoclasts. It is also possible that mineralized bone per se can induce monocytes and tissue macrophages to differentiate into osteoclasts under culture conditions.

At present it is thus generally accepted that the osteoclast is the only cell that is able to resorb mineralized bone. Both

mononuclear and multinuclear osteoclasts can resorb bone, but larger cells seem to be more effective than smaller ones, although there is no direct relationship between the resorption capacity and the number of nuclei (Piper *et al.,* 1992). The number of nuclei in osteoclasts also varies among species, being higher in birds than in mammals.

Osteoclasts in different types of bone have been thought to be similar. There is now some evidence, however, that osteoclasts are not necessarily similar at all sites. Lee *et al.*(1995) described mononuclear cells with ruffled borders on uncalcified septa of the growth plate cartilage. These cells, named septoclasts by the authors, express high levels of cathepsin B but not ED1, which is present in osteoclasts. Septoclasts probably resorb transversal septa of the growth plate just before chondroclasts start resorption of longitudinal calcified septa. Rice *et al.* (1997) also reported two populations of multinucleated osteoclasts. They observed that in growing calvaria, osteoclasts that were next to sutures expressed MMP-9 but were tartrate-resistant acid phosphatase (TRAP) negative. The majority of multinucleated osteoclasts expressed both enzymes. Everts *et al.* (1999) concluded that osteoclasts in calvarial bones were sensitive to matrix metalloproteinase (MMP) inhibitors, whereas osteoclasts in long bones were not. According to present knowledge, chondroclasts that resorb calcified cartilage are similar to osteoclasts, but there are perhaps also other types of resorbing cells present in specific areas of the growing skeleton.

Life Span of the Osteoclast and the Resorption Cycle

In the adult, stem cells for osteoclasts originate from the hematopoetic tissue (for review, see Chapter 7). They share a common differentiation pathway with macrophages until the final differentiation steps. Differentiation is characterized by the sequential expression of different sets of genes. Several cytokines and growth factors are known to affect the differentiation pathway of the osteoclast, and studies have confirmed the central role of the RANK-RANKL-OPG pathway in this process.

After proliferating in bone marrow, mononuclear preosteoclasts are guided to bone surfaces by mechanisms, that are so far unknown. It is not known in detail where and when fusion of mononuclear precursors to multinuclear osteoclasts actually takes place and what are the molecular mechanisms regulating fusion. There are certainly several types of molecular interactions between the membranes of two cells undergoing fusion. Cadherin-mediated cell–cell interactions might play a role in the early phase of osteoclast fusion (Mbalaviele *et al.,* 1995). Syncytin, a captive retroviral envelope protein, has been described to be important in the fusion of placental syncytiotrophoblasts (Mi *et al.,* 2000). It remains to be seen if similar fusion proteins are also mediating the fusion of precursors into multinucleated osteoclasts.

The fusion of mononuclear precursors into multinucleated osteoclasts in the bone marrow mainly takes place in the vicinity of bone surfaces, as multinuclear osteoclasts are seldom observed far away from the bone surface. Somehow, precursors are guided near to those sites that are determined to be resorbed. How this happens, how these sites are determined, and which cells actually make the decision where and when for instance a new remodeling unit is initiated are not known. Strongest candidates for this role are osteocytes and bone lining cells. Both negative and positive regulation between osteocytes and osteoclasts could exist. We have shown that osteocytes secrete a biological activity that inhibits osteoclasts differentiation (Matikainen *et al.,* 2000). Thus, it is possible that healthy osteocytes secrete a factor(s) that prevents osteoclast differentiation and activation, whereas dying osteocytes promote osteoclast activity. There is indirect evidence, indeed, that such mechanisms are operating *in vivo.* Osteocytes that form an internal network that could sense the whole bone as a single unit (Aarden *et al.,* 1994) are the most obvious cells to act as gatekeepers for the local remodeling processes. Learning the molecular details of this complicated biological event will be the challenge of the next decade for bone biologists.

Although it is not known how resorption sites are determined, it is known that the first sign of a forthcoming resorption place on the endosteal surface is the retraction of bone-lining cells (Jones and Boyde, 1976). This retraction uncovers osteoid and after its removal by osteoblasts the osteoclasts can attach to the mineralized surface. The sequence of cellular events needed for bone resorption is called the resorption cycle (see Figs. 1 and 4). One resorption cycle of any individual osteoclast thus involves complicated multistep processes, which include osteoclast attachment, its polarization, formation of the sealing zone, and

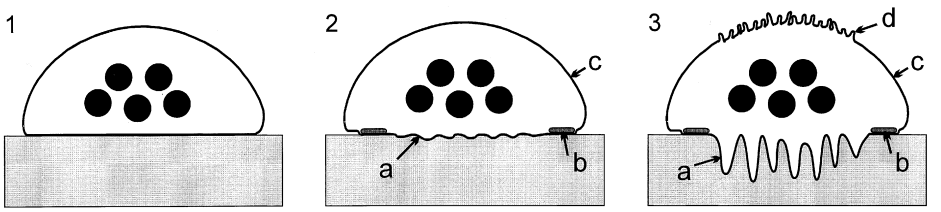

Figure 1 The nonresorbing osteoclast is polarized (1), but immediately after attachment for resorption it shows three different membrane domains (2): ruffled border (a), sealing zone (b), and basal membrane (c). Once matrix degradation has started (3), the fourth membrane domain appears in the basal membrane (d).

resorption itself with final detachment and cell death (Lakkakorpi and Väänänen, 1995; Väänänen *et al.,* 2000). On the basis of *in vitro* studies, one osteoclast can undergo several consecutive resorption cycles before entering the apoptosis pathway (Kanehisa and Heerche, 1988). Following is a short description of each particular phase of the resorption cycle.

Attachment of Osteoclasts to the Bone Surface and Formation of the Sealing Zone

Several lines of evidence have shown that adhesion receptors in osteoclasts play an important functional role in bone resorption. Osteoclasts express at least four integrin extracellular matrix receptors: $\alpha_v\beta_3$ (a classical vitronectin receptor), $\alpha_v\beta_5$ $\alpha_2\beta_1$ (collagen receptor), and $\alpha v\beta 1$, which binds to a variety of extracellular matrix proteins, including vitronectin, collagen, osteopontin, and bone sialoprotein (Nesbit *et al.,* 1993). Antibodies against the vitronectin receptor, Arg-Gly-Asp (RGD) mimetics, and RGD peptides, which block the attachment of the vitronectin receptor to RGD-containing bone matrix proteins, inhibit bone resorption *in vitro* (Sato *et al.,* 1990; Horton *et al.,* 1991; Lakkakorpi *et al.,* 1991) and *in vivo* (Fisher *et al.,* 1993).

Glanzmann's thrombasthenia patients and knock out of the β_3 gene in mice have clarified the importance of the vitronectin receptor in the function of osteoclasts (Djaffar *et al.,* 1993; Hodivala-Dilke *et al.,* 1999; McHugh *et al.,* 2000). β_3 mutants grow normally, indicating that the lack of β_3 does not prevent bone resorption during skeletal growth and modeling. However, in these mice, osteoclasts are morphologically abnormal and do not express normal cytoskeletal organization, including actin ring formation. These observation strongly suggest that the vitronectin receptor, rather than forming a tight cell–matrix contact at the sealing zone, plays a regulatory role in mediating signals between the extracellular matrix and the osteoclast. This function is also supported by observations from echistatin-treated mice (Masarachia *et al.,* 1998; Yamamoto *et al.,* 1998), which did not reveal changes in the sealing zone or ruffled borders. A regulatory role for $\alpha_v\beta_3$ was originally suggested on the basis of its distribution on the plasma membrane of the resorbing osteoclasts (Lakkakorpi *et al.,* 1991). A precise function(s) of $\alpha_v\beta_3$ in osteoclasts remains unknown at the present. The $\alpha_v\beta_3$ integrin could play a role both in the adhesion and migration of osteoclasts (e.g.) by regulation of actin cytoskeleton) and in the endocytosis and transportation of resorption products (Väänänen *et al.,* 2000).

During the mineral dissolution phase, osteoclasts must retain a low pH in the resorption lacuna (see later). The ultrastructure of the resorbing osteoclast (Fig. 2) clearly shows that the plasma membrane of the osteoclast at the sealing zone is tightly attached to the matrix, offering a good structural basis for the isolation of the resorption lacuna from the extracellular fluid. It has also been demonstrated that a pH gradient really exists (Baron *et al.,* 1985), indicating that the sealing around the resorption lacuna is tight enough to maintain the pH gradient. So far the molecular interaction(s) between the plasma membrane and the bone matrix remains unknown. As explained earlier, it is unlikely that $\alpha_v\beta_3$ could mediate this interaction. On the basis of extensive series of morphological and functional studies, Väänänen and Horton (1995) suggested that the osteoclast sealing zone represents a specific type of cell–matrix interaction. Ilvesaro *et al.* (1998) illustrated that the sealing zone might have common features with the epithelial zonula–adherens

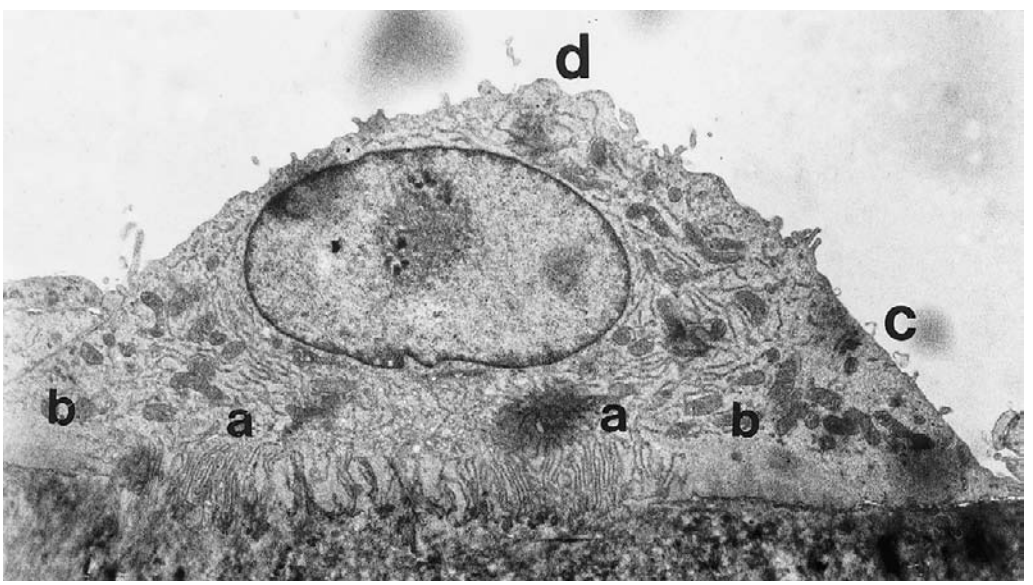

Figure 2 A transmission electron microscopic image of a bone-resorbing osteoclast. a, ruffled border; b, sealing zone; c, basal membrane; d, functional secretory domain. Original magnification: 2500×.

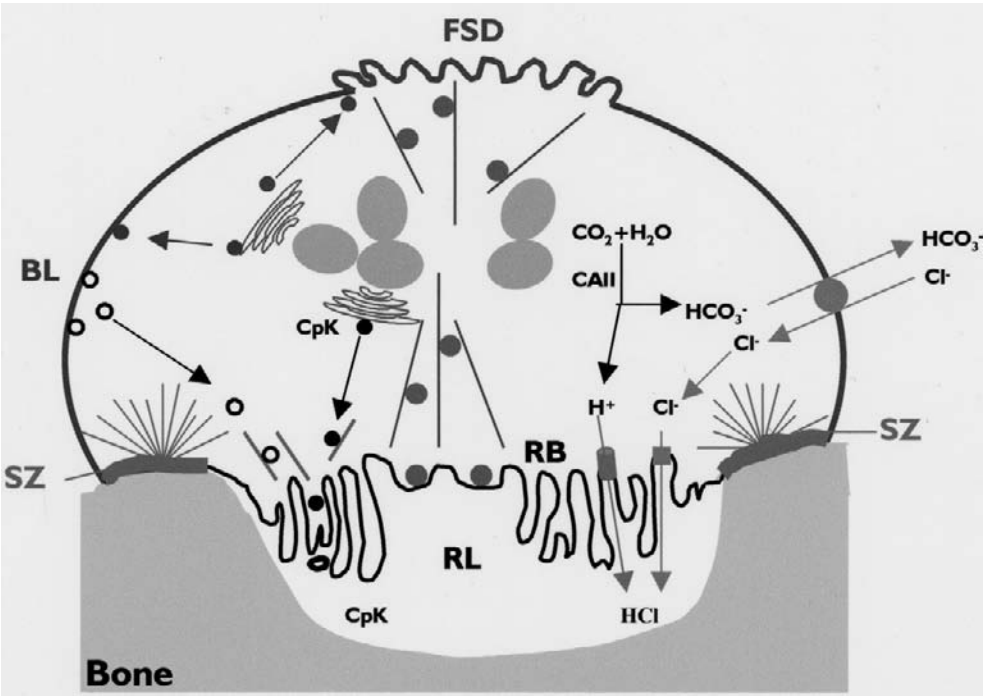

Figure 3 Schematic illustration of a bone-resorbing osteoclast. Intensive intracellular membrane trafficking is involved in the establishment of specific plasma membrane domains and resorption processes. Cathepsin K and protons are secreted vectorially to the resorption lacunae. Transcytotic vesicles are presented as brown full circles. BL, basolateral domain; CAII, carbonic anhydrase II; CpK, cathepsin K; FSD, functional secretory domain; RB, ruffled border; RL, resorption lacunae; SZ, sealing zone. (See also color plate.)

type junction, given that tight sealing could be prevented by a hexapeptide containing the cell adhesion recognition sequence of cadherins.

Resorbing Osteoclasts Are Highly Polarized and Show Four Different Membrane Domains

Osteoclasts cycle between resorbing and nonresorbing phases, which are accompanied by drastic changes in their polarization (Figs. 1–4). Resorbing osteoclasts are highly polarized cells containing several different plasma membrane domains, whereas those osteoclasts that are not resorbing do not reveal clear morphological features of polarity. In resorbing cells the sealing zone itself forms one distinctive membrane domain and simultaneously separates two other membrane domains, the ruffled border (RB) and the basolateral domain (Figs. 1 and 2). In addition to these three membrane domains, morphological and functional studies have revealed a distinct membrane domain in the basal membrane, namely a functional secretory domain (FSD) (Salo *et al.,* 1996). This membrane domain has some characteristic features of the apical membrane domain in epithelial cells. Viral proteins that are usually targeted to the apical domain are in osteoclasts targeted to this domain. In addition, FSD is morphologically different from the rest of the membrane, and it has been also shown to be a target for transcytotic vesicles

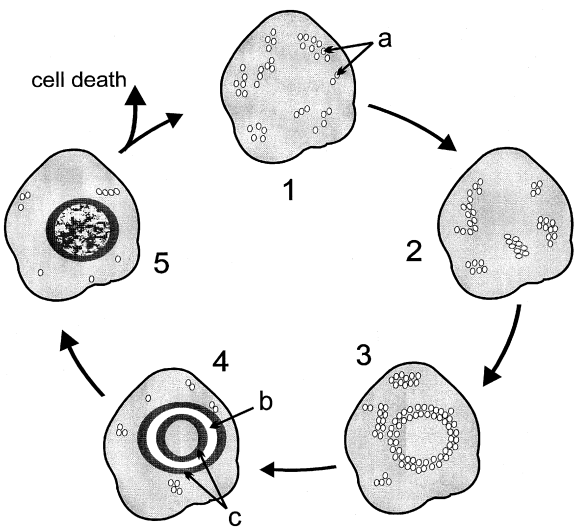

Figure 4 Organization of microfilaments in osteoclasts can be used to recognize different phases of the resorption cycle. When a nonresorbing cell (1) is induced to resorb podosome-type structures (a), gather to certain areas of bone-facing surface (2) and finally form a large circular collection of podosomes (3). In the following step, the individual podosome-type structure diappears (4) and a distinct dense actin ring (b) appears between two broad vinculin rings (c). The vitronectin receptor is tightly colocalized with vinculin in those rings as well as in podosomes. After resorption, cytoskeletal rings disappear in a certain order (5) and the cell can either undergo apoptosis or return to the resting phase.

carrying bone degradation products (Salo *et al.,* 1997; Nesbitt *et al.,* 1997). In resorbing osteoclasts, thick bundles of microtubules connect the RB and the FSD (Mulari *et al.,* manuscript in preparation), and a specific type of exocytotic vesicles, clastosomes, have been described in close association with the FSD (Salo *et al.,* manuscript in preparation). It remains to be seen if these vesicles are associated with the active secretion of resorbed material or if they represent, for instance, a specific type of apoptotic vesicles.

The ruffled border membrane forms the actual "resorbing organ." The characteristics of this unique membrane domain do not perfectly fit any other known plasma membrane domain described so far. The ruffled border membrane is formed by the rapid fusion of acidic intracellular vesicles (Palokangas *et al.,* 1997). Many of the proteins reported to be present at the ruffled border are also found in endosomal and/or lysosomal membranes, including the vacuolar proton pump, mannose-6-phosphate receptor, rab7, and lgp 110 (Baron *et al.,* 1988; Väänänen *et al.,* 1990; Palokangas *et al.,* 1997) (Fig. 5 see also color plate). Functional experiments, for instance, with labeled transferrin and dextran, have further supported the conclusion that the ruffled border represents a plasma membrane domain with typical features of the late endosomal compartment (Palokangas *et al.,* 1997).

Several membrane proteins reveal a nonhomogeneous distribution at the ruffled border. For instance, the vacuolar proton pump (Mattsson *et al.,* 1997), as well as rab7, is concentrated at the lateral edges of the ruffled border (Palokangas *et al.,* 1997). Our functional experiments have also shown that exocytotic vesicles in resorbing osteoclasts are found at the lateral areas of the ruffled border, and endocytotic vesicles mainly bud from the central area of the ruffled border (Zhao *et al.,* 2000). On the basis of these and previous results, we suggest that the ruffled border is composed of two different domains — lateral and central — where exocytosis and endocytosis occur, respectively. This allows for simultaneous proton and enzyme secretion and for the endocytosis of degradation products.

In order to create and maintain specialized membrane domains, as well as to transport products of biosynthetic or secretory machinery, the cell has distinct intracellular trafficking routes, originating from one compartment and reaching another. Intracellular vesicular routes shown to be operating in the resorbing osteoclasts are presented schematically in Fig. 3 (see also color plate). Very little is known at the moment about the molecular mechanisms

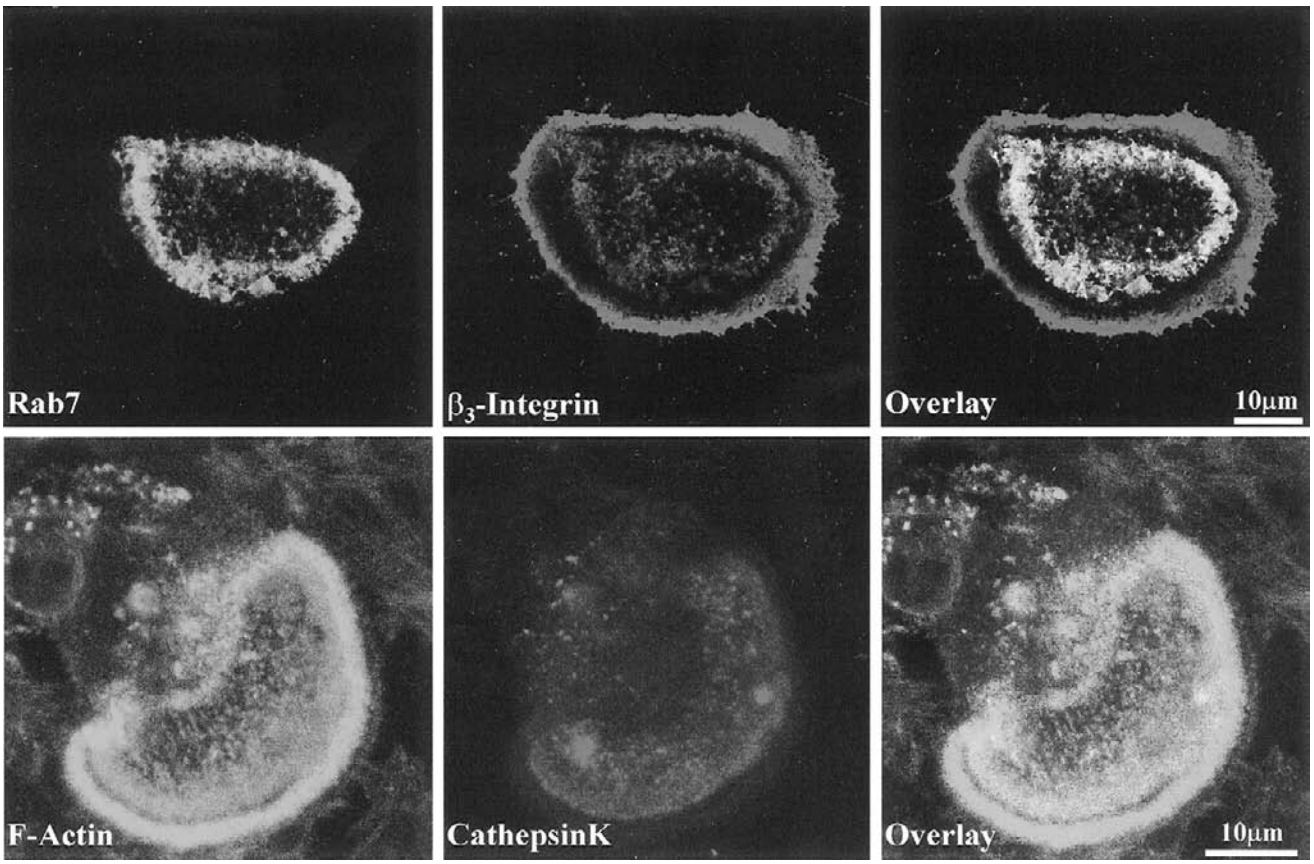

Figure 5 (Top) Laser confocal scanning microscopic images of the localization of rab7 and (β_3, integrin in the resorbing osteoclast. (Bottom) Laser confocal scanning microscopic images of cathepsin K and F-actin in the resorbing osteoclast. (See also color plate.)

regulating these particular vesicular activities in the osteoclasts. It is obvious that these events could be highly specific and may offer new potential targets to inhibit or stimulate bone resorption.

In addition to its role in the organization of membrane domains, the cytoskeleton also undergoes drastic reorganization during cell polarization. In fact, changes in the cytoskeletal organization are the driving force for the formation of different plasma membrane domains. Recent years have clarified to a large degree the connection of cytoskeletal organization to the resorptive activity of osteoclasts.

Cytoskeletal Changes during the Resorption Cycle

Changes in the organization of the cytoskeleton, especially microfilaments and microtubules, are characteristic of the polarization of any cell type. *In vitro* studies of osteoclasts on bone or dentine slices have revealed that the microfilament pattern in osteoclasts undergoes rapid changes when preparing for resorption (Fig. 4) (Kanehisa *et al.,* 1990; Lakkakorpi *et al.,* 1989). In osteoclasts that are not resorbing, polymerized actin is accumulated in podosome-type structures throughout the whole bone-facing surface of the osteoclast (Zambone-Zallone *et al.,* 1988). Thus, at the sites of podosomes, the actin cytoskeleton is anchored via integrins to the extracellular proteins of the matrix. The vitronectin receptor ($\alpha_v\beta_3$ integrin) is the major integrin in the podosomes of osteoclasts (Zambone-Zallone *et al.,* 1988). When resting or moving osteoclasts start preparing for resorption, an intense accumulation of podosomes to the local areas of the bone-facing membrane takes place (Lakkakorpi and Väänänen, 1991). Gradually, podosomes are collected into a large circular structure(s) and simultaneously the density of podosomes increases. Up to this point, vinculin and talin are tightly colocalized with F-actin and with the vitronectin receptor. In the next step, actin forms a dense belt-like structure where individual podosomes cannot be recognized anymore. This actin band (ring) is seen only in cells that are resorbing (Lakkakorpi and Väänänen, 1991). At this point, those proteins, which mediate the association of actin filaments to integrin, form a broad band around the actin band, but still colocalize with vitronectin receptors (Lakkakorpi *et al.,* 1991, 1993). This type of molecular organization at the sealing zone strongly suggests that molecules other than integrins are linking the actin cytoskeleton to the extracellular matrix at the fully developed sealing zone. This is the case not only *in vitro,* osteoclasts in bone also have a similar type of actin ring around the resorption lacuna (Sugiyama and Kusuhara, 1994).

In order to understand the cell biology of the resorption process it is essential to understand in detail how the organization of the cytoskeleton is regulated and how the sealing zone is finally formed. Src-deficient knockout mice (Soriano *et al.,* 1991) offer an interesting model to study the organization of the cytoskeleton and the formation of the sealing zone and ruffled border. These mice, which suffer from osteopetrosis, have a normal number of osteoclasts, which attach to bone but do not form normal ruffled borders (Boyce *et al.,* 1992).

In addition to actin, only a couple of proteins have been localized in the sealing zone. These are PYK2 and $p130^{cas}$ (cas, Crk-associated substrate). PYK2 seems to be a major adhesion-dependent tyrosine kinase in osteoclasts (Duong *et al.,* 1998, Nakamura *et al.,* 1997). When $\alpha_v\beta_3$ is activated by substrate ligation or by some other means, PYK2 is phosphorylated, its kinase activity is increased, and it associates with c-src. Osteoclasts in $src^{-/-}$ mice are not able to form actin rings and, interestingly, the tyrosine phosphorylation of PYK2 is reduced markedly in these cells. $P130^{cas}$ has been shown to be associated with PYK2 and also with actin cytoskeleton. On the basis of these observations, localization of PYK2 and c-src, and the fact that PYK2 is also tightly associated with the cytoskeleton, it has been suggested that the src-dependent tyrosine phosphorylation of PYK2 may be an important regulatory event in formation of the sealing zone (Duong *et al.,* 1999).

Calcitonin and dbcAMP induce a rapid destruction of the actin rings in resorbing osteoclasts (Lakkakorpi and Väänänen, 1990). More recently, Suzuki *et al.* (1996) demonstrated that the effect of calcitonin on the cytoskeleton is mediated by a protein kinase A-dependent pathway. These results suggest that the cytoskeleton could be an important target of the action of calcitonin.

In addition to calcitonin, several other agents, which inhibit osteoclast function, have been shown to cause disruption of the actin rings. These include tyrosine kinase inhibitors such as herbimycin (Tanaka *et al.,* 1995) and an inhibitor of phosphatidylinositol 3-kinase (PI3-kinase), wortmannin (Nakamura *et al.,* 1995). Evidence shows that PI3-kinase is associated with c-src and induces cytoskeletal reorganization in osteoclasts (Grey *et al.,* 2000). Lakkakorpi *et al.* (1997) have also provided evidence on the association of PI3-kinase with the cytoskeleton and $\alpha_v\beta_3$ integrin.

The role of Rho family proteins in cytoskeletal organization has been studied extensively. Zhang *et al.* (1995) demonstrated an important role for Rho p21 protein in regulation of the osteoclast cytoskeleton. Microinjection into osteoclasts of *Clostridium botulinum*-derived ADP-ribosyltransferase, which specifically ADP ribosylates Rho p21, completely disrupted actin rings within 20 min. These studies show that actin ring formation or disruption in osteoclasts is regulated by tyrosine kinase-mediated and PI3-kinase-mediated signals. They also suggest that a Rho p21-mediated pathway is involved in the regulation of the actin cytoskeleton in osteoclasts. More recently, members of the Rho-GTPase subfamily, Rac 1 and Rac 2, were shown to be involved in the organization of the actin cytoskeleton in osteoclasts (Razzouk *et al.,* 1999). Chellaiah *et al.,* (2000) also demonstrated the importance of Rho-A for cytoskeletal organization in osteoclasts.

Burgess *et al.* (1999) offered interesting new information on the resorption cycle of osteoclasts. The authors show that in the presence of RANKL (OPGL), osteoclasts in culture can go through more resorption cycles than in control cultures. Thus, in addition to osteoclast-recruiting activity, RANKL also directly stimulates bone resorption by extending their activity period, at least *in vitro.* Incubation of isolated rat osteoclasts with RANKL stimulated actin ring formation and further resorption markedly after only 30 min of exposure. This study strongly suggests that RANKL could be an important regulator of osteoclast activity. It would be interesting to identify the signal transduction pathways of this biological response downstream of RANK.

Only very few of the proteins participating in the organization of the cytoskeleton during the resorption cycle are known at the moment. We should know many additional details before we can completely understand the regulation of the cytoskeletal changes during the resorption cycle. This would be an important field of osteoclast research during the coming years, especially because it will uncover several new molecular targets for inhibiting bone resorption.

Much less is known about the changes in the organization of microtubules and intermediate filaments during the resorption cycle. In osteoclasts cultured on glass, each nucleus preserves its own microtubule-organizing center during fusion, whereas in myotubes, individual centrioles are eliminated (Moudjou *et al.,* 1989). In resorbing osteoclasts, microtubules form thick bundles in the middle of the cell, originating from the top of the cell and converging toward the ruffled border (Lakkakorpi and Väänänen, 1995). Data suggest that these microtubules actually extend from the ruffled border to the functional secretory domain and are most probably mediating transcytotic trafficking (Mulari *et al.,* 1998). Even less is known about the organization of intermediate filaments and their possible changes during the polarization of osteoclasts.

How Osteoclasts Dissolve Bone Mineral

Bone mineral is mainly crystalline hydroxyapatite, and there are not many biological processes that could be responsible for the solubilization of crystals. In fact, the only process that has been suggested to be able to solubilize hydroxyapatite crystals in the biological environment is low pH. Thus the idea that bone resorption is facilitated by local acidification has been discussed among bone biologists for a long time, and Fallon *et al.* (1984) demonstrated that the resorption lacuna is really acidic. This observation was also confirmed later by other investigators who used the accumulation of acridine orange in acidic compartments as an indicator of low pH (Baron *et al.,* 1985).

First experiments to explain the molecular mechanism of lacunar acidification suggested the presence of gastric-type proton pumps in osteoclasts (Baron *et al.,* 1985; Tuukkanen and Väänänen, 1986). However, it soon became clear that the main type of proton pump in the ruffled border of osteoclasts is a V-type ATPase (Bekker and Gay 1990a; Blair *et al.,* 1989; Väänänen *et al.,* 1990).

V-type ATPases are electrogenic proton pumps that contain several different subunits that form two independently assembled complexes: cytoplasmic and membranebound (for a review, see Stevens and Forgac, 1997). V-type ATPases are found in all mammalian cells and are responsible for the acidification of different intracellular compartments, including endosomes, lysosomes, secretory vesicles, and synaptic vesicles. The membrane-bound complex is composed of at least five different subunits, and the soluble catalytic complex contains at least eight different subunits. In addition, each pump contains several copies of each subunit. Thus the V-type ATPase and mitochondrial F-type ATPase share a common structural architecture.

The level of expression of V-type ATPase is very different among tissues, being very high in adrenal, kidney, and brain. In osteoclasts, it is exceptionally high. In contrast to many other cells, it is found both in various intracellular compartments and in the plasma membrane, namely the ruffled border. In addition to the marked differences in expression level in different cells and tissues, further functional specificity is obtained by slight structural differences of certain subunits. Different isoforms of at least three subunits have been described so far (van Hille *et al.,* 1994; Bartkiewicz *et al.,* 1995; Hernando *et al.,* 1995; Toyomura *et al.,* 2000), and these isoforms are specifically expressed in certain cells giving, at least in theory, a good possibility for remarkable cell and tissue specificity.

Heterogeneity of the 116-kDa subunit has turned out to be of special importance. Three different isoforms of this subunit that stabilize the soluble complex to the membrane complex by as yet unknown mechanisms have been characterized and one of these shows substantial specificity for osteoclasts (Li *et al.,* 1996; Toyomura *et al.,* 2000).

Yamamoto *et al.* (1993) described a patient suffering from craniometaphyseal dysplasia and reported a lack of expression of V-type ATPase in this patient's osteoclasts. Although the specific gene mutation in this patient has not been characterized, studies in other laboratories have shown that a certain number of patients suffering from malignant osteopetrosis have a mutation in the 116-kDa subunit of vacuolar type ATPase (Kornak *et al.,* 2000; Frattini *et al.,* 2000). Further evidence for the importance of this particular subunit is provided by the severe osteopetrotic phenotype in knockout mice of this specific subunit (Li *et al.,* 1999) and also by the fact that oc/oc osteopetrotic mice have a deletion in this subunit (Scimeca *et al.,* 2000).

The low pH in the resorption lacuna is achieved by the action of the proton pump both at the ruffled border and in intracellular vacuoles. Cytoplasmic acidic vacuoles disappear at the time when the ruffled border appears, during the polarization of the cell. It is thus highly likely that the initial acidification of the subcellular space is achieved by the direct exocytosis of acid during the fusion of intracellular

vesicles to form the ruffled border. This ensures rapid initiation of mineral dissolution, and further acidification may be obtained by the direct pumping of protons from the cytoplasm to the resorption lacuna.

In vitro studies with isolated osteoclasts were first used to show the functional importance of the proton pump for mineral dissolution. Sundquist *et al.* (1990) studied the effect of bafilomycin A1 on osteoclast function and observed that it effectively blocked bone resorption without affecting cell adhesion or viability. Antisense oligonucleotides against different subunits of the proton pump complex were then used to confirm the role of the proton pump in osteoclast function (Laitala and Väänänen, 1994). *In situ* hybridization studies using nonradioactive probes and laser confocal microscopy revealed a polarized distribution of mRNAs of vacuolar proton pumps in resorbing osteoclasts (Laitala-Leinonen *et al.,* 1996). Thus osteoclasts seem to offer an excellent dynamic model for studies of mRNA polarization.

An elegant *in vivo* study using local administration of bafilomycin A1 confirmed that inhibition of the vacuolar proton pump can potentially be used to inhibit bone resorption (Sundquist and Marks, 1995). The development of new types of vacuolar proton pump inhibitors with improved specificity and selectivity (Keeling *et al.,* 1998; Visentin *et al.,* 2000) has opened a whole new opportunity for treating osteoporosis and other bone metabolic bone diseases.

In addition to the proton pump, several other molecules have critical functions in the acidification of the resorption lacuna. Minkin *et al.* (1972) showed that bone resorption in mouse calvarial cultures could be inhibited by carbonic anhydrase inhibitors and decrease of CA II expression by using antisense technology leads to the inhibition of bone resorption in cultured rat osteoclasts (Laitala and Väänänen, 1994). In humans, CA II deficiency causes nonfunctional osteoclasts and osteopetrosis (Sly and Hu, 1995), and aging CA II deficient mice also show mild osteopetrosis (Peng *et al.,* 2000). To date, only one isoenzyme of the large carbonic anhydrase family containing at least 14 different members has been found in the osteoclast, namely CA II. It is likely, however, that other members of this gene family are also present in osteoclasts.

Pumping of protons through the ruffled border is balanced by the secretion of anions (see Fig. 3). The presence of a high number of chloride channels in the ruffled border membrane of osteoclasts has been shown (Blair and Schlesinger, 1990). The outflow of chloride anions through the ruffled border is most likely compensated by the action of a HCO_3/Cl exchanger in the basal membrane.

It has been suggested that there is also another mechanism for proton extrusion, besides V-ATPase, in resorbing osteoclasts (Nordström *et al.,* 1995). This could be the Na/H antiporter, as its inhibition blocks bone resorption *in vitro* (Hall *et al.,* 1992). It is not yet known how this antiporter is distributed in resorbing and polarized osteoclasts and what is its specific role in the acidification of resorption lacunae as such. Information now available supports the conclusion that it has a role in the early phases of the resorption cycle (Hall *et al.,* 1992). The basolateral membrane of resorbing osteoclasts also contains a high concentration of Na/K-ATPase (Baron *et al.,* 1986) and Ca-ATPase (Bekker and Gay, 1990b).

How Osteoclasts Degrade Organic Matrix

After solubilization of the mineral phase, the organic matrix is degraded. Roles of two major classes of proteolytic enzymes—lysosomal cysteine proteinases and MMPs—have been studied most extensively. The question of the role of proteolytic enzymes in bone resorption can be divided into at least three subquestions. First, what are the proteolytic enzymes, which are needed to remove unmineralized osteoid from the site of future resorption? Second, what are the proteolytic enzymes, which take part in the degradation of organic matrix in the resorption lacuna? Third, is there intracellular matrix degradation in osteoclasts and what proteolytic enzymes, if any, are responsible for this final degradation process?

Sakamoto *et al.* (1982) and Chambers *et al.* (1985) suggested that osteoblast-derived collagenase (MMP-1) plays a major role in the degradation of bone covering osteoid. Removal of the osteoid layer seems to be a necessary or even obligatory step for the future action of osteoclasts. Although the role of osteoblasts seems to be essential in this early phase of bone resorption, it has not been shown definitively that osteoblasts *in situ* are responsible for the production of all proteolytic enzymes necessary for osteoid degradation. It has not been possible to rule out the possibility that some proteinases necessary for matrix degradation have been produced and stored in the matrix already during bone formation. The stimulus for bone resorption may lead to activation of a local population of osteoblasts, which then secrete the factors that are needed to activate matrix-buried proteinases.

In addition to MMP-1, a membrane-bound matrix metalloproteinase (MT1-MMP) clearly has a role in bone turnover. MT1-MMP-deficient mice develop dwarfism and osteopenia among other connective tissue disorders (Holmbeck *et al.,* 1999). The analysis of deficient mice, however, suggests that the primary defects are in bone-forming cells rather than in osteoclasts. However some studies have indicated a high expression of MT1-MMP in osteoclasts (Sato *et al.,* 1997; Pap *et al.,* 1999). It appears to be localized in specific areas of cell attachment, but at the moment there are no direct data pointing to the specific function of MT1-MMP in the resorption processes. In conclusion, it seems evident that proteinases in osteoblastic cells play an important role in the regulation of bone turnover, but specific mechanisms remain to be elucidated.

It is obvious that a substantial degradation of collagen and other bone matrix proteins during bone resorption takes place in the extracellular resorption lacuna. Two enzymes,

cathepsin K and MMP-9, have been suggested to be important in this process. Data that cathepsin K is a major proteinase in the degradation of bone matrix in the resorption lacuna are now very convincing. First of all, it is highly expressed in osteoclasts and is also secreted into the resorption lacuna (Inaoka *et al.,* 1995; Drake *et al.,* 1996; Tezuka *et al.,* 1994b; Littlewood-Evans *et al.,*1997). Second, it has been shown that it can degrade insoluble type I collagen, and inhibition of its enzymatic activity in *in vitro* and *in vivo* models prevents matrix degradation (Bossard *et al.,* 1996; Votta *et al.,* 1997). Third, deletion of the cathepsin K gene in mice leads to osteopetrosis (Saftig *et al.,* 1998; Gowen *et al.,* 1999). Finally, human gene mutations of cathepsin K lead to pycnodysostosis (Gelb *et al.,* 1996; Johnson *et al.,* 1996).

These data have encouraged several researchers and pharmaceutical companies to try to develop specific inhibitors of cathepsin K to be used in the treatment of osteoporosis. Because mineral dissolution is not prevented by such inhibitors, it is possible that inhibition of this enzyme could lead to the accumulation of an unmineralized matrix. This may be an important contraindication to the long-term use of cathepsin K inhibitors in the treatment of bone diseases.

A number of other lysosomal cysteine proteinases—cathepsins B, D, L, and S—have been suggested to play a role in osteoclasts, and data exist showing that at least B and L are also secreted into the resorption lacuna (Goto *et al.,* 1994). However, extracellular localization of cathepsin B was not observed in the inhibitor studies of Kakegawa (1993) and Hill *et al.* (1994). Taken together, these results suggest that cathepsin L has a role in extracellular matrix digestion, whereas cathepsin B acts only intracellularly. If this is the case, one may find it difficult to explain why a major part of cathepsin B is secreted into the resorption lacunae. It is possible that many of the earlier inhibitor studies were actually complicated by the inhibition of cathepsin K, which was discovered later. Thus, many of the earlier studies on the role of different cathepsins in bone must now be reevaluated.

Several studies have shown a high expression of MMP-9 in osteoclasts, both at the mRNA and at the protein level (Wucherpfennig *et al.,* 1994; Reponen *et al.,* 1994; Tezuka *et al.,* 1994a; Okada *et al.,* 1995). Although there is no direct evidence for the secretion of MMP-9 into resorption lacunae, it is likely to occur. When demineralized bone particles were incubated with MMP-9, the degradation of collagen fragments was observed by electron microscopy (Okada *et al.,* 1995). This suggests that under conditions present in bone matrix, it is possible that MMP-9 could digest collagen even without the presence of collagenase. MMP-9 knockout mice show an interesting phenotype of transient osteopetrosis (Vu *et al.,* 1998). This and some other studies suggest that the role of MMP-9 in different populations of osteoclasts could be different, and furthermore that osteoclasts may be a more herogeneous cell population than previously thought (Everts *et al.,* 1999).

In conclusion, evidence indicates that several MMPs and lysosomal and nonlysosomal proteinases, especially cathepsin K (and perhaps other cysteine proteinases), play a major role in matrix degradation (Everts *et al.,* 1998; Xia *et al.,* 1999). The coordinated action of several proteinases may be needed to solubilize completely fibrillar type I collagen and other bone matrix proteins. Some of these proteinases obviously act mainly extracellularly and some intracellularly or both. Extracellular matrix degradation may be enhanced further by the production of free oxygen radicals directly into resorption lacunae.

How Bone Degradation Products Are Removed from the Resorption Lacuna

During bone resorption the ruffled border membrane is continuously in very close contact with the bone matrix. Thus, it is somewhat misleading to speak about a resorption lacuna, which is only a very narrow space between the cell membrane and the matrix components. This tentatively suggests that degradation products must be somehow removed continuously from the resorption space in order to allow the resorption process to continue. Theoretically, there are two different routes for resorption products. They can either be released continuously from the resorption lacuna beneath the sealing zone or be transcytosed through the resorbing cell.

Salo *et al.* (1994) provided first evidence for the transcytosis of bone degradation products in vesicles through the resorbing osteoclasts from the ruffled border to the functional secretory domain of the basal membrane. This finding has now been confirmed by demonstrating fragments of collagen and other matrix proteins in the transcytotic vesicles (Salo *et al.,* 1997; Nesbit *et al.,* 1997). We have described new vesicle-like structures, called clastosomes, that appear in polarized cells on the FSD area (Salo *et al.,* manuscript in preparation). It is of interest that the number of these structures is tightly linked to the resorption activity of osteoclasts.

When matrix degradation products are endocytosed, it is possible that further degradation of matrix molecules takes place during the transcytosis. Results have shown that TRAP is localized in transcytotic vesicles in resorbing osteoclasts. They also showed that TRAP can generate highly destructive reactive oxygen species able to destroy collagen and other proteins (Halleen *et al.,* 1999). This suggests a new function for TRAP in the final destruction of matrix degradation products during transcytosis. The observed phenotype of mild osteopetrosis in TRAP knockout mice (Hayman *et al.,* 1996) is in good agreement with this type of new role for TRAP in bone resorption.

Stenbeck and Horton (2000) suggested that the sealing zone is a more dynamic structure than previously thought and could be loose enough to allow the diffusion of molecules from the extracellular fluid into the resorption lacuna.

On the basis of their findings, the authors concluded that the sealing zone may also allow diffusion of the resorption products out from the lacuna. However, there is no direct evidence for this at the moment. It also remains to be seen if inorganic ions and degradation products of matrix proteins follow the same pathway or if parallel routes exist.

What Happens to Osteoclasts after Resorption

In vitro studies have shown that an osteoclast can go through more than one resorption cycle (Kanehisa and Heersche, 1988; Lakkakorpi and Väänänen, 1991). Unfortunately, we do not yet know if this also happens *in vivo* or if the osteoclast continues its original resorption cycle as long as it is functional. Regardless, there must be a mechanism that destroys multinucleated osteoclasts *in situ.* There are at least two different routes that the multinucleated osteoclast can take after it has fulfilled its resorption task. It can undergo fission into mononuclear cells or it can die.

Very little evidence exists to support the idea that multinucleated cells are able to undergo fission back to mononuclear cells. Solari *et al.* (1995) have provided some *in vitro* evidence that mononuclear cells can be formed from multinucleated giant cells, but this study remains to be confirmed. Most likely these postmitotic cells are removed by apoptosis after stopping resorption, and in fact there is now a lot of evidence that supports this conclusion. At present, little is known about the molecular mechanisms that regulate osteoclast apoptosis *in vivo.* However, the induction of apoptosis in osteoclasts can be used to inhibit bone resorption and prevent bone loss. It is well established that both aminobisphosphonates and clodronate induce apoptosis in osteoclasts (Hughes *et al.,* 1995; Selander *et al.,* 1996b), but their mechanisms of action are different. Their kinetics are also quite different (Selander *et al.,* 1996a). Aminobisphosphonates inhibit protein prenylation, which leads to disturbances in intracellular vesicular trafficking (Luckman *et al.,* 1998). Apoptosis is most probably secondary to this effect and is observed clearly after the inhibition of bone resorption. In contrast, with clodronate, apoptosis in osteoclasts seems to be the primary reason for the inhibition of bone resorption (Selander *et al.,* 1996ba).

In addition to bisphosphonates, estrogen and calcitonin have been suggested to regulate osteoclast apoptosis (Hughes *et al.,* 1996; Selander *et al.,* 1996a). It is quite evident that cell–matrix interactions are also important signals for osteoclast survival. The importance of the right extracellular milieu for the osteoclast phenotype and for survival is seen clearly when one compares their sensitivity to extracellular calcium. A moderate concentration of extracellular calcium has been shown to promote apoptosis in osteoclasts cultured on an artificial substrate (Lorget *et al.,* 2000), whereas if cultured on bone, the cells can tolerate high concentrations of extracellular calcium (Lakkakorpi *et al.,* 1996).

It is most likely that the molecular pathways leading to apoptosis in osteoclasts are similar to those described in other cells. It is possible, however, that the highly specific phenotype of the osteoclast also includes cell-specific features of cell survival and death.

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CHAPTER 9

Integrin and Calcitonin Receptor Signaling in the Regulation of the Cytoskeleton and Function of Osteoclasts

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Introduction

The maintenance of normal bone mass during adult life depends on a balance between osteoblastic bone formation and osteoclastic bone destruction. Bone resorption is primarily carried out by osteoclasts, which are multinucleated, terminally differentiated cells derived from the monocyte/macrophage lineage (Roodman, 1999; Suda *et al.,* 1997; Teitelbaum, 2000). The rate of osteoclastic bone resorption is regulated by osteoclast number and function. Osteoclastogenesis is controlled by the proliferation and homing of the progenitors to bone, their differentiation and fusion to form multinucleated cells. A comprehensive review on the regulation of osteoclast generation has been discussed in a previous chapter. Osteoclast function starts with adhesion to the bone matrix, leading to cytoskeletal reorganization that is important for the migration of these cells to and between the resorption sites and their polarization during the resorption process (Duong *et al.,* 2000a). Cell polarization is initiated by cell attachment to the bone surface and forms a tight sealing zone (or "clear zone") enclosing the resorption lacunae. This sealing attachment structure is highly enriched in filamentous actin and is organized as a ring surrounding a convoluted membrane area, called ruffled border, which is formed as a result of directional insertion of vesicles for the active secretion of protons and lysosomal enzymes toward the bone surface (Baron *et al.,* 1985, 1988; Väänänen *et al.,* 2000). During active resorption, the degraded bone matrix is either processed extracellularly or in part endocytosed into the osteoclast and degraded within secondary lysosomes and in part transported through the cell by transcytosis and secreted at the basal membrane (Nesbitt and Horton, 1997; Salo *et al.,* 1997). Detailed description on the cytoskeletal organization associated with osteoclastic bone resorption is also discussed in another chapter.

This chapter focuses on information regarding the signaling pathways that mediate cytoskeletal organization during osteoclast migration and polarization. Spontaneous mutations and gene knockouts in mice have identified many genes that regulate various stages of osteoclast development. The myeloid and B lymphoid transcription factor PU.1, macrophage colony-stimulating factor (M-CSF or CSF-1), c-fos, p50/p52 subunits of NF-κB, RANK, and its soluble receptor, osteprotegerin (OPG), were shown to be essential for osteoclast differentiation (Suda *et al.,* 1997;

Teitelbaum, 2000). However, pharmacological or genetic methods used to disrupt osteoclast function (Rodan and Martin, 2000; Teitelbaum, 2000) identified many molecules that are involved either in the degradation of bone matrix proteins, such as cathepsin K or metalloproteinases (MMPs), or in the regulation of acidification, including carbonic anhydrase type II, tartrate resistant acid phosphatase (TRAP), the a3 subunit of the vacuolar H^+-ATPase (Li *et al.,* 1999; Scimeca *et al.,* 2000; Frattini *et al.,* 2000), and, very recently, the CIC-7 chloride channel (Kornak *et al.,* 2000). Another class of molecules appeared to be important in modulating osteoclastic cytoskeletal organization, including the calcitonin receptor and the adhesion receptor $\alpha_v\beta_3$. Calcitonin has been considered as an important therapeutic agent to acutely block bone resorption in osteoporosis therapy (Rodan and Martin, 2000). More recently, small molecular weight inhibitors of $\alpha_v\beta_3$ integrin have emerged as attractive orally available agents for antiosteoporosis therapy (Hartman and Duggan, 2000; Lark *et al.,* 1999). This chapter discusses the signal transduction pathways mediated by these two receptors in the context of their regulation of cytoskeletal reorganization during osteoclastic bone resorption.

Adhesion and Cytoskeletal Organization in Osteclasts—$\alpha_v\beta_3$ Integrins

Integrins, a superfamily of heterodimeric transmembrane receptors, mediate cell–matrix and cell–cell interactions. Integrin-mediated adhesion and signaling regulate a variety of cell functions, including bone resorption, platelet aggregation, leukocyte homing and activation, tumor cell growth and metastases, cell survival and apoptosis, and cellular responses to mechanical stress (Clark and Brugge, 1995; Schlaepfer *et al.,* 1999). In addition to cell adhesion, the assembly of these receptors also organizes extracellular matrices, modulates cell shape changes, and participates in cell spreading and motility (Wennerberg *et al.,* 1996; Wu *et al.,* 1996).

Upon ligand binding, integrins usually undergo receptor clustering, leading to the formation of focal adhesion contacts, where these receptors are linked to intracellular cytoskeletal complexes and bundles of actin filaments. It has long been recognized that the short cytoplasmic domains of the α and β integrin subunits can recruit a variety of cytoskeletal and signaling molecules. Integrin-mediated signaling has been shown to change phosphoinositide metabolism, raise intracellular calcium, and induce tyrosine or serine phosphorylation of signaling molecules (Giancotti and Ruoslahti, 1999). Models of integrin-stimulated tyrosine phosphorylation and signaling pathways have been discussed extensively elsewhere (Giancotti and Ruoslahti, 1999; Schlaepfer *et al.,* 1999). The involvement of several signaling and adapter molecules, such as c-Src, PYK2, $p130^{Cas}$, and c-Cbl in integrin function in osteoclasts, is discussed in the following sections.

Bone consists largely of type I collagen (>90%) and of noncollagenous proteins interacting with a mineral phase of hydroxylapatite. Adhesion of osteoclasts to the bone surface involves the interaction of integrins with extracellular matrix proteins within the bone matrix. Osteoclasts express very high levels of the vitronectin receptor $\alpha_v\beta_3$ (Duong *et al.,* 2000a; Horton, 1997; Rodan and Martin, 2000). Mammalian osteoclasts also lower levels of the collagen/laminin receptor $\alpha_2\beta_1$ and the vitronectin/fibronectin receptor $\alpha_v\beta_1$. Rat osteoclasts adhere in an $\alpha_v\beta_3$ dependent manner to extracellular matrix (ECM) proteins containing RGD sequences, including vitronectin, osteopontin, bone sialoprotein, and a cryptic RGD site in denatured collagen type I (Flores *et al.,* 1996). More recently, it was reported that rat osteoclasts adhere to native collagen type I using $\alpha_2\beta_1$ integrin, surprisingly in an RGD-dependent manner (Helfrich *et al.,* 1996). Moreover, osteoclastic bone resorption is partially inhibited by both anti-α_2 and anti-β_1 antibodies (Nakamura *et al.,* 1996). To date, it is still unclear the physiological ECM substrate of $\alpha_v\beta_3$ integrins in bone.

$\alpha_v\beta_3$-Mediated Osteoclast Function

The first evidence that $\alpha_v\beta_3$ may play an important role in osteoclast function was obtained when a monoclonal antibody raised against osteoclasts inhibited bone resorption *in vitro* (Chambers *et al.,* 1986), whose antigen was later identified to be the $\alpha_v\beta_3$ integrin (Davies *et al.,* 1989). Furthermore, osteoclastic bone resorption *in vitro* can be inhibited by RGD-containing peptides and disintegrins or by blocking antibodies to $\alpha_v\beta_3$ (Horton *et al.,* 1991; King *et al.,* 1994; Sato *et al.,* 1990).

Inhibition of $\alpha_v\beta_3$ integrins can also block bone resorption *in vivo.* In the thyro-parathyroidectomized (TPTX) model, coinfusion of disintegrins such as echistatin or kinstrin and parathyroid hormone (PTH) completely blocked the PTH-induced increase in serum calcium (Fisher *et al.,* 1993; King *et al.,* 1994). Echistatin was also shown to inhibit bone loss in hyperparathyroid mice maintained on a low calcium diet. In the bones of these animals, echistatin colocalizes with $\alpha_v\beta_3$ integrins in osteoclasts (Masarachia *et al.,* 1998). Additional *in vivo* studies demonstrated that echistatin or RGD peptidomimetics inhibit bone resorption in ovariectomized rodents by blocking the function of $\alpha_v\beta_3$ integrins (Engleman *et al.,* 1997; Yamamoto *et al.,* 1998). Moreover, infusion of the anti-rat β_3 integrin subunit antibody blocked the effect of PTH on serum calcium in TPTX rats (Crippes *et al.,* 1996). Further compelling evidence for the role of $\alpha_v\beta_3$ integrin in osteoclast function has been provided by the targeted disruption of the β_3 integrin subunit in mice, which induces late onset osteopetrosis, 3 to 6 months after birth (McHugh *et al.,* 2000).

Although these findings indicate that the $\alpha_v\beta_3$ integrin has an important rate-limiting function in bone resorption, its mechanism of action in the osteoclast is far from being fully understood. Recognition of extracellular matrix components by osteoclasts is an important step in initiating

osteoclast function. Several studies have demonstrated that integrin-mediated cell adhesion to vitronectin, fibronectin, or collagen was reported to induce cell spreading and actin rearrangement in osteoclasts. The $\alpha_v\beta_3$ integrin plays a role in the adhesion and spreading of osteoclasts on bone (Lakkakorpi and Väänänen, 1991; Nesbitt *et al.,* 1993). Expression of the $\alpha_v\beta_3$ integrin could also be detected in mononucleated osteoclast precursors, and $\alpha_v\beta_3$ was found to mediate the migration of the precursors, necessary for their fusion, during osteoclast differentiation in culture (Nakamura *et al.,* 1998b). Furthermore, the $\alpha_v\beta_3$ integrin plays a role in the regulation of two processes required for effective osteoclastic bone resorption: cell migration and maintenance of the sealing zone (Nakamura *et al.,* 1999).

The presence of the RGD sequence in osteopontin, a bone matrix protein produced by osteoblasts and osteoclasts, led to the suggestion that attachment at the sealing zone may be mediated by integrins. It was reported that $\alpha_v\beta_3$ is found in the sealing zone of resorbing osteoclasts (Hultenby *et al.,* 1993; Reinholt *et al.,* 1990) and in the podosomes (Zambonin Zallone *et al.,* 1989). Other investigators, using different antibodies, could not detect $\alpha_v\beta_3$ in the sealing zone by confocal and electron microscopy (Lakkakorpi *et al.,* 1993; Nakamura *et al.,* 1999). These discrepant results suggest the need for further studies. All investigators, however, agree that the receptor is found in basolateral membranes, in a few intracellular vesicles, and at a much lower level in the ruffled border region of the resorbing osteoclasts.

In echistatin-treated mice, where bone resorption was inhibited, the number of osteoclasts on the bone surface was unchanged rather than decreased, suggesting that osteoclast inhibition by $\alpha_v\beta_3$ integrin antagonists is not due to the detachment of osteoclasts from the bone surface (Masarachia *et al.,* 1998; Yamamoto *et al.,* 1998). Consistent with this observation, the osteoclast number was not reduced in the bones of $\beta_3^{-/-}$ mice (McHugh *et al.,* 2000). These data suggest, as indicated by the analysis of the integrin repertoire of osteoclasts, that in the absence of functional $\alpha_v\beta_3$ receptors, adhesion per se is not compromised and that other integrins, or different attachment proteins, participate in this process. However, $\alpha_v\beta_3$ may well be critical for cell motility via its signaling ability (Nakamura *et al.,* 2001; Sanjay *et al.,* 2001). Ligands (antagonists) of $\alpha_v\beta_3$ may therefore inhibit osteoclastic bone resorption *in vivo* by a different mechanism, such as interfering with the integrin-dependent cytoskeletal organization, required for osteoclast migration and efficient resorption (Nakamura *et al.,* 1999).

Adhesion-Dependent Signal Transduction in Osteoclasts

Target disruption of the β_3 integrin subunit results in an osteopetrotic phenotype (McHugh *et al.,* 2000). Osteoclasts isolated from these mice fail to spread, do not form actin rings, have abnormal ruffled membranes, and exhibit reduced bone resorption activity *in vitro.* Thus, $\alpha_v\beta_3$ plays a pivotal in the resorptive process, through its functions in cell adhesion-dependent signal transduction and in cell motility. Indeed, $\alpha_v\beta_3$ transmits bone matrix-derived signals, ultimately activating intracellular events, which regulate cytoskeletal organization essential for osteoclast migration and polarization. Research interests have been focused on identifying the hierarchy of the $\alpha_v\beta_3$-regulated cascade of signaling and structural proteins required for this cytoskeletal organization. One of the earliest events initiated by integrin–ligand engagement is the elevation of intracellular calcium. In rat, mouse, and human osteoclasts, RGD-containing peptides trigger a transient increase in intracellular calcium apparently mobilized from intracellular stores (Paniccia *et al.,* 1995; Zimolo *et al.,* 1994), and this event is independent of the presence or activation of c-Src (Sanjay *et al.,* 2001). Studies using both genetic and biochemical approaches, such as immunolocalization and -coprecipitation, have partially elucidated the $\alpha_v\beta_3$-associated molecular complex in osteoclasts. Schematic illustration of the recruitment of these signaling molecules to $\alpha_v\beta_3$ integrins in resting and activated osteoclasts is shown in Fig. 1, (see also color plate), although the exact relationship between these proteins and α_v and β_3 subunits is still not firmly established.

c-Src Regulates $\alpha_v\beta_3$ Integrin-Mediated Cytoskeletal Organization and Cell Motility

Src kinases play an important role in cell adhesion and migration, in cell cycle control, and in cell proliferation and differentiation (Thomas and Brugge, 1997). Moreover, novel roles for Src kinases in the control of cell survival and angiogenesis have emerged (Schlessinger, 2000). In bone, the tyrosine kinase c-Src was found to be essential for osteoclast-resorbing activity and/or motility. Targeted disruption of c-Src in mice induces osteopetrosis due to a loss of osteoclast function, without a reduction in osteoclast number (Soriano *et al.,* 1991). Morphologically, osteoclasts in *Src*$^{-/-}$ mice are not able to form a ruffled border, but appear to form sealing zones on bone surfaces (Boyce *et al.,* 1992). Src is highly expressed in osteoclasts, along with at least four other Src family members (Hck, Fyn, Lyn, and Yes) (Horne *et al.,* 1992; Lowell *et al.,* 1996). Hck partly compensates for the absence of Src, as the double mutant *Src*$^{-/-}$*/Hck*$^{-/-}$ mouse is more severely osteopetrotic than the *Src*$^{-/-}$ mouse, despite the fact that the single mutant *hck*$^{-/-}$ mouse is apparently not osteopetrotic (Lowell *et al.,* 1996). However, the transgenic expression of kinase-deficient Src in *Src*$^{-/-}$ mice partially rescued osteoclast function, indicating that full Src tyrosine kinase activity is not absolutely required for mediating osteoclast function, with its role as an adaptor protein to recruit downstream signaling molecules (Schwartzberg *et al.,* 1997) being also essential in its function in osteoclasts (Sanjay *et al.,* 2001).

c-Src is associated with the plasma membrane and multiple intracellular organelles (Horne *et al.,* 1992; Tanaka *et al.,* 1996). It is also concentrated in the actin ring and the cell periphery (Sanjay *et al.,* 2001), regions that are involved in osteoclast attachment and migration. This suggests that the absence of Src might compromise these aspects of os-

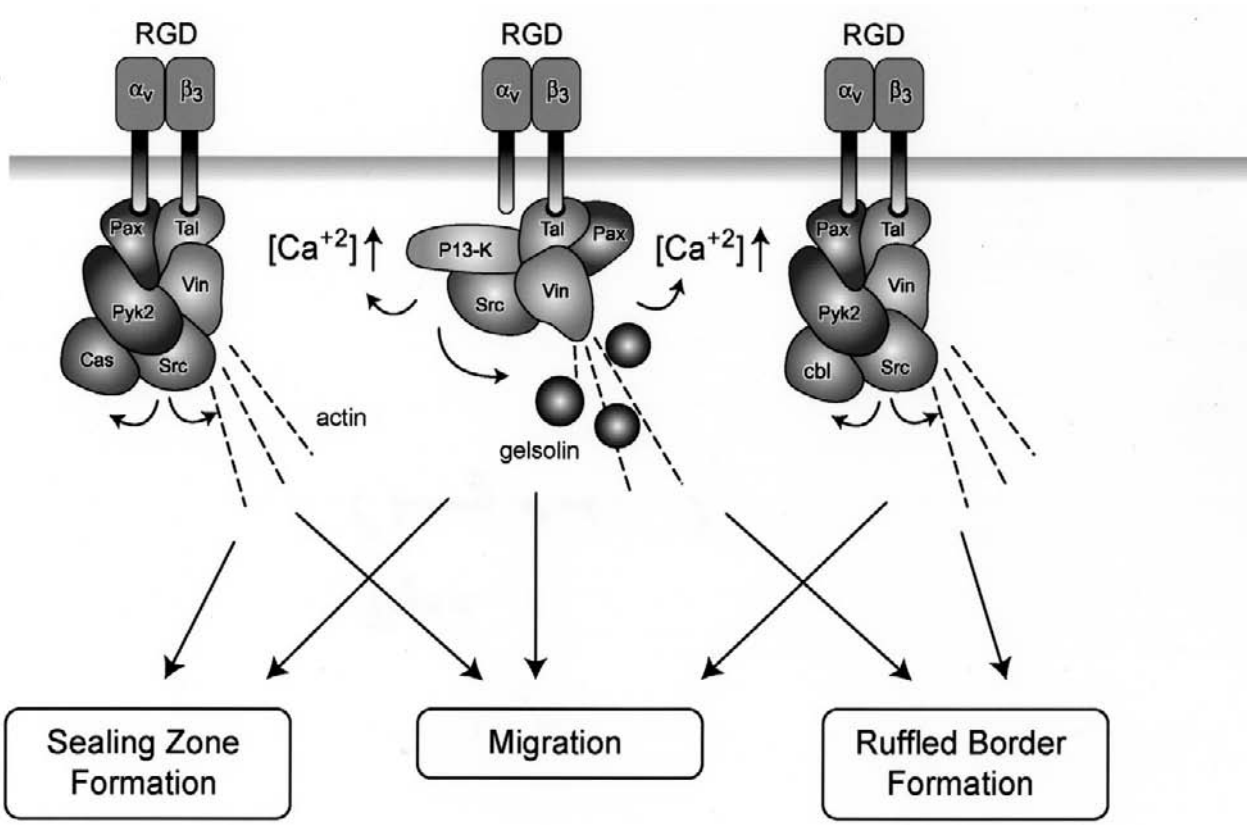

Figure 1 Schematic summary of the current hypothesis regarding the role of the $\alpha_v\beta_3$ integrin-mediated signaling pathway(s) involved in osteoclast cytoskeletal organization during migration and polarization. (See also color plate.)

teoclast function. Indeed, *Src*$^{-/-}$ osteoclasts generated *in vitro* do not resorb bone and exhibit a profound defect in cell adhesion and spreading on vitronectin, suggesting that c-Src plays an important role in adhesion-dependent cytoskeletal organization in osteoclasts (Lakkakorpi *et al.,* 2001; Nakamura *et al.,* 2001). Although $\alpha_v\beta_3$ integrin expression and ligand-binding affinity are not altered in *Src*$^{-/-}$ osteoclasts, the lack of c-Src results in the pronounced aggregation of $\alpha_v\beta_3$ integrins (Fig. 2, see also color plate) and their downstream effectors, such as Pyk2, p130^{Cas}, and paxillin, in the basal surface of resorbing osteoclasts (Lakkakorpi *et al.,* 2001). This thus suggests that c-Src might not be important for the initial recruitment of $\alpha_v\beta_3$-dependent downstream effectors, but it seems to mediate the turnover of the integrin-associated complex of signaling and cytoskeletal molecules. This hypothesis has been supported strongly by the finding that Src$^{-/-}$ osteoclasts do indeed demonstrate significant decreases in their ability to migrate over a substrate, at least *in vitro* (Sanjay *et al.,* 2001). It is therefore conceivable that the decreased bone resorption in *Src*$^{-/-}$ osteoclasts is due in part to low motility of the cells.

Pyk2 and c-Src Coregulate $\alpha_v\beta_3$ Integrin-Mediated Signals

Pyk2 has been identified as a major adhesion-dependent tyrosine kinase in osteoclasts, both *in vivo* and *in vitro* (Duong *et al.,* 1998). Pyk2 is a member of the focal adhesion kinase (FAK) family, highly expressed in cells of the central nervous system and cells of hematopoietic lineage (Schlaepfer *et al.,* 1999). Pyk2 and FAK share about 45% overall amino acid identity and have a high degree of sequence conservation surrounding binding sites of SH2 and SH3 domain-containing proteins. Although the presence of FAK has been reported in osteoclasts (Berry *et al.,* 1994; Tanaka *et al.,* 1995), Pyk2 is expressed at much higher levels than FAK in osteoclasts (Duong *et al.,* 1998). Ligation of $\alpha_v\beta_3$ integrins either by ligand binding or by antibody-mediated clustering results in an increase in Pyk2 tyrosine phosphorylation (Duong *et al.,* 1998). Moreover, Pyk2 colocalizes with F-actin (Fig. 3, see also color plate), paxillin, and vinculin in podosomes and in sealing zones of resorbing osteoclasts on bone (Duong *et al.,* 1998). These observations strongly implicate Pyk2 as a downstream effector in $\alpha_v\beta_3$-dependent signaling in mediating cytoskeletal organization associated with osteoclast adhesion, spreading, migration, and sealing zone formation.

More recently, osteoclasts infected with adenovirus expressing Pyk2 antisense have a significant reduction in Pyk2 expression (Duong *et al.,* 2000b). Similar to *Src*$^{-/-}$ osteoclasts, these cells do not resorb bone accompanied with defects in cell adhesion, spreading, and sealing zone organization, suggesting that Pyk2 is also important for cytoskeletal organization in osteoclasts (Duong *et al.,* 2000b). Consistent with the observation just mentioned,

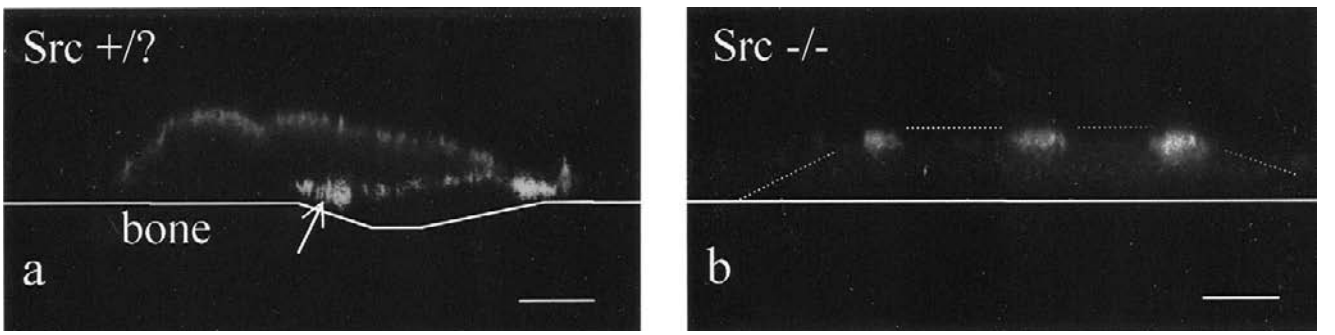

Figure 2 Pronounced aggregation of $\alpha_v\beta_3$ integrin (in red) and F-actin (in green) in the basal membrane of Src-deficient osteoclasts (b) as compared to the normal distribution of the receptor in wild-type osteoclasts on bone (a) (Lakkakorpi *et al.,* 2001). (See also color plate.)

mice that lack Pyk2 were shown to develop osteopetrosis with age similar to that of $\beta_3^{-/-}$ mice (Sims *et al.,* 1999). Furthermore, a double knockout of Src and Pyk2 in mice is more severely osteopetrotic than either of the single mutants (Sims *et al.,* 1999), suggesting that these two proteins might interact during normal osteoclast functioning.

Indeed, tyrosine phosphorylation and kinase activity of Pyk2 are reduced markedly in preosteoclasts derived from *Src*$^{-/-}$ mice (Duong *et al.,* 1998). This is in contrast with the findings of Sanjay *et al.* (2001), who reported that, upon adhesion, *Src*$^{-/-}$ osteoclasts demonstrated levels of Pyk2 phosphorylation comparable to those found in wild-type osteoclasts. Reasons for this discrepancy are not known, but it should be noted that the cells used in these two studies were at different stages of differentiation. Moreover, in adherent osteoclasts, Pyk2 is tightly associated with c-Src via its SH2 domain. An increase in intracellular calcium that results from the engagement or cross-linking of the $\alpha_v\beta_3$ integrin is suggested to activate Pyk2 by inducing the autophosphorylation of Tyr402. This phosphorylated residue binds to the Src SH2 domain (Dikic *et al.,* 1996), displacing the inhibitory Src phosphotyrosine 527 and activating Src, leading to further recruitment and activation of Src-mediated downstream signals. At the same time, activation of Pyk2 following integrin–ligand engagement results in the recruitment of cytoskeletal proteins in a similar manner as FAK (Schlaepfer *et al.,* 1999). The N-terminal domain of FAK was shown to interact with the cytoplasmic domain of the β integrin subunit. On addition to binding to Src, the autophosphorylation sites in FAK and Pyk2 are capable of binding to SH2 domains of phospholipase C-γ (Nakamura *et al.,* 2001; Schlaepfer *et al.,* 1999; Zhang *et al.,* 1999a). Furthermore, the C-terminal domain of FAK and Pyk2 contains binding sites for Grb-2, p130^{Cas}, and paxillin (Schlaepfer *et al.,* 1999). Together, the association and activation of c-Src and Pyk2 could initiate a cascade of activation and recruitment of additional signaling and structural molecules to the $\alpha_v\beta_3$ integrin-associated protein complex, which are important for modulating the actin cytoskeleton in osteoclasts.

ASSOCIATION OF PYK2 WITH SRC AND C-CBL DOWNSTREAM OF INTEGRINS

Engagement of the $\alpha_v\beta_3$ integrin induces the formation of a signaling complex that contains not only c-Src and Pyk2, but also c-Cbl (Sanjay *et al.,* 2001). The product of the protooncogene cCbl is a 120-kDa adaptor protein that gets tyrosine phosphorylated in response to the activation of various signaling pathways, including M-CSF stimulation in monocytes and v-Src transformation and EGF or PDGF

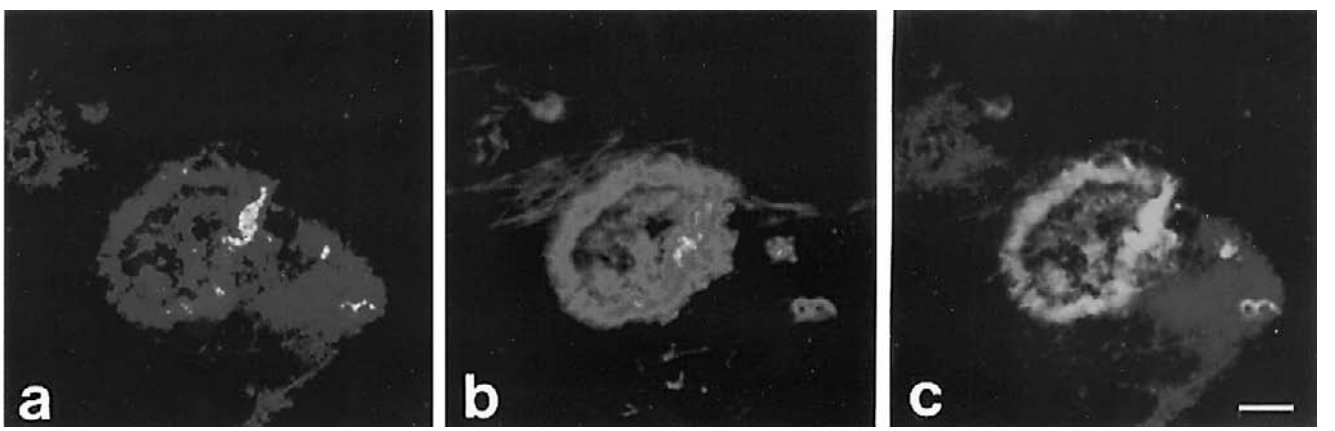

Figure 3 Localization of Pyk2 in the sealing zone of osteoclasts on bone. Confocal images of double immunostaining for Pyk2 (a, in red) and F-actin (b, in green) and their overlay image (c, in yellow) in an osteoclast during resorption (Duong *et al.,* 1998) (See also color plate.)

activation in fibroblasts (Tanaka *et al.,* 1995). Tanaka *et al.* (1996) reported that the level of tyrosine phosphorylation of c-Cbl immunoprecipitated from *Src*$^{-/-}$ osteoclasts is reduced markedly, compared with that found in wild-type osteoclasts (Tanaka *et al.,* 1996). Furthermore, c-Src is found to associate and colocalize with c-Cbl in the membranes of intracellular vesicles in osteoclasts (Tanaka *et al.,* 1996). c-Cbl is a negative regulator of several receptor and nonreceptor tyrosine kinases (Miyake *et al.,* 1997). Consistent with this function, overexpression of Cbl results in decreased Src kinase activity. Interestingly, equivalent inhibitory effects are seen with any fragment of Cbl that contains the N-terminal phosphotyrosine-binding (PTB) domain, regardless of the presence of the Src-binding, proline-rich region (Sanjay *et al.,* 2001). The inhibition of Src kinase activity and the binding to Src of v-Cbl (which lacks the proline-rich domain of Cbl and therefore will not bind to the Src SH3 domain) require the presence of phosphorylated Src Tyr416, the autophosphorylation site of Src on the activation loop of the kinase domain (Sanjay *et al.,* 2001).

In HEK293 cells, overexpression of the Cbl constructs also causes decreases in cell adhesion in parallel with their effects on Src kinase activity. Thus, c-Cbl and fragments of Cbl that contain the PTB domain reduce cell adhesion, whereas the C-terminal half of Cbl, which contains the Src SH3-binding, proline-rich region but not the PTB domain, does not (Sanjay *et al.,* 2001). Interestingly, the absence of either c-Src or c-Cbl causes a decrease in both cell motility (the rapid extension and retraction of lamellipodia) and directional migration of authentic osteoclasts isolated from Src$^{-/-}$ or Cbl$^{-/-}$ mice (Sanjay *et al.,* 2001), and freshly plated osteoclasts from Src$^{-/-}$ mice fail to disassemble podosomes efficiently as the cell spreads (Sanjay *et al.,* 2001), suggesting that the Pyk2/Src/Cbl complex may be required for the disassembly of attachment structures. The increased stability of podosomes could well explain the decreased motility of the Src$^{-/-}$ and Cbl$^{-/-}$ osteoclasts.

Another recently described function of c-Cbl could contribute to the regulation and stability of podosomes in osteoclasts. It has been shown that Cbl acts as a ubiquitin ligase, targeting ubiquitin-conjugating enzymes to the kinases that Cbl binds to and downregulates, with the result that the kinases are ubiquitinated and degraded by the proteosome (Yokouchi *et al.,* 1999; Joazeiro *et al.,* 1999; Levkovitz *et al.,* 1999). Because active, but not inactive, Src is ubiquitinated and degraded via the proteosome (Hakak and Martin, 1999), it could therefore be that once Cbl is bound to the Pyk2/Src complex, it recruits the ubiquitinating system to the podosome, leading to the ubiquitination and degradation of the other components of the complex, thereby participating in the turnover of podosome components that is required to ensure cell motility.

$\alpha_v\beta_3$ Integrin-Mediated Association of Pyk2 with Src and p130^{Cas}

Another integrin-dependent signaling adaptor characterized in osteoclasts is p130^{Cas} (Cas, Crk associated substrate). In a number of different cell types, p130^{Cas} has been shown to be substrates for FAK, Pyk2, Src kinase, or Abl (Schlaepfer *et al.,* 1999). In osteoclasts, p130^{Cas}, via its SH3 domain, can directly associate with the proline-rich motifs in the C-terminal domains of FAK and Pyk2. Similar to Pyk2, p130^{Cas} was shown to be highly tyrosine phosphorylated upon osteoclast adhesion to extracellular matrix substrates of $\alpha_v\beta_v$ integrins (Lakkakorpi *et al.,* 1999; Nakamura *et al.,* 1998a). In addition, p130^{Cas} localizes to the actin ring formed in osteoclasts on glass and to the sealing zone on bone (Lakkakorpi *et al.,* 1999; Nakamura *et al.,* 1998a). In adhering osteoclasts, p130^{Cas} is found to be constitutively associated with Pyk2, suggesting that this adapter molecule participates in the integrin-PYK2 signaling pathway (Lakkakorpi *et al.,* 1999).

Much less is known about what signals are generated by phosphorylation of the p130^{Cas} adaptor protein. In fibroblasts, integrin-stimulated tyrosine phosphorylation of p130^{Cas} has been shown to promote binding to the SH2 domain of either Crk (Vuori *et al.,* 1996) or Nck (Schlaepfer *et al.,* 1997). More recently, expression of the SH3 domain of p130^{Cas} can inhibit FAK-mediated cell motility (Cary *et al.,* 1998), whereas overexpression of Crk has been shown to promote cell migration in a Rac- and Ras-independent manner (Klemke *et al.,* 1998). Moreover, the downstream components of this Rac-mediated migration pathway appear to involve phosphatidyl inositol 3-kinase (PI3-kinase) (Shaw *et al.,* 1997). In osteoclasts, the PI3-kinase-dependent target was found to be the cytoskeletal-associated protein gelsolin (Chellaiah *et al.,* 1998).

$\alpha_v\beta_3$ Integrin-Dependent Activation of PI3-kinase

In avian osteoclasts, $\alpha_v\beta_3$ is associated with the signaling molecule PI3-kinase and c-Src (Hruska *et al.,* 1995). Interaction of $\alpha_v\beta_3$ with osteopontin resulted in increased PI3-kinase activity and association with Triton-insoluble gelsolin (Chellaiah *et al.,* 1998). In murine osteoclasts formed in culture, PI3-kinase was found to translocate into the cytoskeleton upon osteoclast attachment to the bone surface (Lakkakorpi *et al.,* 1997). In addition, potent inhibitors of PI3-kinase, such as wortmannin, inhibit mammalian osteoclastic bone resorption *in vitro* and *in vivo* (Nakamura *et al.,* 1995).

Gelsolin, an actin-binding protein, is known to regulate the length of F-actin *in vitro,* and thus cell shape and motility (Cunningham *et al.,* 1991). More recently, gelsolin-deficient mice were generated that express mild defects during hemostasis, inflammation, and possibly skin remodeling (Witke *et al.,* 1995). Gelsolin$^{-/-}$ mice have normal tooth eruption and bone development, with only modest thickened calcified cartilage trabeculae due to delayed cartilage resorption and very mild and progressive osteopetrosis (Chellaiah *et al.,* 2000). However, osteoclasts isolated from gelsolin$^{-/-}$ mice lack podosomes and actin rings and have reduced cell motility (Chellaiah *et al.,* 2000).

In conclusion, there is little doubt today that integrins present in the osteoclast membrane, and particularly the

$\alpha_v\beta_3$ integrin, play a critical role in osteoclast biology. This involves not only the adhesion itself, but also the regulation of outside-in signaling, which ensures the proper organization of the cytoskeleton, and of inside-out signaling, which modulates the affinity of the receptors for their substrates. These two regulatory modes are essential in ensuring the assembly and disassembly of the attachment structures (podosomes), a cyclic process necessary for efficient cell motility

Calcitonin and the Cytoskeleton

Because of its potent inhibitory effects on osteoclast activity, calcitonin has long been recognized as a potential therapeutic agent for the treatment of diseases that are characterized by increased bone resorption, such as osteoporosis, Paget's disease, and late-stage malignancies. Signaling mechanisms downstream of the calcitonin receptor have therefore been of great interest. A comprehensive discussion of calcitonin-induced signaling appears elsewhere in this volume, and this section focuses on what is known of the interaction of calcitonin-activated signaling events with attachment-related signaling.

In situ, calcitonin causes reduced contact of osteoclasts with the bone surface and altered osteoclast morphology (Holtrop *et al.,* 1974; Kallio *et al.,* 1972). While *in vitro,* calcitonin-treated osteoclasts retract and become less mobile (Chambers and Magnus, 1982; Chambers *et al.,* 1984; Zaidi *et al.,* 1990). These effects suggest that some of the key targets of calcitonin signaling are involved in cell attachment and cytoskeletal function, possibly in relation with integrins and/or their signaling function.

The calcitonin receptor, a G protein-coupled receptor that has been cloned from several species and cell types, couples to multiple heterotrimeric G proteins (G_s, $G_{i/o}$, and G_q)(Chabre *et al.,* 1992; Chen *et al.,* 1998; Force *et al.,* 1992; Shyu *et al.,* 1996; 1999). For technical reasons, much of the recent characterization of signaling downstream of the calcitonin receptor has been conducted in cells other than osteoclasts, particularly cell lines that express recombinant calcitonin receptor. Because it couples to multiple G proteins, the proximal signaling mechanisms that are activated by calcitonin include many classical GPCR-activated effectors, such as adenylyl cyclase and protein kinase A (PKA), phospholipases C, D, and A2, and protein kinase C (PKC). In HEK 293 cells that express the rabbit calcitonin receptor, calcitonin induces phosphorylation and activation of the extracellular signal-regulated kinases, Erk1 and Erk2 (Chen *et al.,* 1998; Naro *et al.,* 1998), via mechanisms that involve the $\beta\gamma$ subunits of pertussis tox-insensitive G_i as well as pertussis toxin-insensitive signaling via phospholipase C, PKC, and elevated intracellular calcium ($[Ca^{2+}]_i$)

It has been shown in the same cell line that calcitonin induces tyrosine phosphorylation and association of FAK, paxillin, and HEF1, a member of the $p130^{Cas}$ family (Zhang *et al.,* 1999b), which are components of cellular adhesion complexes. Interestingly, while these cells express both HEF1 and $p130^{Cas}$, only HEF1 is phosphorylated and associates with FAK and paxillin. This response to calcitonin is independent of adenylyl cyclase/PKA and of pertussis toxin-sensitive mechanisms and appears to be mediated by the pertussis toxin-insensitive PKC/$[Ca^{2+}]_i$ signaling pathway. The relevance of the FAK/paxillin/HEF1 phosphorylation to integrins and the actin cytoskeleton is demonstrated by its requirement for integrin attachment to the substratum and its sensitivity to agents (cytochalasin D, latrunculin A) that disrupt actin filaments (Zhang *et al.,* 2000). The calcitonin-induced tyrosine phosphorylation of paxillin and HEF1 is enhanced by the overexpression of c-Src and is strongly inhibited by the overexpression of a dominant negative kinase-dead Src, indicating that c-Src is required at some point in the coupling mechanism. Interestingly, the dominant-negative Src has little effect on the calcitonin-induced phosphorylation of Erk1/2, in contrast to what has been reported for some other G protein coupled receptors (Della Rocca *et al.,* 1997,1999), indicating that some aspects of calcitonin-induced signaling may be significantly different from other better studied G protein-coupled receptors. These or similar events could well play important roles in mediating the calcitonin-induced changes in cell adhesion and motility in osteoclasts.

Therapeutic Implications

Although the mechanism and action of calcitonin in blocking osteoclast function are not fully understood, the relative selective expression of the calcitonin receptor in osteoclasts and its well accepted role in regulating osteoclastic cytoskeleton have made calcitonin an attractive therapeutic agent for many years. Calcitonin of human, pig, salmon, and eel has been used in both injected and intranasal form in the treatment of osteoporosis and Paget's disease. However, calcitonin-induced downregulation of the receptors in osteoclasts has been observed, resulting in the hormone-induced resistance of osteoclasts in bone resorption. It therefore remains to be seen whether this problem can be overcome for therapeutic application of this class of receptor. Identification of the downstream signaling pathway of calcitonin receptor would further our understanding on how calcitonin blocks bone resorption and regulates calcium hemostasis. As discussed here, it is possible that much of the effects of calcitonin on the osteoclast are due to its ability to generate intracellular signals that interfere with the normal regulation of the cytoskeleton, adhesion, and/or cell motility, thereby converging on similar functional targets as the integrin signaling pathways.

The findings that gene deletion of $\alpha_v\beta_3$ integrin and its downstream effectors, as well as the use of inhibitors of this integrin, results in inhibition of bone resorption *in vivo* in rodent models point to these molecules as potential therapeutic targets for osteoporosis therapy. $\alpha_v\beta_3$ integrin is highly and somewhat selectively expressed in osteoclasts across species. β_3 knockout mice appear to have normal development and growth, besides the defects

related to $\alpha_v\beta_3$ integrin associated osteopetrosis and $\alpha_{IIb}\beta_3$ integrin-associated bleeding. Orally active RGD mimetics have been reported to successfully block bone resorption in rodents without notable adverse effects. This raises the possibilities of developing safe and effective therapeutic agents for osteoporosis based on interfering with the interaction of the osteoclast $\alpha_v\beta_3$ integrin with its physiological ligands.

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CHAPTER 10

Apoptosis in Bone Cells

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Introduction

Apoptosis is a form of individual cell suicide that was originally defined by a series of morphological changes in nuclear chromatin and cytoplasm (Kerr *et al.,* 1972; Wyllie *et al.,* 1980). Cells undergoing apoptosis contract, lose attachment to their neighbors, and break up into fragments, apoptotic bodies, which get phagocytosed quickly by surrounding cells. The Greek derivation of apoptosis depicts petals falling from flowers or leaves falling from trees. Thus, the "apo" (as in apocrine) describes the apparent extrusion of dying cells into spaces around them (see later) and the "ptosis" (as in drooping of the upper eyelid) describes them falling out or disappearing from the tissue.

Apoptosis is an important regulatory program in which activation or suppression of many factors, including oncogenes, tumor suppressor genes, growth factors, cytokines, and integrins, can determine a cell's fate (see Chao and Korsmeyer, 1998; Earnshaw *et al.,* 1999; Desagher and Martinou, 2000; Green, 2000; Strasser *et al.,* 2000; Hengartner, 2000). It controls cell numbers in populations of neural, mesenchymal, and epithelial cells during embryonic development; facilitates deletion of superfluous tissue, such as soft tissue between developing fingers; and accounts for some of the cell loss from regenerating epithelial surfaces in adult tissues, such as the skin and alimentary tract.

Since the first version of this chapter was published in 1996, there has been an explosion of information on the significance and molecular regulation of apoptosis in bone (Manolagas, 2000; Weinstein and Manolagas, 2000). It is now evident that apoptosis is as important as its functional opposite, mitosis, for the growth and maintenance of the skeleton. This second edition of the chapter reflects these new developments and is an amalgam of the efforts of two groups of investigators: one (Drs. Boyce and Xing) working on osteoclast apoptosis and one (Drs. Jilka, Bellido, Weinstein, Parfitt, and Manolagas) studying the apoptosis of osteoblasts and osteocytes and its role in the pathophysiology and therapeutic management of osteoporosis. After briefly sketching the features and molecular regulation of apoptosis, we will review its occurrence and regulation in bone cells, its significance for bone development and maintenance, and its importance in the etiology and treatment of bone diseases.

General Features and Regulation of Apoptosis

Morphologic Features and Detection Techniques

The most dramatic changes during apoptosis are seen in the nucleus and begin with clumping of chromatin into dense aggregates around the nuclear membrane (Fig. 1A, see also color plate), rather than the even dispersal seen typically in viable cells (Fig. 1B, see also color plate). This is followed by further chromatin condensation, disintegration of the nucleus, and the formation of numerous balls of condensed chromatin within the cytoplasm (Fig. 1C, see

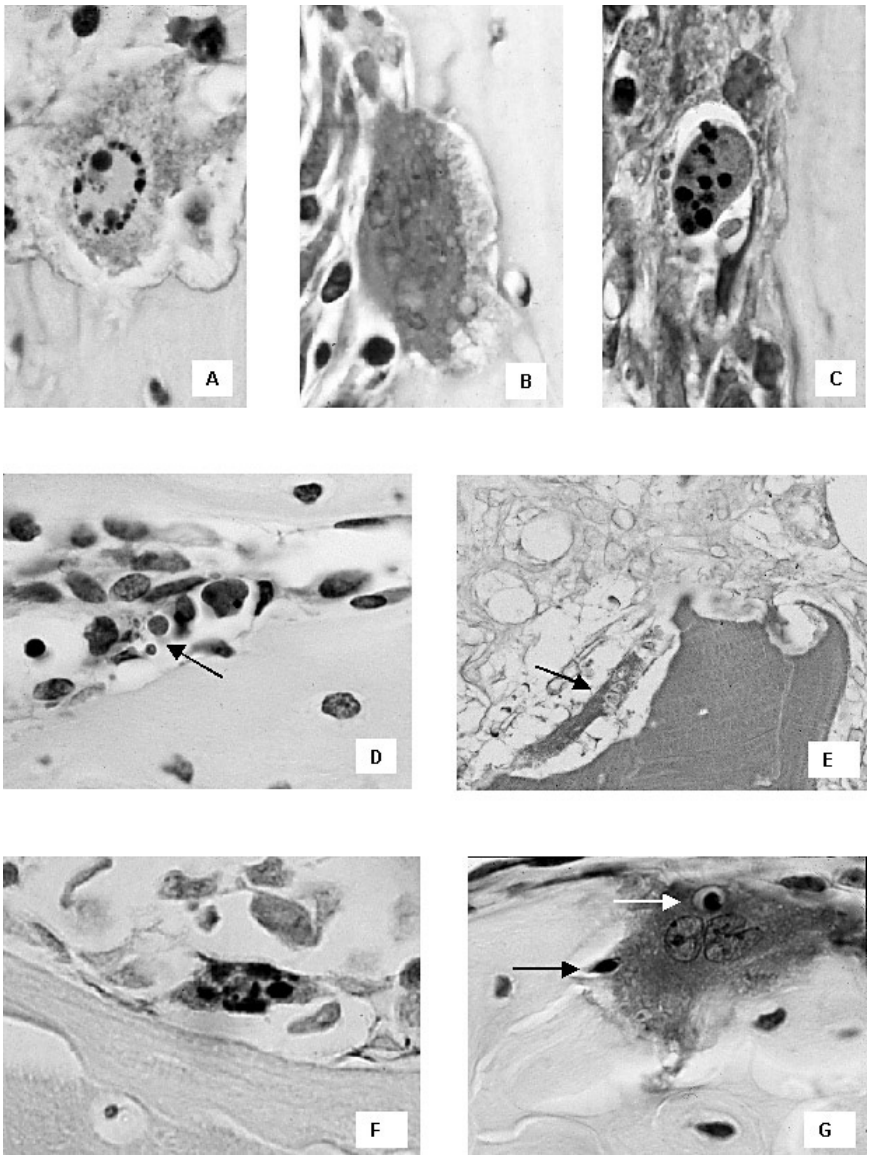

Figure 1 Apoptosis of osteoclasts. (A) Peripheral clumping of chromatin around the nuclear membrane of an osteoclast from a TRAP SV40 Tag transgenic mouse (Boyce *et al.,* 1995). H&E, orange G and phloxine. (B) Actively resorbing osteoclast with normal nuclear and cytoplasmic morphology and ruffled border. TRAP staining, normal mouse following treatment with IL-1. (C) Osteoclast showing classical nuclear and cytoplasmic morphologic features of apoptosis. Note that the cell has withdrawn from the bone surface, contracted, has more intense TRAP staining than the osteoclast in B, and that all of its nuclei have condensed and fragmented simultaneously. TRAP staining. Normal mouse following treatment with IL-1. (D) TRAP-positive apoptotic bodies adjacent to the bone surface (arrow). Note that some do not have fragments of nuclei in them and thus would not be recognized as parts of an osteoclast in an H&E-stained section. TRAP staining, ovariectomized mouse following treatment with estrogen. (E) Necrotic osteoclast in a resorption lacuna (arrow). Note that the nuclei and cytoplasm have not contracted. H&E, orange G and phloxine. Human bone, ischemic necrosis. (F) TUNEL staining of an apoptotic osteoclast. Note the strong positive dark brown signal in all of the contracted nuclei of the cell and no signal in adjacent viable cells. Methyl green counterstain. TRAP SV40 Tag transgenic mouse (Boyce *et al.,* 1995). (G) Osteoclast containing the nucleus of an engulfed apoptotic osteocyte (top arrow). Note the condensed nuclear chromatin of this cell and of the osteocyte about to be engulfed (lower arrow) in comparison with the normal evenly dispersed nuclear chromatin of the viable osteocytes below and to the right of the osteoclast. TRAP staining. Ovariectomized mouse. (See also color plate.)

also color plate). These nuclear changes are accompanied by cell shrinkage due to fluid movement out of the cell and loss of contact with neighboring cells or matrix (Fig. 1C)—a feature that has been used to collect apoptotic cells for analysis in *in vitro* assays (Wyllie *et al.,* 1980; Arends *et al.,* 1990; Hughes *et al.,* 1995b; Hughes *et al.,* 1996; Jilka *et al.,* 1998). As apoptosis progresses, numerous cell surface convolutions form and the cell disintegrates into multiple membrane-bound, condensed apoptotic bodies (Fig. 1D, see also color plate) that typically are phagocytosed rapidly by neighboring cells. The whole process can take a few minutes in some cells (e.g., cytotoxic T-cell-induced apoptosis) to several hours; but in others, DNA fragmentation can begin 2 days prior to cellular disintegration (Pompeiano *et al.,* 1998). Apoptosis differs from ischemic necrosis in that it typically affects single cells rather than groups of cells and, unlike in necrosis (Fig. 1E, see also color plate), apoptotic cells and their nuclei do not swell nor does their destruction attract inflammatory cells.

Caspase-activated DNases (CAD) (Nagata, 2000) are activated early in the apoptosis process and they split genomic DNA at nucleosomes into fragments of varying sizes, giving rise to characteristic "ladders" that are seen on gel electrophoresis (Wyllie *et al.,* 1980; Arends *et al.,* 1990; Kaufmann *et al.,* 2000). The subsequent nuclear fragmentation and condensation can be visualized using acridine orange, Höescht dyes, and propidium iodide by their bright fluorescence upon binding to DNA (Fig. 2A, see also color plate) (Arndt-Jovin and Jovin, 1977; Arends *et al.,* 1990) and in cells transfected with green fluorescent protein containing a nuclear localization sequence (Fig. 2B, see also color plate)—a useful tool for studying apoptosis in cells cotransfected with genes of interest (Bellido *et al.,* 2000; Kousteni *et al.,* 2001).

Degraded DNA can also be detected enzymatically and quantified (Stadelmann and Lassmann, 2000) using TUNEL (TdT-mediated dUTP-biotin nick end labeling) (Gavrieli *et al.,* 1992), ISNT (*in situ* nick translation) (Gold *et al.,* 1993) and ISEL (*in situ* nick end labeling) (Wijsman *et al.,* 1993; Ansari *et al.,* 1993) (Fig. 1F, Fig. 2C-H, see also color plate). The latter is 10-fold more sensitive than TUNEL and can even detect cells undergoing DNA repair; thus it can less specific (Gold *et al.,* 1994). These DNA labeling methods can also identify cells dying by necrosis (Grasl-Kraupp *et al.,* 1995), but unlike apoptosis, necrosis is rarely focal.

Regulation of Apoptosis

Two main pathways appear to initiate apoptosis. One signals cells to die as a consequence of ligand interaction with so-called death receptors on the cell surface, many of which are members of the tumor necrosis factor (TNF) receptor superfamily. The second is activated by a set of molecules from the mitochondria following a variety of stimuli, including oxidative stress and loss of survival signals generated by growth factors and cytokines, as well as loss of attachment to the extracellular matrix—a process called anoikis (Frisch and Ruoslahti, 1997). Both pathways activate a family of proteolytic enzymes called caspases that, by cleaving specific substrates, cause the morphological changes described earlier. The events underlying the regulation of caspase activation are depicted in Fig. 3 (see also color plate).

Death Receptors (DR)

DR have been studied most thoroughly in cells of the immune system and include CD95 (Fas/APO-1), and the TNF receptors (Krammer, 2000). The cytoplasmic tail of CD95 contains a death domain that binds adapter proteins, such as FADD (Fas-associated death domain protein) following receptor activation. FADD has a death effector domain (DED) that recruits DED-containing proteins, such as procaspase-8. TNF and TRAIL (TNF-related apoptosis inducing ligand), which binds to DR5, induce a similar reaction following binding to their receptor in susceptible cells, except that an additional adaptor, TRADD, links the tail of the receptor with FADD. Although the predominant effect of Fas ligand/CD95 and TRAIL/DR5 binding is to trigger cell death, binding of TNF to its receptors can result in additional diverse effects, including NF-κB activation, which can inhibit, rather than stimulate apoptosis (Van Antwerp *et al.,* 1996) in some cell types, including osteoclasts.

Caspases

These are highly conserved cysteine proteases that cleave substrates at aspartate residues (Earnshaw *et al.,* 1999). The focal point of apoptosis control is the proteolytic conversion of inactive procaspases, primarily procaspase-8 and -9 (Hengartner, 2000), to the proteolytically active form. This conversion may be a direct result of death receptor activation or the release of caspase-activating factors such as cytochrome c from mitochondria. Cytochrome c binds to Apaf-1 complexed to procaspase-9, resulting in its activation. Caspases-8 and -9 cleave and activate the effector caspases-3, -6, and-7, which cleave substrates in the nucleus and cytoplasm. For example, effector caspases activate CAD to initiate DNA fragmentation; they also cleave cytoskeletal proteins, such as fodrin and gelsolin (Kothakota *et al.,* 1997; Janicke *et al.,* 1998), to change cell shape.

Regulation of Caspase Activity

Members of the Bcl-2 family of proteins control the release of caspase-activating proteins from the mitochondria. Proapoptotic members of the family include Bad, Bax, and Bid; antiapoptotic members include Bcl-2 and Bcl-x_L (Chao and Korsmeyer, 1998; Kelekar and Thompson, 1998). Because members of the Bcl-2 family can form either homodimers or heterodimers, changes in their biosynthesis and/or activity can shift the balance of the cell between an anti- and a proapoptotic state. Thus, dimers of antiapoptotic members, e.g., Bcl-2, bind to the outer membrane of the

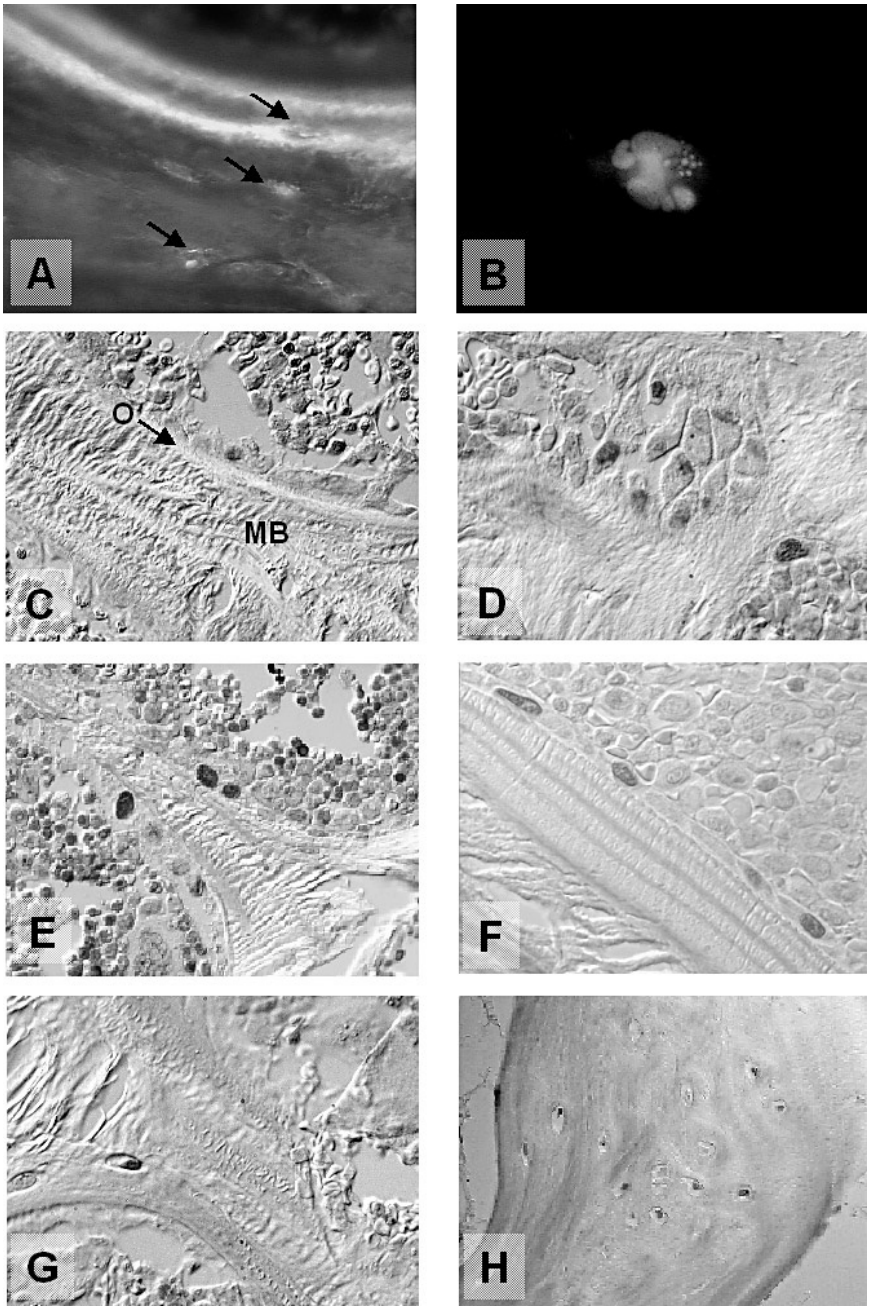

Figure 2 Apoptosis osteoblasts and osteocytes. (A) Transiliac biopsy from a patient with glucocorticoid-induced osteoporosis stained with Höescht dye. Note the apoptotic osteocytes (arrows) recently buried in a new packet of bone outlined by yellow tetracycline labeling. (B) Nuclear fragmentation in osteoblastic OB-6 cells transfected with green fluorescent protein containing a nuclear localization sequence, treated with etoposide for 6 hr, and observed under epifluorescence illumination. (C–H) Apoptotic osteoblasts and osteocytes detected by ISEL staining of nondecalcified sections of vertebral bone of a normal 4-month-old mouse (C) an ovariectomized mouse (D) a prednisolone-treated mouse (E and G) a transiliac bone biopsy from a patient with glucocorticoid-induced osteoporosis (F) and a femoral head obtained during total hip replacement because of glucocorticoid-induced osteonecrosis (H) (C) Note the juxtaposition of labeled osteoblasts (brown) to osteoid ("O") interspersed with unaffected viable (blue) members of the osteoblast team. Mineralized bone is indicated by "MB." (See also color plate.)

mitochondria and prevent the release of caspase activators, whereas dimers of proapoptotic members (e.g., Bax) or, in some cases, heterodimers (e.g., Bad • Bcl-2) promote an increase in membrane permeability. Interaction of Bad with 14–3–3 protein, in cooperation with the phosphorylation of Bad, inactivates the proapoptotic function of Bad by causing its release from Bcl-2 and preventing its interaction with the mitochondrial membrane (Datta *et al.,* 2000). Apoptosis

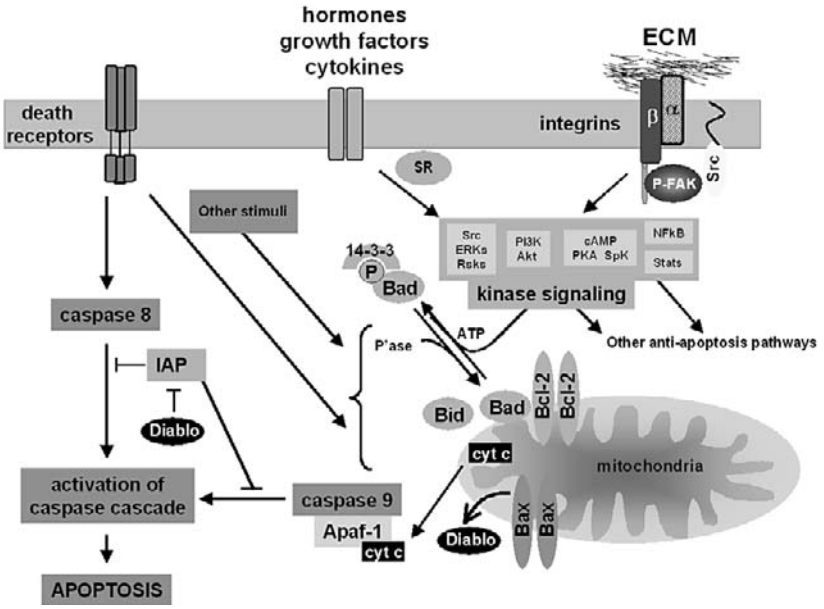

Figure 3 Basic mechanisms of apoptosis regulation. See text for details. SR, steroid hormone receptors; P'ase, protein phosphatase; ERK, extracellular signal-regulated kinases; Rsks, MAPK-activated ribosomal S6 kinases; PI3K, phosphatidyl inositol-3-kinase; PKA, protein kinase A; SpK, sphingosine kinase; Stats, signal transducers and activators of transcription. (See also color plate.)

stimulation, however, can be mediated by the dephosphorylation of phospho-Bad via the activation of specific phosphatases (Desagher and Martinou, 2000).

Inhibitors of apoptosis proteins (IAPs), including XIAP, c-IAP1, c-IAP2, and survivin, specifically bind to and inhibit caspases (Roy *et al.,* 1997; Deveraux *et al.,* 1998), thus providing a second mechanism of negative control of apoptosis. They also function as ligases to ubiquinate interacting proteins, thereby facilitating their degradation by proteosomes (Yang *et al.,* 2000), and their action is overcome during apoptosis via the release of Smac/DIABLO from mitochondria, which binds to and inactivates IAPs (Green, 2000).

Survival Signaling via Integrins and Growth Factor Receptors

Binding of integrins with extracellular matrix proteins fosters the assembly of the focal adhesion complex comprising cytoskeletal and catalytic proteins (Giancotti and Ruoslahti, 1999). The most important of the latter are focal adhesion kinase (FAK) and Src-family kinases. Assembly of focal adhesion complexes results in the autophosphorylation of FAK, which in turn activates phosphatidyl inositol-3-kinase (PI3-kinase) and/or the MAP kinase cascade to provide a constant inhibitory brake on the execution of apoptosis (Ilic *et al.,* 1998), perhaps via Bad phosphorylation. FAK signaling may also inhibit apoptosis by the induction of IAP synthesis (Sonoda *et al.,* 2000). Growth factor/cytokine receptor activation also generates survival signals via the activation of intracellular kinase cascades, including Stats, ERK, Akt, and PI3-kinase, as illustrated in Fig. 3.

Chondrocyte Apoptosis

Apoptosis has a major role in three areas of chondrocyte biology: the shaping of long bones during development, endochondral ossification, and the loss of articular cartilage in degenerative and inflammatory bone diseases.

Limb Development

Long bones form from limb buds under the control of genes expressed by primitive mesenchymal cells that interact with overlying ectodermal cells (Rowe and Fallon, 1982; Shubin *et al.,* 1997; Panganiban *et al.,* 1997). They are first formed of a cartilage analag, which is sculpted to determine the shape and length of developing limb elements relative to one another. This sculpting involves both proliferation and apoptosis of chondrocytes in a tightly regulated process in which FGFs, BMPs, sonic hedgehog, and *Hox* genes play major regulatory roles (Macias *et al.,* 1997; Chen and Zhao, 1998; Buckland *et al.,* 1998). The list of genes involved in the regulation of limb development is growing rapidly and consequently the genetic basis of many human skeletal anomalies is being revealed (Mundlos

and Olsen, 1997a; Innis and Mortlock, 1998). These are described in detail in Chapter 3.

Endothelial cells invade the perichondrium near the middle of these elementary bone structures, inducing apoptosis of chondrocytes adjacent to them in the perichondrium. Osteoblasts form from mesenchymal cells behind the advancing endothelial cells laying down bone matrix and forming a primary center of ossification in the central parts of limbs into which circulating hematopoietic precursors cells migrate and form the bone marrow of the medullary cavity. Osteoclasts are not required for this process because it occurs in their absence in RANKL and RANK knockout mice (Hofbauer *et al.,* 2000), but the signaling molecules released by endothelial and other mesenchymal cells that mediate the massive chondrocyte apoptosis that takes place have yet to be determined.

Endochondral Ossification

An epiphyseal growth plate forms from the physis near the ends of long bones and maintains endochondral bone formation until epiphyses close at varying times after puberty in humans. A secondary center of ossification forms in the physis adjacent to the growth plate following massive chondrocyte apoptosis, leaving a rim of articular cartilage at the end of the bone. Proliferating chondroblasts in growth plates give rise to the hypertrophic chondrocytes around which the cartilage calcifies. Some of these hypertrophic chondrocytes undergo apoptosis before they removed by chondroclasts (Lewinson and Silbermann, 1992) and some survive to be covered by new bone laid down by osteoblasts at the primary spongiosa.

Parathyroid hormone related peptide (PTHrP) and Indian hedgehog (Ihh) play central roles in the regulation of endochondral ossification (Vortkamp *et al.,* 1996; Lanske *et al.,* 1996). PTHrP appears to control the life span of hypertrophic chondrocytes, at least during the early stages of endochondral ossification before the formation of the secondary ossification center when it is involved in direct signaling between perichondrial cells at the ends of long bones and underlying proliferating chondroblasts and prehypertrophic and hypertrophic chondrocytes. It prevents premature apoptosis of hypertrophic chondrocytes in a complex autocrine and paracrine signaling mechanism involving Ihh upstream and Bcl-2 downstream (Weir *et al.,* 1996; Zerega *et al.,* 1999). This action of PTHrP was discovered following the analysis of bones from mice with loss or gain of function. PTHrP knockout mice have thinner than normal growth plates with reduced numbers of hypertrophic chondrocytes and growth retardation (Karaplis *et al.,* 1994; Lee *et al.,* 1996), whereas transgenic mice overexpressing PTHrP have thick growth plates and increased numbers of hypertrophic chondrocytes (Weir *et al.,* 1996). Thickened growth plates and delayed apoptosis of hypertrophic chondrocytes were also observed in mice lacking matrix metalloproteinase-9 (MMP-9)/gelatinase B, a defect that was rescued by the transplantation of chondroclast precursors and which also healed spontaneously beginning 3 weeks after birth (Vu *et al.,* 1998).

Degenerative and Inflammatory Joint Disease

Chondrocyte viability is sustained in healthy cartilage through integrin-mediated survival signaling. *In vitro* studies indicate that chondrocytes require continuous contact with one another for their survival (Ishizaki *et al.,* 1994), which can be maintained by cytokines, such as IL-1, TNF and interferon-γ (Blanco *et al.,* 1995; Kuhn *et al.,* 2000). However, in other *in vitro* studies, IL-1 and TNF, induced apoptosis of chondrocytes (Nuttall *et al.,* 2000; Fischer *et al.,* 2000), an effect also seen with nitric oxide (Blanco *et al.,* 1995; Amin and Abramson, 1998) and prostaglandin E_2 (PGE_2) (Miwa *et al.,* 2000). All of these agents are released by activated immune cells and synoviocytes in joints of patients with a variety of inflammatory joint diseases. Finally, IL-1 promotes the release of matrix-degrading enzymes, such as hexosaminidase, by chondrocytes, which could promote their apoptosis (Shikhman *et al.,* 2000).

In rheumatoid arthritis, activated synoviocytes produce degradative enzymes, as well as inflammatory cytokines. Destruction of the cartilage matrix results in anoikis (Nuttall *et al.,* 2000). Specific inhibitors of caspase-3 and-7, but not caspase-1 (Lee *et al.,* 2000), prevent apoptosis of chondrocytes *in vitro,* raising the possibility that such inhibitors might be efficacious in the prevention of degenerative or inflammatory joint diseases. Activated synoviocytes also have a greatly increased expression of the death factor, Fas/Apo-1, but unlike many other cell types, these hyperplastic cells do not die, presumably because the levels of Fas ligand in synovial fluid are extremely low. Delivery of Fas ligand (Yao *et al.,* 2000) or Fas-associated death domain (FADD) protein (Kobayashi *et al.,* 2000) to inflamed joints of arthritic animals induced synoviocyte apoptosis and amelioration of the arthritis. Chondrocyte apoptosis was unaffected, suggesting a new approach to treatment of this disease.

Several studies have documented increased chondrocyte apoptosis in the articular cartilage of osteoarthritic patients compared to normal subjects (Hashimoto *et al.,* 1998; Kirsch *et al.,* 2000; Kobayashi *et al.,* 2000; Kim *et al.,* 2000), a change that is associated with the expression of annexin V (Kirsch *et al.,* 2000) and Fas (Kim *et al.,* 2000). However, further studies are required to determine if chondrocyte apoptosis is the cause of cartilage erosion or a consequence of the matrix degeneration that characterizes osteoarthritis.

Apoptosis of Osteoclasts

Apoptosis of osteoclasts was first recognized following targeting of the simian virus 40 large T antigen to the osteoclast in transgenic mice using the TRAP promoter, and following withdrawal of M-CSF from osteoclasts in culture (Boyce *et al.,* 1995; Fuller *et al.,* 1993). Morphologic

changes consistent with osteoclast apoptosis had been described previously in several publications on the effects of bisphosphonates and estrogen on osteoclasts, but the cellular changes were not recognized as apoptosis at the time (Rowe and Hausmann, 1976; Liu *et al.,* 1982; Flanagan and Chambers, 1989; Sato and Grasser, 1990; Liu and Howard, 1991).

Morphology of Apoptotic Osteoclasts

Osteoclasts undergoing apoptosis have two striking features: stronger cytoplasmic TRAP staining than viable osteoclasts (Figs. 1B and 1C) and simultaneous apoptosis of all their nuclei (Fig. 1C) (Boyce *et al.,* 1995; Hughes *et al.,* 1995b). Retention of cytoplasmic organelle function and enzyme activity is typical of cells undergoing apoptosis. The intense TRAP staining may reflect cytoplasmic contraction and concentration of TRAP within the cells or decreased secretion of TRAP or a combination of both. It facilitates recognition not only of intact apoptotic osteoclasts in tissue sections, but also of fragments of osteoclasts following disintegration of the dead cells (Fig. 1D). It may account for the relatively high percentage (0.3%) of osteoclasts with classic morphologic features of apoptotic cells seen in sections of normal mouse bone (Hughes *et al.,* 1995b) compared with the low percentage of cells with such morphologic features in regenerating tissues, such as large intestine [0.05% in enterocytes (Lee and Bernstein, 1993)] and liver [0.01% in hepatocytes (Bursch *et al.,* 1990)].

Simultaneous death of nuclei contrasts with their progressive accumulation in osteoclasts as mononuclear cells are incorporated into formed osteoclasts by cytoplasmic fusion. It suggests that the theoretical survival of osteoclasts for very long periods through continuous recruitment of new nuclei and shedding of older nuclei is unlikely and that the intracellular signaling controlling osteoclast apoptosis is a highly coordinated process that leads to the demise of the whole cell. Individual nuclei of phagocytosed apoptotic osteocytes are observed frequently in actively resorbing osteoclasts (Fig. 1G, see also color plate).

Osteoclast Apoptosis in Bone-Remodeling Units

The osteoclast number increases dramatically following daily injections of high doses of IL-1 over the calvarial bones of 2- to 3-week-old mice (Boyce *et al.,* 1989), and resorption is followed by the deposition of abundant new bone matrix in a sequence similar to that seen in normal bone remodeling. Thirteen percent of the osteoclasts are apoptotic (Wright *et al.,* 1994), with most of them being at the reversal site between the advancing resorption front (where apoptotic osteoclasts were not seen) and the ensuing new bone formation. Osteoclast apoptosis has been confirmed at the reversal site in bone remodeling units in other animal models and in patients with increased bone turnover (Wright *et al.,* 1994), with the number being close to that seen in long bones of normal mice (0.3%) (Hughes *et al.,* 1995b).

The high percentage of apoptotic osteoclasts observed in mouse calvariae 4–6 days after cessation of IL-1 injections may be due to abrupt reduction in the local concentration of prostaglandins or other antiapoptotic factors. This mechanism might be similar to that seen following the reintroduction of calcium to the diet of calcium-deficient rats (Liu *et al.,* 1982; Wright *et al.,* 1995) and mice when there is a sharp fall in the blood concentrations of 1,25(OH)2 vitamin D3 and parathyroid hormone (PTH) (Liu *et al.,* 1982), both of which prevent osteoclast apoptosis. On the basis of these findings, Parfitt *et al.* (1996) proposed that osteoclast precursors are recruited to the advancing resorption front to maintain the youngest and presumably most active osteoclasts for forward progression of the basic multicellular unit (BMU) of bone remodeling. Older osteoclasts are left behind to complete lateral and downward progression, and these ultimately undergo apoptosis. The depth to which they resorb, and thus the removal or survival of trabeculae, is dependent on the timing of their apoptosis, which can be hastened pharmacologically (e.g., by bisphosphonates or estrogen) or delayed by sex steroid deficiency. Delayed apoptosis could also explain not only the deep resorption lacunae seen typically in Paget's disease, but also the large number of osteoclast nuclei, which might be related to increased Bcl-2 expression (Mee, 1999).

The regulation of osteoclast viability in remodeling units remains poorly understood, but it is possible that the resorptive process itself activates proapoptotic pathways. For example, exposure to the millimolar calcium ion concentrations found in resorption lacunae could induce osteoclast apoptosis (Lorget *et al.,* 2000), an effect that may be attenuated by IL-6 (Adebanjo *et al.,* 1998). Release of chloride and hydrogen ions, the latter through a proton pump on the ruffled border membrane, is required for this release of calcium, and although failure of ruffled border formation is not associated with increased osteoclast apoptosis, specific inhibition of the vacuolar H(+)-ATPase involved in proton release is (Okahashi *et al.,* 1997).

Regulation of Osteoclast Apoptosis.

Like many other factors that stimulate bone resorption, PTH and $1,25(OH)_2$ vitamin D3, prevent osteoclast apoptosis most likely by stimulating expression of RANKL and decreasing expression of osteoprotegerin (OPG) by stromal cells, and the relative local concentrations of these two recently described regulators of most aspects of osteoclast function are likely to be important determinants of osteoclast survival.

RANKL AND M-CSF

Since the initial observations of osteoclast apoptosis, several positive and negative regulators of osteoclast life span have been identified. These include factors such as

M-CSF (Fuller *et al.,* 1993) and RANKL (Lacey *et al.,* 2000), which are also required for osteoclast formation, and OPG, the most potent negative regulator of osteoclasts identified to date. The precise mechanisms whereby M-CSF and RANKL mediate osteoclast survival remain unclear, but studies suggest that the PI3-kinase/Akt signaling pathway may be involved (Wong *et al.,* 1999), and results in increased expression of the antiapoptotic genes, Bcl-2, Bcl-xL (Lacey *et al.,* 2000), and XIAP (Kanaoka *et al.,* 2000).

Following binding of RANKL to RANK on the surface of osteoclasts, TRAF 6, a member of the TNF receptor activator family, binds to the cytoplasmic domain of RANK; *c-src* expression is increased and Src binds to TRAF 6 (Lacey *et al.,* 2000). PI3-kinase binds to Src and Akt binds to PI3-kinase. Akt subsequently phosphorylates Bad and caspase-9 (Wong *et al.,* 1999), thus preventing activation of the apoptosis cascade. A similar signaling cascade is activated when IL-1 binds to its receptor on osteoclasts (Wong *et al.,* 1999). In contrast to M-CSF, RANKL does not appear to induce expression of XIAP (Kanaoka *et al.,* 2000) or Bcl-x_L (Jimi *et al.,* 1999). In addition to activating the PI-3- kinase pathway, RANKL also activates NF-κB (Jimi *et al.,* 1998) to promote osteoclast survival, consistent with findings that inhibitors of NF-κB induce osteoclast apoptosis (Ozaki *et al.,* 1997). This signaling pathway is essential for RANKL-mediated osteoclast formation (Xing *et al.,* 1998) and requires expression of the p50 and p52 subunits of NF-κB (Iotsova *et al.,* 1997; Franzoso *et al.,* 1997).

Src plays an important role in the antiapoptotic action of RANKL because withdrawal of RANKL causes significantly more apoptosis of osteoclasts generated from *src* knockout mice than those from wild-type mice (Wong *et al.,* 1999). The role of Src in RANKL-mediated osteoclast survival, however, is not essential because *src* mutant mice have no increase in osteoclast apoptosis *in vivo* (Xing *et al.,* 2001) and their osteoclast numbers are actually increased (Boyce *et al.,* 1992). However, overexpression of a truncated *src* transgene lacking the kinase domain in *src* knockout mice caused a marked increase in osteoclast apoptosis and more severe osteopetrosis (Xing *et al.,* 2001). This increased osteoclast apoptosis may be due to a dominant-negative action of the truncated protein via interference with survival-related functions of other Src family members that likely substitute for Src in this role in *src* mutant mice. M-CSF-mediated osteoclast survival, however, involves C-jun/AP-1 signaling rather than Src (Wong *et al.,* 1999).

The antiapoptotic effects of RANKL are opposed by OPG (Lacey *et al.,* 2000), a product of stromal/osteoblastic cells that prevents RANKL–RANK interaction. Thus, when OPG is in excess, osteoclasts will die due to loss of antiapoptotic RANKL signaling. Studies suggest that as stromal/osteoblastic cells differentiate into osteoblasts, the OPG/RANKL ratio increases, suggesting that mature osteoblasts might have a negative regulatory role in the survival of osteoclasts (Gori *et al.,* 2000).

Integrin Binding

The PI3-kinase antiapoptotic pathway is also activated as a consequence of integrin-mediated signaling when osteoclasts bind to bone matrix (Rani *et al.,* 1997). This pathway also initiates and maintains signals that reorganize the osteoclast cytoskeleton for ruffled border formation, for which Src is essential (Boyce *et al.,* 1992). However, it is not known if Src, which is also activated after integrin binding, is involved in this antiapoptotic mechanism, but it is worth noting that reorganization of the cytoskeleton and contraction of cells without leakage of their contents are major features of apoptosis that prevent the initiation of an acute inflammatory reaction.

The vitronectin receptor, integrin $\alpha_v\beta3$, mediates osteoclast adhesion to bone matrix (Horton, 1997). Because osteoclasts require tight adhesion to bone matrix to resorb effectively, interruption of adhesion could lead to initiation of the death pathway in a manner similar to that reported in other cell types (Frisch and Ruoslahti, 1997). Treatment with antisense oligonucleotides to the α_v gene not only inhibits osteoclast adhesion and bone resorption, it also promotes osteoclast apoptosis that is associated with reduced expression of Bcl-2 (Villanova *et al.,* 1999). Loss of adhesion, inhibition of resorption, and induction of osteoclast apoptosis have also been reported with nonspecific matrix receptor-inhibiting proteins containing RGD sequences (Rani *et al.,* 1997; Rodan and Rodan, 1997). Although the suspension of osteoclasts in culture medium is associated with a twofold increase in their apoptosis (Sakai *et al.,* 2000), loss of adhesion does not inevitably trigger an apoptosis signal. For example, the antivitronectin receptor agents echistatin and $\alpha_v\beta3$ monoclonal antibody do not induce osteoclast apoptosis (Wesolowski *et al.,* 1995; Villanova *et al.,* 1999).

Cytokines and Other Proinflammatory Agents

The production of cytokines and other proinflammatory agents, such as prostaglandins and nitric oxide, is increased in a variety of conditions associated with increased bone resorption, including estrogen deficiency and chronic inflammatory bone diseases. Most of these agents stimulate stromal cell RANKL expression and promote bone resorption indirectly by multiple mechanisms, including prevention of osteoclast apoptosis. IL-1 also appears to have direct effects on osteoclasts to prevent their apoptosis, although it is not clear whether this is mediated by NF-κB (Jimi *et al.,* 1998) or ERK (Miyazaki *et al.,* 2000) activation. Intracellular domains of the receptors for IL-1 and TNF on osteoclasts (similar to RANKL), bind TRAF 6, which leads to activation of NF-κB and prevention of osteoclast apoptosis (Jimi *et al.,* 1998). The Src/PI3-kinase/AKT pathway is activated in osteoclasts in response to IL-1 (Wong *et al.,* 1999), but it has not been determined if it is also involved in TNF prevention of apoptosis. IL-6 mediates its antiapoptotic signaling through gp130, but the mechanism of this effect in osteoclasts remains unknown. The effects of nitric oxide on osteoclasts are controversial and are discussed in Chapter 55.

Even though it can stimulate bone resorption, nitric oxide appears to promote apoptosis of osteoclasts (Kanaoka *et al.,* 2000) and their precursors (van't Hof and Ralston, 1997).

Calcitonin causes osteoclasts to lose attachment to substrates (Kallio *et al.,* 1972) and inhibit their resorptive activity, which can be resumed upon withdrawal of the hormone (Chambers and Moore, 1983). However, it does not induce osteoclasts to die (Selander *et al.,* 1996; Kanaoka *et al.,* 2000). Indeed, calcitonin protects them from nitric oxide-induced apoptosis, an effect that involves protein kinase A and is associated with the inhibition of caspase-3-like protease activity (Kanaoka *et al.,* 2000). Thus, apoptosis is not an obligatory sequel of inhibition of osteoclastic resorption.

Vitamin K_2, but not vitamin K_1 (Kameda *et al.,* 1996), induces osteoclast apoptosis. Vitamin K_2 has a geranylgeranyl side chain and its effect is associated with increased superoxide and peroxide free radical production (Sakagami *et al.,* 2000), but given that osteoclasts produce and respond positively to free radicals (Garrett *et al.,* 1990), the mechanism of action of vitamin K_2 will require further study.

Apoptosis of Osteoblasts and Osteocytes

Osteoblasts with the condensed chromatin and/or nuclear fragmentation that characterizes the late stages of apoptosis have been reported in murine calvariae and rat fracture callus, but such cells are rare (Furtwangler *et al.,* 1985; Landry *et al.,* 1997). Using TUNEL, however, apoptotic osteoblasts were demonstrated in fracture callus (Landry *et al.,* 1997; Olmedo *et al.,* 1999; Olmedo *et al.,* 2000) and in the osteogenic front of developing sutures in murine calvaria (Rice *et al.,* 1999; Opperman *et al.,* 2000).

Unambiguous identification of apoptotic osteoblasts in remodeling cancellous bone is more difficult because of the proximity of the bone surface to the complex cellular architecture of the marrow. Therefore, specific criteria must be used when enumerating apoptotic osteoblasts in cancellous bone. They must be juxtaposed to osteoid and to other osteoblasts because bone formation is carried out by teams of osteoblasts and they should have a cuboidal morphology to distinguish them from nearby marrow cells and lining cells (Figs. 2C–2F). When analyzed with the TUNEL method, 0.5 to 1.0% of osteoblasts in vertebral cancellous bone of adult mice exhibit DNA strand breaks (Weinstein *et al.,* 1998; Jilka *et al.,* 1998; Silvestrini *et al.,* 1998) and 2–10% are labeled using the highly sensitive ISEL procedure (Plotkin *et al.,* 1999; Jilka *et al.,* 1999b). DNA labeling is highly specific, as evidenced by the lack of any staining in the nuclei of nearby osteoblasts. Most of the labeled osteoblast nuclei are round and only a few have evidence of chromatin condensation (Figs. 2C–2F). Thus, they likely represent cells in the early stage of apoptosis. The demonstration of apoptotic osteoblasts supports Parfitt's earlier contention that the majority of these cells die during bone remodeling, with less than 50% of the originally recruited team of osteoblasts surviving as lining cells or osteocytes

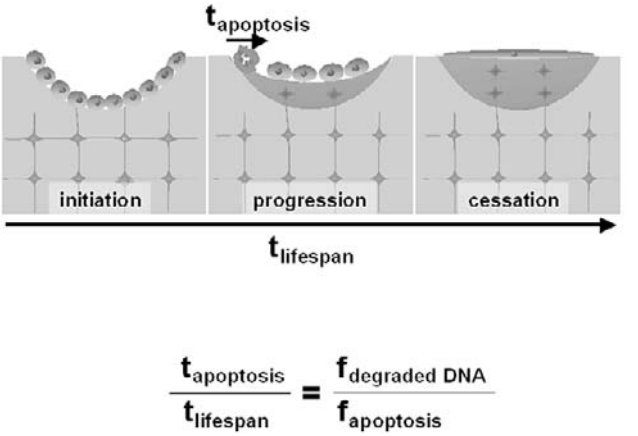

Figure 4 Osteoblast fate and apoptosis. A team of osteoblasts assembles at the floor of the resorption cavity at the beginning of the process ("initiation"). During matrix synthesis ("progression"), some osteoblasts become entombed as osteocytes and some die by apoptosis. When matrix synthesis ceases ("cessation"), all that remain of the original team of osteoblasts are osteocytes and lining cells. The equation shown can be used to calculate the fraction of osteoblasts that die by apoptosis ($f_{apoptosis}$) based on the observed prevalence of osteoblasts exhibiting ISEL labeling ($f_{degraded\ DNA} \sim 0.05$), osteoblast life span [$t_{life\ span}$ = wall width divided by mineral appositional rate = 200–400 hr in vertebral bone of adult mice (Jilka *et al.,* 1999b)], and the number of hours that apoptotic osteoblasts exhibit ISEL labeling, $t_{apoptosis}$. In other cells the latter can be as high as 40–50 hr (Pompeiano *et al.,* 1998), but is likely to be significantly less for osteoblasts because they cannot be recognized after they detach from the extracellular matrix. (See also color plate.)

(Parfitt, 1990) (Fig. 4, see also color plate). Thus, unlike osteoclasts, osteoblasts have three possible fates. Only a small number of osteoblasts exhibit signs of apoptosis because the process is fleeting. Using the equation shown in Fig. 4 (Jilka *et al.,* 1998), it can be shown that the observed prevalence of apoptotic osteoblasts (2–10%) means that 50–80% of them die between the initiation and the completion of bone formation, consistent with Parfitt's estimate (Parfitt, 1990).

Condensed chromatin and degraded DNA (Figs. 2A, 2G, and 2H) have been demonstrated in osteocytes (Noble *et al.,* 1997; Rice *et al.,* 1999; Shibahara *et al.,* 2000; Stevens *et al.,* 2000; Verborgt *et al.,* 2000; Silvestrini *et al.,* 2000). Like osteoblasts, the number of osteocytes undergoing apoptosis is increased by the loss of sex steroids (Tomkinson *et al.,* 1997, 1998; Kousteni *et al.,* 2001), as well as glucocorticoid excess (Weinstein *et al.,* 1998, 2000b) (Figs. 2G and 2H). In distinction to osteoblasts, osteocyte apoptosis represents cumulative death because the cellular debris is not accessible to phagocytic scavenger cells. Indeed, degraded DNA can be detected in osteocyte lacunae of necrotic human bone long after the initial insult of glucocorticoid excess (Weinstein *et al.,* 2000b). The existence of empty osteocyte lacunae has been taken previously as evidence of osteocyte death (Frost, 1960), but in some circumstances this may be an artifact due to loss of the loosely adherent pyknotic cells and cellular debris during processing for histological examination (Wong *et al.,* 1987).

Regulation of Osteoblast and Osteocyte Apoptosis

Activation of death receptors with TNF or CD95 ligand stimulates osteoblast apoptosis *in vitro* (Kitajima *et al.,* 1996; Jilka *et al.,* 1998; Tsuboi *et al.,* 1999; Urayama *et al.,* 2000). Although CD95 and its ligand are expressed by osteoblasts and osteocytes *in vivo* (Hatakeyama *et al.,* 2000), their role in physiologic apoptosis of these cells is unknown.

INTEGRINS

Intracellular antiapoptosis signals are generated in osteoblasts upon integrin binding to extracellular matrix, as evidenced by the induction of apoptosis when binding to fibronectin or collagen is prevented *in vitro* (Globus *et al.,* 1998; Jilka *et al.,* 1999a). A periodic loss of integrin signaling may occur *in vivo* due to dynamic alterations in integrin adhesion migration and matrix assembly as osteoblasts rise above the cement line, as during bone formation (Fig. 4). Thus, it is tempting to suggest that the very process of bone formation is responsible for the induction of apoptosis (Jilka *et al.,* 1999a).

GROWTH FACTORS AND CYTOKINES

Most growth factors and cytokines produced in the bone microenvironment inhibit osteoblast apoptosis, including IGFs (Hill *et al.,* 1997), TGFβ, and IL-6- type cytokines (Jilka *et al.,* 1998). Interestingly, FGF inhibits apoptosis in primary cultures of dividing preosteoblastic cells (Hill *et al.,* 1997), but stimulates the apoptosis of mature osteoblasts (Mansukhani *et al.,* 2000), suggesting differentiation- or cell cycle-dependent effects of this factor. Activation of CD40 by its ligand CD154 (expressed by monocytes and T cells) also inhibits the apoptosis of osteoblasts and osteocytes (Ahuja *et al.,* 1999).

The antiapoptotic effect of IL-6 type cytokines on osteoblastic cells requires cyclin kinase inhibitor $p21^{WAF1,CIP1,SDI1}$, the synthesis of which is stimulated by STAT phosphorylation (Bellido *et al.,* 1998). Although not yet demonstrated in osteoblastic cells, the antiapoptotic effects of IGFs and FGF in other cells involve PI3-kinase and Akt–mediated phosphorylation of Bad (Lizcano *et al.,* 2000; Zhou *et al.,* 2000). IGFs also stimulate the synthesis of $p21^{WAF1,CIP1,SDI1}$ and Bcl-2 (Pugazhenthi *et al.,* 1999; Martelli *et al.,* 2000). Finally, IGFs and FGF may upregulate calbindin-D_{28k} (Wernyj *et al.,* 1999), which binds to and inhibits caspase-3 and blocks TNF-induced apoptosis in osteoblastic cells (Bellido *et al.,* 2000).

MATRIX METALLOPROTEINASES

Increased osteocyte and osteoblast apoptosis was observed in mice bearing a targeted mutation of the Col1a1 gene ($col1A1^{r/r}$ mice) that made collagen resistant to proteolytic attack by MMPs (Zhao *et al.,* 2000). This finding suggests that tonic MMP-mediated pericellular degradation of type I collagen provides survival signals to osteoblasts and osteocytes, perhaps via exposure of cryptic integrin-binding sites following cleavage of collagen (Messent *et al.,* 1998) or release of growth factors associated with the collagenous matrix.

Because of their location, osteocytes and their canalicular system provide the most likely means by which the skeleton detects sustained changes in mechanical forces. Perception of these changes in turn leads to adaptive changes in bone strength. Likewise, osteocytes likely perceive fatigue-induced bone microdamage (Parfitt *et al.,* 1996; Parfitt, 1996), and their apoptosis might provide site-specific signals its repair (Verborgt *et al.,* 2000). Induction of microcracks in rat ulnae by fatigue loading induced apoptosis of osteocytes adjacent to microcracks, but not in distant osteocytes. More importantly, resorption of the affected sites followed. Very high strains also increased osteocyte apoptosis in rat ulnae (Noble *et al.,* 1998).

Regulation of Bone Cell Apoptosis by Sex Steroids

Loss of estrogens leads to an increased rate of remodeling. This alone accounts for the initial decrease in bone mineral density due to expansion of the remodeling space, but it cannot explain the imbalance between formation and resorption that leads to progressive bone loss. A potential explanation for this imbalance is that estrogen promotes osteoclast apoptosis (Hughes *et al.,* 1996; Kameda *et al.,* 1997), but prevents apoptosis of osteoblasts (Kousteni *et al.,* 2001). Loss of these complementary actions in estrogen deficiency could account for the deeper than normal erosion cavities created by osteoclasts (Parfitt *et al.,* 1996; Eriksen *et al.,* 1999) and the reduction in wall thickness. Moreover, estrogen deficiency increases the prevalence of osteocyte apoptosis (Tomkinson *et al.,* 1997, 1998; Kousteni *et al.,* 2001), which might impair the ability of the osteocyte/canalicular mechanosensory network to repair microdamage, thus contributing further to bone fragility.

Estrogen promotes the apoptosis of osteoclasts (Hughes *et al.,* 1996) and their precursors (Shevde and Pike, 1996; Hughes *et al.,* 1996) in mixed cell cultures, as well as in cultures of isolated osteoclasts (Kameda *et al.,* 1997; Bellido *et al.,* 1999) or preosteoclastic cells, in some (Zecchi-Orlandini *et al.,* 1999; Sunyer *et al.,* 1999), but not all studies (Arnett *et al.,* 1996). The proapoptotic effect is associated with a reduced expression of IL-1R1 mRNA, and increased IL-1 decoy receptor expression (Sunyer *et al.,* 1999). However, in murine bone marrow cocultures, the proapoptotic effect of 17β-estradiol, as well as tamoxifen, seems to be mediated by TGFβ (Hughes *et al.,* 1996). Increased TGFβ, whether produced by stromal cells (Oursler *et al.,* 1991) or B lymphocytes (Weitzmann *et al.,* 2000), can directly stimulate the apoptosis of osteoclasts and osteoclast progenitors. It may also act indirectly via the stimulation of OPG synthesis (Takai *et al.,* 1998, Murakami *et al.,* 1998), which would reduce RANKL-mediated antiapoptotic signaling. The effects of TGFβ on osteoclasts,

however, are not straightforward, and further study is required to determine its role in normal and disease states. For example, in the absence of stromal cells, TGFβ (Fuller *et al.,* 2000b), activin (Fuller *et al.,* 2000a), and BMP-2 (Koide *et al.,* 1999) act directly on osteoclast precursors to promote their differentiation, and transgenic mice overexpressing TGFβ have an osteoporotic phenotype (Erlebacher and Derynck, 1996).

Testosterone, like estrogen, indirectly promotes osteoclast apoptosis *in vitro* and *in vivo* (Hughes *et al.,* 1995a). Its effect is also prevented by the anti-TGFβ antibody, but the proapoptotic effect of testosterone is observed approximately 4 hr later than that of estrogen (Dai and Boyce, 1997). In these cultures, testosterone is converted to estradiol within 4 hr, which presumably then promotes the formation of TGFβ, thus accounting for the delay. Dihydrotestosterone, which cannot be converted to estrogen, did not promote osteoclast apoptosis in mixed cultures (B. F. Boyce, unpublished observations); however, it did in osteoclast cultures devoid of stromal/osteoblastic cells (S. C. Manolagas *et al.,* unpublished observations).

The increase in osteoblast and osteocyte apoptosis following loss of sex steroids is due to loss of survival signals induced directly by estrogens and androgens via a newly discovered nongenotropic activity of the estrogen receptor (ER) and AR (Kousteni *et al.,* 2001). Upon activation with ligand, ER and AR stimulate a Src/Shc/ERK signaling pathway that prevents apoptosis induced by TNF, dexamethasone, etoposide, or anoikis. Strikingly, either estrogens or androgens activate the antiapoptotic activity of both ER and AR with similar efficiency. This activity could be eliminated by nuclear, but not by membrane, targeting of the ER. More important, it can be dissociated from its transcriptional activity with peptide antagonists of ER activity and by synthetic ER ligands.

Induction of Bone Cell Apoptosis by Glucocorticoids

High dose glucocorticoid treatment causes rapid bone loss via transiently increased resorption and reduced osteoblast number and bone formation rate (Dempster, 1989; Weinstein *et al.,* 1998). The rapid bone loss is due to increased osteoclast activity and/or life span, which may be caused by suppression of OPG synthesis and increase in RANKL (Hofbauer *et al.,* 1999). Increased osteoclastogenesis, however, is not involved because bone marrow of glucocorticoid-treated mice exhibits a decline in osteoclast progenitors even after brief glucocorticoid exposure (Weinstein *et al.,* 2000a). In rats, glucocorticoids increase bone mass, in contrast to humans and mice. This atypical situation has been attributed to the glucocorticoid-induced apoptosis of rat osteoclasts (Dempster *et al.,* 1997).

The decrease in osteoblast number and bone formation rate in glucocorticoid excess may be explained in part by the increased prevalence of osteoblast apoptosis observed in murine vertebral bone and human iliac bone (Weinstein *et al.,* 1998; Gohel *et al.,* 1999) (Figs. 2E and 2F). A decrease in osteoblast progenitors may also contribute (Weinstein *et al.,* 1998). Increased osteocyte apoptosis has also been documented in bone of mice and humans receiving glucocorticoids (Figs. 2A, 2G, and 2H). In fact, osteocytes with condensed chromatin have been observed in between the tetracycline labeling that demarcates sites of bone formation, indicating that these cells died immediately after entombment in the bone matrix (Fig. 2A).

Glucocorticoid-induced osteoporosis is often complicated by the *in situ* death of portions of bone, a process called osteonecrosis (Mankin, 1992). Studies of femoral heads from patients with glucocorticoid excess, but not with alcoholic, traumatic, or sickle cell osteonecrosis, revealed abundant apoptotic osteocytes and cells lining cancellous bone juxtaposed to the subchondral fracture crescent—a ribbon-like zone of collapsed trabeculae (Weinstein *et al.,* 2000b). In these five patients, signs of marrow inflammation and necrosis, such as hyperemia, round cell infiltration, or lipid cyst formation, were absent in contrast to the high frequency of these features in patients with sickle cell disease and femoral osteonecrosis. Therefore, in femoral head osteonecrosis due exclusively to glucocorticoid excess, marrow and bone necrosis are not inextricably linked to collapse of the joint. Thus, glucocorticoid-induced "osteonecrosis" may actually be osteocyte apoptosis, a cumulative and unrepairable defect that would disrupt the mechanosensory osteocyte–canalicular network. This situation would promote collapse of the femoral head and explain the correlation between total steroid dose and the incidence of avascular necrosis of bone (Felson and Anderson, 1987), as well as the occurrence of osteonecrosis after cessation of steroid therapy.

Glucocorticoids induce apoptosis of murine and rat calvarial cells and murine MLOY4 osteocyte-like cells (Plotkin *et al.,* 1999; Gohel *et al.,* 1999) via the glucocorticoid receptor. This effect was prevented by the receptor antagonist RU486, as well as overexpression of 11β-hydroxysteroid dehydrogenase type 2, which inactivates glucocorticoids (O'Brien *et al.,* 2000), suggesting that it is due to direct actions on osteoblasts/osteocytes rather than indirect actions of the steroid on the gastrointestinal tract, kidneys, parathyroid glands, or gonads. The molecular mechanism of glucocorticoid-induced apoptosis of osteoblasts and osteocytes is unknown. It may involve the suppression of survival factors such as IGFs (Cheng *et al.,* 1998), IL-6 type cytokines (Tobler *et al.,* 1992), integrins, and MMPs (Meikle *et al.,* 1992; Partridge *et al.,* 1996).

Regulation of Osteoblast and Osteocyte Apoptosis by PTH

Intermittent administration of PTH increases bone mass due to an increase in osteoblast number and bone formation rate (Dempster *et al.,* 1993). The precise mechanism is unclear but possibilities include increased osteoblast

precursor proliferation, increased osteoblast life span, and reactivation of lining cells. Studies in mice have revealed that it may be due to an approximately 10-fold reduction in osteoblast, as well as osteocyte, apoptosis (Jilka *et al.,* 1999b). Increased bone formation and osteoblast apoptosis suppression were also noted in mice expressing a constitutively active PTH/PTHrP receptor in osteoblastic cells (Calvi *et al.,* 2001). The ability to inhibit apoptosis provides a rational explanation for the efficacy of intermittent PTH administration in the prevention of glucocorticoid-induced bone loss and increased fragility (Lane *et al.,* 1998).

In vitro studies indicate that the antiapoptotic effect of PTH is due to G_s-activated cAMP production, and protein kinase A-mediated Bad phosphorylation (Jilka, unpublished observations). Although the PTH/PTHrP receptor delivers proapoptotic signals when coupled to G_q in human embryonic kidney cells (Turner *et al.,* 1998), PTH fails to activate G_q in osteoblastic cells expressing both G_s and G_q (Schwindinger *et al.,* 1998). The anabolic effects of other cAMP-inducing agents, such as PGE, may likewise be due to decreased osteoblast apoptosis (Jee and Ma, 1997; Machwate *et al.,* 1998a, 1999). Indeed, cell-permeable analogs of cAMP, as well as agents that stimulate cAMP production such as PGE and calcitonin (CT), inhibit the apoptosis of cultured osteoblastic and osteocytic cells (Machwate *et al.,* 1998b; Plotkin *et al.,* 1999). However, unlike PTH, PGE-stimulated cAMP activates sphingosine kinase to suppress apoptosis in periosteal osteoblastic cells (Machwate *et al.,* 1998b). The CT-mediated suppression of MLOY4 osteocytic cells involves ERK activation, most likely via a cAMP-activated pathway as reported in other cells (Gutkind, 1998). Besides activation of intracellular antiapoptotic pathways, PTH may exert its antiapoptotic effects via stimulation of synthesis of MMPs and/or IGFs that exert their own survival effects (Meikle *et al.,* 1992; Watson *et al.,* 1995; Pfeilschifter *et al.,* 1995; Partridge *et al.,* 1996; Hill *et al.,* 1997). Indeed, IGF-1 is required for the anabolic effect of PTH (Bikle *et al.,* 2000; Miyakoshi *et al.,* 2000).

A PTH receptor (CPTHR) that recognizes a sequence in the C-terminal portion of PTH(1–84) has been shown to stimulate the apoptosis of osteocytic cells (Divieti *et al.,* 2001). Because of the high level of circulating carboxy-terminal fragments of PTH in renal osteodystrophy, activation of this receptor could be responsible for the loss of osteocyte viability seen in this condition (Bonucci and Gherardi, 1977).

Effects of Bisphosphonates on Bone Cell Apoptosis

Bisphosphonates have been used since the mid-1970s to inhibit pathologic bone resorption. It has been shown that the induction of apoptosis, rather than death by necrosis, is the characteristic morphologic effect of bisphosphonates on osteoclasts *in vitro* and *in vivo* (Hughes *et al.,* 1995b), which could account for much of their inhibitory action. The number of osteoclasts with morphologic features of apoptosis seen in bone sections of mice treated in short-term experiments with bisphosphonates are high (up to 26% of osteoclasts) (Hughes *et al.,* 1995b). In most circumstances, macrophages or other adjacent cells remove apoptotic cells rapidly. The high number of apoptotic and nonapoptotic osteoclasts seen in these experiments may reflect bisphosphonate-induced impairment of macrophage phagocytic function and short-term stimulation of osteoclast formation (Fisher *et al.,* 2000).

The proapoptotic effect of aminobisphosphonates on osteoclasts is due to inhibition of the function of enzymes that mediate cholesterol synthesis in the mevalonic acid pathway (Luckman *et al.,* 1998; Coxon *et al.,* 2000). A final step in this pathway is prenylation of small proteins, such as ras and rho, which are involved in cell survival. In contrast, nonaminobisphosphonates, such as clodronate and etidronate, are metabolized to toxic ATP-like molecules whose precise mechanism of induction of osteoclast apoptosis remains unclear. These studies and the description of other possible mechanism of action of bisphosphonates are described in detail in Chapter 78. However, it is worth noting that statins also inhibit enzymes in the mevalonate pathway, and not only induce osteoclast apoptosis (Coxon *et al.,* 1998), but also prevent glucocorticoid-induced osteonecrosis (Cui *et al.,* 1997), presumably by a mechanism similar to that of bisphosphonates. The concentrations of these drugs that induce osteoclast apoptosis are higher than those shown to stimulate osteoblasts *in vitro* and increase bone formation *in vivo* (Mundy *et al.,* 1999).

Bisphosphonates may do more than kill osteoclasts and prevent further bone erosion. They may also possess anabolic activity as evidenced by an increase in wall thickness after long-term treatment (Storm *et al.,* 1993; Balena *et al.,* 1993; Chavassieux *et al.,* 1997). In addition, they appear to decrease fracture incidence disproportional to their effect on bone mass, suggesting another effect on bone strength unrelated to effects on bone resorption or formation (Weinstein, 2000). Bisphosphonates such as alendronate are an effective therapy for glucocorticoid-induced osteoporosis (Reid, 1997; Gonnelli *et al.,* 1997), an effect that may be due in part to the prevention of increased apoptosis of osteoblasts and osteocytes (Plotkin *et al.,* 1999). Thus, part of the antifracture efficacy of bisphosphonates may be due to the inhibition of osteocyte apoptosis via preservation of the integrity of the canalicular mechanosensory network.

The antiapoptotic activity of bisphosphonates is exerted at concentrations 3–4 orders of magnitude lower than required for stimulating osteoclast apoptosis (Hughes *et al.,* 1995b; Plotkin *et al.,* 1999). Moreover, a bisphosphonate that lacks antiresorptive activity (IG9402) (Van Beek *et al.,* 1996; Brown *et al.,* 1998) also suppresses osteocyte/osteoblast apoptosis (Plotkin *et al.,* 1999), indicating that bisphosphonates modulate different apoptosis regulating pathways in osteoblasts/osteocytes and osteoclasts. Indeed, like sex steroids, the antiapoptotic effect of bisphosphonates is trig-

gered by the stimulation of ERK phosphorylation (Plotkin *et al.,* 1999). Importantly, activation of this pathway appears to be mediated by the opening of connexin-43 hemichannels in the plasma membrane (Plotkin *et al.,* 2000).

Summary

Apoptosis of chondrocytes, osteoclasts, osteoblasts, and osteocytes plays a critical role in the development and maintenance of the skeleton. Alterations in bone cell life span contribute to the pathogenesis of limb development and growth plate defects, erosive joint disease, and the bone loss that results from sex steroid deficiency and glucocorticoid excess. Moreover, the ability of PTH to influence osteoblast life span may account for at least part of its anabolic effect on the skeleton—an action that may also extend to bisphosphonates. Further work is needed to identify the factors controlling bone cell survival and death during bone remodeling. It may then be possible to develop pharmacologic agents to modulate apoptosis so as to preserve, or even enhance, bone mass and strength.

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CHAPTER 11

Involvement of Nuclear Architecture in Regulating Gene Expression in Bone Cells

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Introduction

Skeletal development and bone remodeling require stringent control of gene activation and suppression in response to physiological cues. The fidelity of skeletal gene expression necessitates integrating a broad spectrum of regulatory signals that govern the commitment of osteoprogenitor stem cells to the bone cell lineage and proliferation and differentiation of osteoblasts, as well as maintenance of the bone phenotype in osteocytes residing in a mineralized bone extracellular matrix. To accommodate the requirements for short-term developmental and sustained phenotypic expression of cell growth and bone-related genes, it is necessary to identify and functionally characterize the promoter regulatory elements as well as cognate protein/DNA and protein/protein interactions that determine the extent to which genes are transcribed. However, it is becoming increasingly evident that the catalogue of regulatory elements and proteins is insufficient to support transcriptional control in the nucleus of intact cells and tissues. Rather, gene regulatory mechanisms must be understood within the context of the subnuclear organization of nucleic acids and regulatory proteins.

There is growing appreciation that transcriptional control, as it is operative *in vivo,* requires multiple levels of nuclear organization (Figs. 1 and 2). It is essential to package 2.5 yards of DNA as chromatin within the limited confines of the nucleus (Fig. 1, see also color plate). Gene promoter elements must be rendered competent for protein/DNA and protein/protein interactions in a manner that permits binding and functional activities of primary transcription factors as well as coactivators and corepressors. Less understood but pivotally relevant to physiological control is the localization of the regulatory machinery for gene expression and replication at subcellular sites where the macromolecular complexes that support DNA and RNA synthesis are localized (Fig. 2, see also color plate).

This chapter focuses on contributions by several indices of nuclear architecture to the control of gene expression in bone cells. This chapter presents cellular, biochemical, molecular, and genetic evidence for linkages of developmental and tissue-specific gene expression with the organization of transcriptional regulatory machinery in subnuclear compartments. Using the promoter of the bone-specific osteocalcin gene and the skeletal-specific core binding factor alpha (CBFA; also known as runt-related transcription factor, Runx) transcription factor as paradigms, this chapter addresses mechanisms that functionally organize the regulatory machinery for transcriptional activation and suppression during skeletal development and remodeling. It also provides evidence for consequences that result from perturbations in nuclear structure: gene expression interrelationships in skeletal disease.

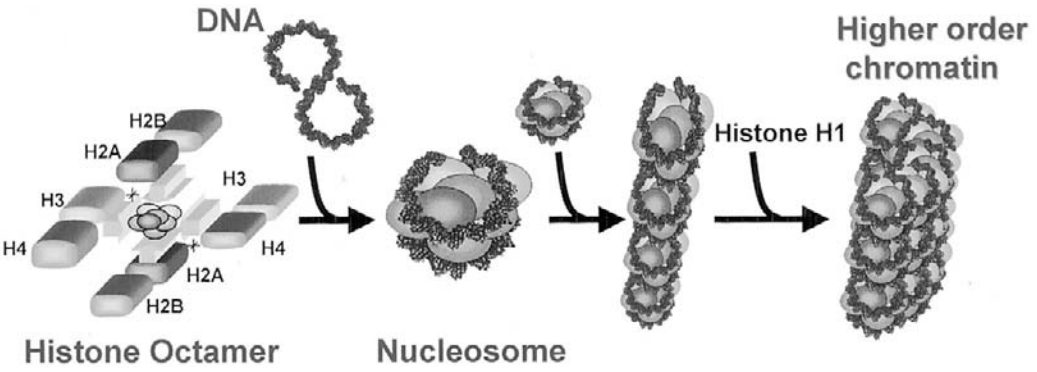

Figure 1 Levels of chromatin organization. DNA (blue coil) is packaged by histone octamers (ovals in gold tones) to form nucleosomes. Histone octamers form by the assembly of two histone H3 and two histone H4 proteins to organize a central H3/H4 tetramer, while two heterodimers of histone H2A and H2B interact with, respectively, the top and bottom of the tetramer. Multiple nucleosomes form a beads on a string structure (10 nm fiber) and, in the presence of histone H1, nucleosomes will form higher order chromatin structures (30 nm fiber) presumably by interdigitation and/or coiling of nucleosomal DNA. (See also color plate.)

Gene Expression within the Three-Dimensional Context of Nuclear Architecture: Multiple Levels of Nuclear Organization Support Fidelity of Gene Regulation

While the mechanisms that control gene expression remain to be formally defined, there is growing awareness that the fidelity of gene regulation necessitates the coordination of transcription factor metabolism and the spatial organization of genes and regulatory proteins within the three-dimensional context of nuclear architecture. The parameters of nuclear architecture include the sequence of gene regulatory elements, chromatin structure, and higher order organization of the transcriptional regulatory machinery into subnuclear domains. All of these parameters involve mechanisms that include transcription factor synthesis, nuclear import and retention, posttranslational modifications of factors, and directing factors to subnuclear sites that support gene expression (Figs. 2 and 3). Remodeling of chromatin and nucleosome organization to accommodate requirements for protein–DNA and protein–protein interactions at promoter elements are essential modifications for both activation of genes and physiological control of transcription. The reconfiguration of gene promoters and assembly of specialized subnuclear domains reflect the orchestration of both regulated and regulatory mechanisms (Fig. 4, see also color plate). There are analogous and complex regulatory requirements for processing of gene transcripts. Here it has been similarly demonstrated that the regulatory components of splicing and export of messenger RNA to the cytoplasm are dependent on the architectural organization of nucleic acids and regulatory proteins.

From a biological perspective, each parameter of factor metabolism requires stringent control and must be linked to structure–function interrelationships that mediate transcription and processing of gene transcripts. However, rather than representing regulatory obstacles, the complexities of nuclear biochemistry and morphology provide the required specificity for physiological responsiveness to a broad spectrum of signaling pathways to modulate transcription under diverse circumstances. Equally important, evidence is accruing that modifications in nuclear architecture and nuclear structure–function interrelationships accompany and appear to be causally related to compromised gene expression under pathological conditions.

Multiple levels of genomic organization that contribute to transcription are illustrated schematically in Fig. 4. Additional levels of nuclear organization are reflected by the subnuclear localization of factors that mediate transcription, processing of gene transcripts, DNA replication, and DNA repair at discrete domains (Fig. 2).

Sequence Organization: A Blueprint for Responsiveness to Regulatory Cues

Appreciation is accruing for the high density of information in both regulatory and mRNA-coding sequences of cell growth and phenotypic genes. The modular organizations of promoter elements provide blueprints for responsiveness to a broad spectrum of regulatory cues that support competency for transient developmental and homeostatic control as well as sustained commitments to tissue-specific gene expression. Overlapping recognition elements expand the options for responsiveness to signaling cascades that mediate mutually exclusive protein/DNA and protein/protein interactions. Splice variants for gene transcripts further enhance the specificity of gene expression. However, it must be acknowledged that the linear order of genes and flanking regulatory elements is necessary but insufficient to support expression in a biological context. There is a requirement to integrate the regulatory information at independent promoter elements and selectively utilize subsets of promoter regulatory information to control the extent to which genes are activated and/or suppressed.

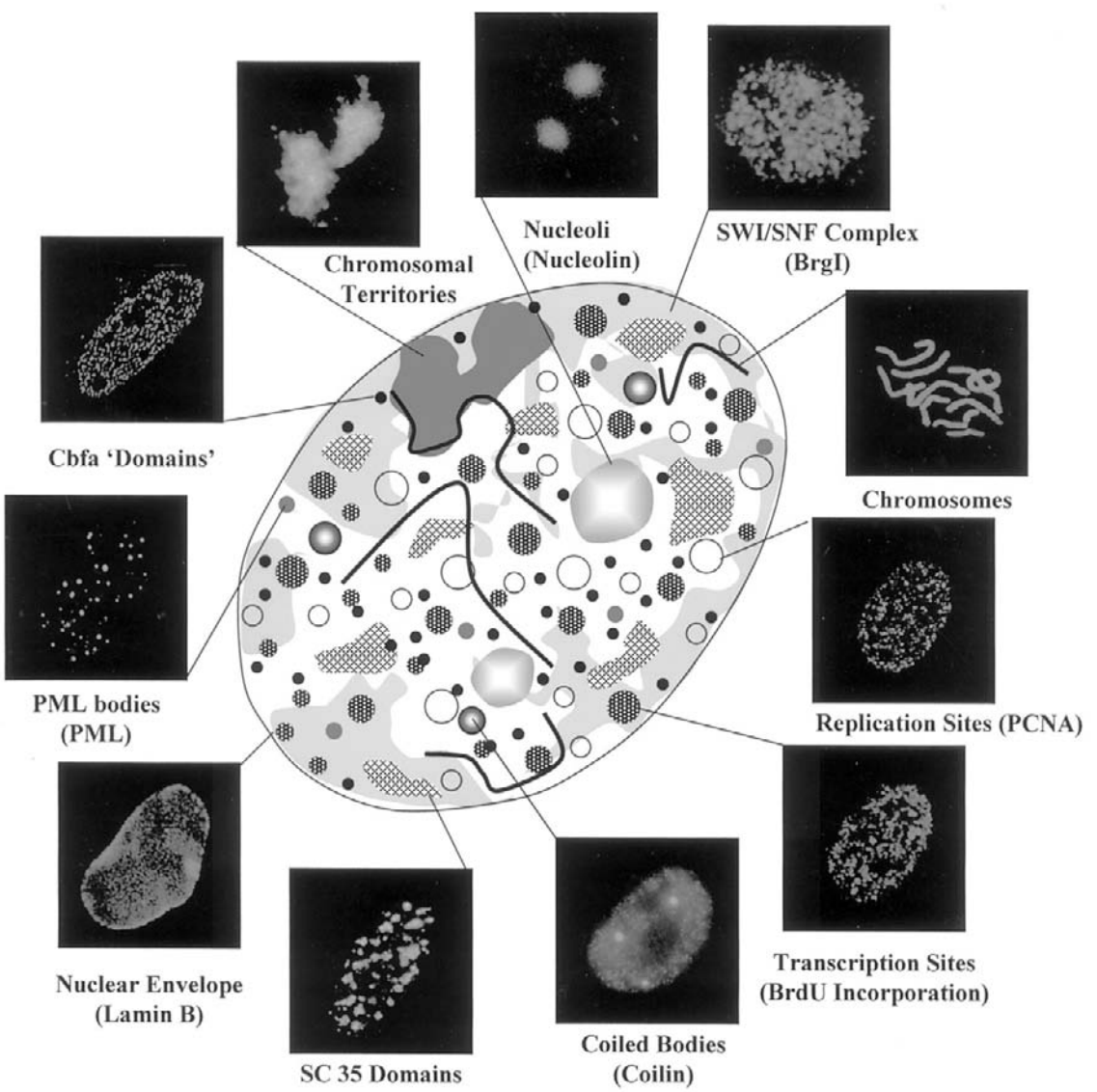

Figure 2 Subnuclear compartmentalization of nucleic acids and regulatory proteins into specialized domains. Nuclear functions are organized into distinct, nonoverlapping subnuclear domains (illustrated schematically in the center). Immunofluorescence microscopy of the nucleus *in situ* has revealed the distinct subnuclear distribution of vital nuclear processes, including (but not limited to) DNA replication sites (Ma *et al.,* 1998); chromatin remodeling, e.g., mediated by the SWI/SNF complex (Reyes *et al.,* 1997) and Cbfa factors (Javed *et al.,* 1999; Prince *et al.,* 2001; Zeng *et al.,* 1998); structural parameters of the nucleus, such as the nuclear envelope, chromosomes, and chromosomal territories (Ma *et al.,* 1999); Cbfa domains for transcriptional control of tissue-specific genes; and RNA synthesis and processing involving, for example, transcription sites (Wei *et al.,* 1999), SC35 domains (reviewed in Shopland and Lawrence, 2000), coiled bodies (Platani *et al.,* 2000), and nucleoli (Dundr *et al.,* 2000). Subnuclear PML bodies of unknown function (McNeil *et al.,* 2000) have been examined in numerous cell types. All of these domains are associated with the nuclear matrix. Each panel is reproduced with permission from the journal and authors of the indicated references. (See also color plate.)

Chromatin Organization: Packaging Genomic DNA in a Manner That Controls Access to Genetic Information

Chromatin structure and nucleosome organization provide architectural linkages between gene organization and components of transcriptional control (Fig. 1). During the past three decades, biochemical and structural analyses have defined the dimensions and conformational properties of the nucleosome, the primary unit of chromatin structure. Each nucleosome consists of approximately 200bp of DNA wrapped in two turns around an octameric protein core containing two copies each of histones H2A, H2B, H3, and H4. A fifth histone, the linker histone H1, binds to the nucleosome and promotes the organization of nucleosomes into a higher order structure, the 30-nm fiber. Nucleosomal organization reduces distances between promoter elements, thereby supporting interactions between the modular components of transcriptional control. The higher order chromatin structure further reduces nucleotide distances

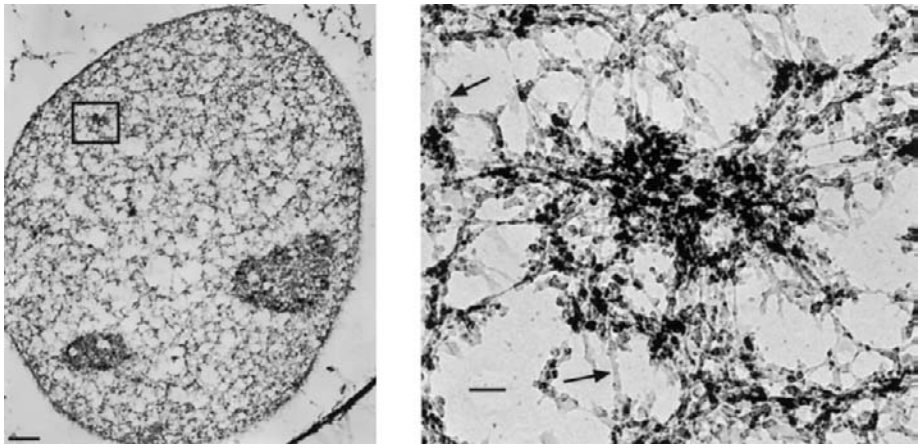

Figure 3 Electron micrograph of the nuclear matrix. A resinless preparation of the cell reveals the nuclear matrix subnuclear domain. (Left) Nuclear matrix intermediary filaments distributed throughout the nucleus. The two denser areas represent the nucleoli. Bar: 1 μm. (Right) The boxed area is enlarged. A higher magnification of the nuclear matrix reveals an interchromatin granule cluster where the processing of RNA transcripts occurs, as well as the anastomosing network of fibers and filaments that support structural and regulatory activities within the nucleus. Arrows point to 10-nm filaments, which may provide an underlying structure of the nuclear matrix. Bar: 100 nm. Reproduced in part from Nickerson (2001) with permission.

between regulatory sequences. Folding of nucleosome arrays into solenoid-type structures provides a potential for interactions that support synergism between promoter elements and responsiveness to multiple signaling pathways.

It has been well established that the presence of nucleosomes generally blocks the accessibility of transcription factors to their cognate-binding sequences (Workman and Kingston, 1998). Extensive analyses of chromatin structure have indicated that the most active genes exhibit increased nuclease sensitivity at promoter and enhancer elements. These domains generally reflect alterations in classical nucleosomal organization and binding of specific nuclear factors. Thus, nuclease digestion has been widely used to probe structures *in vivo* and *in vitro* based on the premise that the chromatin accessibility to nuclease digestion reflects chromatin access to nuclear regulatory molecules. Chromatin immunoprecipitation assays that utilize antibodies to acetylated histones or transcription factors provide the basis for defining the presence of modified histones in chromatin complexes at single nucleotide resolution within gene regulatory elements.

Changes in chromatin organization have been documented under many biological conditions where modifications of gene expression are necessary for the execution of physiological control. Transient changes in chromatin structure accompany and are linked functionally to developmental and homeostatic-related control of gene expression. Long-term changes occur when the commitment to phenotype-specific gene expression occurs with differentiation. However, superimposed on the remodeling of chromatin structure and nucleosome organization that renders genes transcriptionally active are additional alterations in the packaging of DNA as chromatin to support steriod hormone responsive enhancement or dampening of transcription.

During the past several years there have been major advances in the ability to experimentally address the molecular mechanisms that mediate chromatin remodeling. A family of proteins and protein complexes have been described in yeast and in mammalian cells (Cote *et al.,* 1994; Imbalzano, 1998; Kwon *et al.,* 1994; Peterson *et al.,* 1998; Vignali *et al.,* 2000) that promote transcription by altering chromatin structure (Vignali *et al.,* 2000). These alterations render DNA sequences containing regulatory elements accessible for binding cognate transcription factors and mediate protein–protein interactions that influence the structural and functional properties of chromatin. Although the mechanisms by which these complexes function remain to be formally defined, there is general agreement that the increase in DNA sequence accessibility does not require the removal of histones (Cote *et al.,* 1998; Lorch *et al.,* 1998; Schnitzler *et al.,* 1998). Rather, multiple lines of evidence suggest that remodeling of the nucleosomal structure involves alterations in histone–DNA and/or histone–histone interactions.

All chromatin remodeling complexes that have to date been reported include a subunit containing ATPase activity (Cairns *et al.,* 1994, 1996; Cote *et al.,* 1994; Dingwall *et al.,* 1995; Ito *et al.,* 1997; Kwon *et al.,* 1994; LeRoy *et al.,* 1998; Randazzo *et al.,* 1994; Tsukiyama *et al.,* 1994, 1999; Tsukiyama and Wu, 1995; Varga-Weisz *et al.,* 1997; Wade *et al.,* 1998; Wang *et al.,* 1996a, b; Xue *et al.,* 1998; Zhang *et al.,* 1998) (Table I) and have been shown to be critical for modifying nucleosomal organization. Because these subunits share significant homology, it has been suggested that they belong to a new family of proteins with a function that has been highly conserved throughout evolution (Vignali *et al.,* 2000).

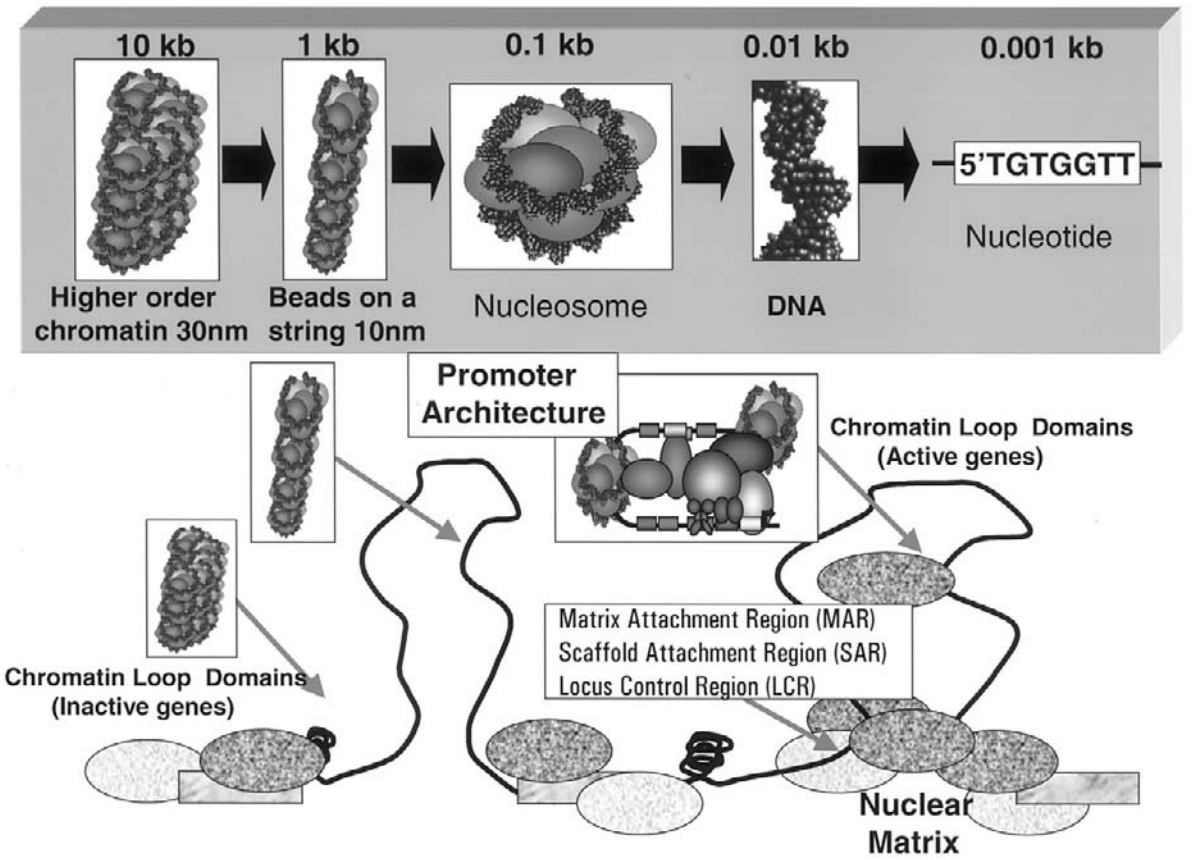

Figure 4 Chromatin organization within the nucleus. (Top) Different levels of DNA organization at different lengths of scale [10 kb to single basepair resolution (0.001 kb)]. The *in situ* organization of DNA within the nucleus involves formation of chromatin loop domains with various degrees of chromatin packaging, including highly condensed chromatin (inactive genes) and partially extended loops near transcriptionally active genes (lower). Anchorage of these loop domains to dynamic architectural complexes (referred to as the nuclear matrix) maintains chromatin in distinct topological states, which accommodate the independent regulation of genes in different chromosomal regions. Distinct conformations of chromatin and posttranslational modifications of histones located in gene promoters support a three-dimensional organization (promoter architecture) that permits specific regulatory protein/protein interactions between distal and proximal regions. (See also color plate.)

Posttranslational modifications of histones have also been implicated in the physiological control of chromatin structure for the past three decades. However, recent findings have functionally linked histone acetylation with changes in nucleosomal structure that alter the accessibility to specific regulatory elements (Workman and Kingston, 1998). For example, acetylation of the N termini of nucleosomal histones has been directly correlated with transcriptional activation. Moreover, it has been observed that core histone hyperacetylation enhances the binding of most transcription factors to nucleosomes (Ura *et al.,* 1997; Vettese-Dadey *et al.,* 1994, 1996). Nevertheless, there have been reports that chromatin hyperacetylation blocks steroid hormone transcriptional enhancement and steriod-dependent nucleosomal alterations (Bresnick *et al.,* 1990, 1991; Montecino *et al.,* 1999). Within this context, it has been shown that hyperacetylation of nuclear proteins alters the chromatin organization of the bone tissue

Table I Nucleosome Remodeling Complexes

Complex	Subunit with ATPase activity	Origin
SWI/SNF	SWI2/SNF2	Yeast
SWI/SNF	BRG 1 BRM	Human
BRAHMA	BRM	*Drosophila*
RSC	STHI	Yeast
NURF	ISWI	*Drosophila*
ACF	ISWI	*Drosophila*
CHRAC	ISWI	*Drosophila*
RSF	hSNF2h	Human
yISWI	yISWI1 yISWI2	Yeast
hNURD	hCHD3 hCHD4	Human
Mi-2	xCHD3/4	*Xenopus*

specific osteocalcin gene promoter in a manner that prevents vitamin D-mediated transcriptional upregulation. By combining nuclease accessibility, indirect end labeling, and ligation-mediated polymerase chain reaction (PCR) analysis, it was demonstrated that protein–DNA interactions that promote formation of a distal DNase I hypersensitive site do not occur under conditions of hyperacetylation (Montecino *et al.,* 1999).

A major breakthrough in addressing the physiological role of histone acetylation experimentally came with the purification and subsequent cloning of the catalytic subunits of yeast and mammalian nuclear histone acetyltransferases (HAT) (Table II). Gcn5 encodes a 55-kDa protein in yeast that acetylates both histones H3 and H4 (Brownell *et al.,* 1996). It has been suggested that Gcn5 is part of a larger protein complex designated SAGA (Grant *et al.,* 1997) that includes proteins that are also present in complexes involved in transcriptional regulation (Workman and Kingston, 1998). Other proteins that contain nuclear HAT activity are p300 and its related homologue CBP (Ogryzko *et al.,* 1996). These two proteins function as transcriptional adaptors that interact with several transcription factors, including CREB, Jun, Fos, Myb, and Myo D, as well as with nuclear steroid hormone receptors (Arany *et al.,* 1994; Arias *et al.,* 1994; Bannister *et al.,* 1995; Chakravarti *et al.,* 1996; Chrivia *et al.,* 1993; Dai *et al.,* 1996; Kamei *et al.,* 1996; Kwok *et al.,* 1994; Oelgeschlager *et al.,* 1996; Yuan *et al.,* 1996). In addition, human and yeast TAFII250 have HAT activity (Mizzen *et al.,* 1996). TAFII250 is part of the TFIID complex that recognizes the TATA sequence at the promoter region of most genes and initiates the formation of transcription preinitiation complexes. The presence of HAT activity in this complex suggests that histone acetylation may be a requirement for transcription factor interaction with nucleosomal DNA.

P/CAF, another protein that contains HAT activity, is highly homologous to both the yeast Gcn5 and the human homologue hGNC5. P/CAF interacts with p300 and CBP to form functional complexes (Yang *et al.,* 1996). These findings are consistent with the formation of complexes containing multiple HAT activities that can accommodate requirements for specificity of histone acetylation under different biological conditions.

Table II Histone Acetyltransferase (HAT)

HAT	Origin
Gcn5	*Tetrahymena* Yeast Mammalian
PCAF	Human
p300/CBP	Human
TAFII250	Human
	Drosophila Yeast
SRC-1	Human
ACTR	Human
Esa1	Yeast
Tip60	Human

Nuclear HAT activity appears to be critical during steroid hormone-dependent transcriptional activation. It has been reported that coactivation factors that include ACTR contain HAT activity (Chen *et al.,* 1997) and recruit p300 and P/CAF to ligand-bound nuclear hormone receptors. This is an example of a multiprotein complex containing three different HAT activities (p300/CBP, P/CAF, and ACTR) that contribute to modifications of nucleosomal histones that are linked functionally to competency for chromatin remodeling that occurs during ligand-dependent transcriptional regulation (Fondell *et al.,* 1996).

For histone acetylation to be a physiologically relevant component of transcriptional control there is a requirement for a cellular mechanism to reverse this posttranslational modification. Histone deacetylases (HDACs), which remove acetate moieties from histone proteins enzymatically, have been studied extensively during the past several years. Multiple forms of this enzyme have been identified and characterized in several organisms (Brosch *et al.,* 1992; Georgieva *et al.,* 1991; Grabher *et al.,* 1994; Lechner *et al.,* 1996; Lopez-Rodas *et al.,* 1992; reviewed in Khochbin *et al*., 2001). The mammalian form, designated HDAC1, was found to be homologous to the yeast form designated Rpd3 (Taunton *et al.,* 1996). It has been reported that the unliganded thyroid and retinoid receptors can mediate transcriptional repression via interaction with corepressor molecules that include SMRT and N-CoR (Chen and Evans, 1995; Horlein *et al.,* 1995). These corepressors can nucleate the formation of high molecular weight complexes containing HDAC activity. These findings indicate that, in general, histone acetylation correlates with activation and suppression of gene expression, confirming that remodeling of chromatin structure and nucleosome organization is obligatory for biological control of transcription.

Histone phosphorylation contributes to modifications in histone–DNA and histone–histone interactions that influence nucleosome placement and chromatin organization. Kinases that mediate histone phosphorylation respond to regulatory information transduced through signaling pathways in a biologically specific manner. The biologically responsive reconfiguration of chromatin is frequently accompanied by the phosphatase-dependent dephosphorylation of histones that occurs in the absence of protein degradation. Other posttranslational modifications of histones that influence chromatin organization include methylation, ubiquitination, and poly ADP ribosylation. The extensive utilization of methylation in biological control is reflected by the methylation of transcription factors and DNA. DNA methylation is frequently associated with transcriptional repression.

Higher Order Nuclear Organization: Interrelationships of Transcriptional Regulatory Machinery with Nuclear Architecture

The necessity for both nuclear architecture and biochemical control to regulate gene expression is becoming increasingly evident. An ordered organization of nucleic acids and regulatory proteins to assemble and sustain macromolecular complexes that provide the machinery for transcription requires stringent, multistep mechanisms. Each component of transcriptional control is governed by responsiveness to an integrated series of cellular signaling pathways. Each gene promoter selectively exercises options for regulating factor interactions that activate or repress transcription. All transcriptional control is operative *in vivo* under conditions where, despite low representation of promoter regulatory elements and cognate factors, a critical concentration is essential for a threshold that can initiate sequence-specific interactions and functional activity.

Historically, there was a dichotomy between pursuit of nuclear morphology and transcriptional control. However, the growing experimental evidence indicating that components of gene regulatory mechanisms are associated architecturally strengthens the nuclear structure–function paradigm. Are all regulatory events that control gene expression linked architecturally? Can genetic evidence formally establish consequential relationships between nuclear structure and transcription? What are the mechanisms that direct genes and regulatory factors to subnuclear sites that support transcription? How are boundaries established that compartmentalize components of gene expression to specific subnuclear domains? Can the regulated and regulatory parameters of nuclear structure–function interrelationships be distinguished? These are key questions that must be addressed experimentally to validate components of gene expression that have been implicated as dependent on nuclear morphology. From a biological perspective, it is important to determine if breaches in nuclear organization are related to compromised gene expression in diseases that include cancer where incurred mutations abrogate transcriptional control.

Nuclear Matrix: A Scaffold for the Architectural Organization of Regulatory Complexes

The identification (Berezney and Coffey, 1975) and *in situ* visualization (Fey *et al.,* 1984; Nickerson *et al.,* 1990) of the nuclear matrix (Fig. 3), together with the characterization of a chromosome scaffold (Lebkowski and Laemmli, 1982), were bases for pursuing the control of gene expression within the three-dimensional context of nuclear architecture.

The anastomosing network of fibers and filaments that constitute the nuclear matrix supports the structural properties of the nucleus as a cellular organelle and accommodates modifications in gene expression associated with proliferation, differentiation, and changes necessary to sustain phenotypic requirements in specialized cells (Bidwell *et al.,* 1994; Dworetzky *et al.,* 1990; Getzenberg and Coffey, 1990; Nickerson *et al.,* 1990). Regulatory functions of the nuclear matrix include but are by no means restricted to DNA replication (Berezney and Coffey, 1975), gene location (Zeng *et al.,* 1997), imposition of physical constraints on chromatin structure that support formation of loop domains, concentration and targeting of transcription factors (Dworetzky *et al.,* 1992; Nelkin *et al.,* 1980; Robinson *et al.,* 1982; Schaack *et al.,* 1990; Stief *et al.,* 1989; van Wijnen *et al.,* 1993), RNA processing and transport of gene transcripts (Blencowe *et al.,* 1994; Carter *et al.,* 1993; Lawrence *et al.,* 1989; Spector, 1990; Zeitlin *et al.,* 1987), and posttranslational modifications of chromosomal proteins, as well as imprinting and modifications of chromatin structure (Davie, 1997).

Initial correlations between the representation of nuclear matrix proteins and phenotypic properties of cells supported involvement of the nuclear matrix in regulatory activities (Bidwell *et al.,* 1994; Dworetzky *et al.,* 1990; Fey and Penman, 1988; Getzenberg and Coffey, 1990; Nickerson *et al.,* 1990). Additional evidence for participation of the nuclear matrix in gene expression came from reports of qualitative and quantitative changes in the representation of nuclear matrix proteins during the differentiation of normal diploid cells (Dworetzky *et al.,* 1990) and in tumor cells (Bidwell *et al.,* 1994; Getzenberg *et al.,* 1991, 1996; Keesee *et al.,* 1994; Khanuja *et al.,* 1993; Kumara-Siri *et al.,* 1986; Long and Ochs, 1983; Partin *et al.,* 1993; Stuurman *et al.,* 1989). More direct evidence for functional linkages between nuclear architecture and transcriptional control was provided by demonstrations that cell growth (Dworetzky *et al.,* 1992; Schaack *et al.,* 1990; van Wijnen *et al.,* 1993) and phenotypic (Dickinson *et al.,* 1992; Merriman *et al.,* 1995; Nardozza *et al.,* 1996) regulatory factors are nuclear matrix associated and by modifications in the partitioning of transcription factors between the nuclear matrix and the nonmatrix nuclear fraction when changes in gene expression occur (van Wijnen *et al.,* 1993).

Contributions of the nuclear matrix to control of gene expression is further supported by involvement in regulatory events that mediate histone modifications (Hendzel *et al.,* 1994), chromatin remodeling (Cote *et al.,* 1994; Imbalzano, 1998; Kwon *et al.,* 1994; Peterson *et al.,* 1998; Workman and Kingston, 1998), and processing of gene transcripts (Blencowe *et al.,* 1994; Carter *et al.,* 1993; Ciejek *et al.,* 1982; Iborra *et al.,* 1998; Jackson *et al.,* 1998; Nickerson *et al.,* 1995; Pombo and Cook, 1996; Wei *et al.,* 1999). Instead of addressing chromatin remodeling and transcriptional activation as complex but independent mechanisms, it is biologically meaningful to investigate the control of genome packaging and expression as interrelated processes that are operative in relation to nuclear architecture (Fig. 4). Taken together with findings that indicate important components of the machinery for both gene transcription and replication are confined to nuclear matrix-associated subnuclear domains (Iborra *et al.,* 1998; Jackson *et al.,* 1998; Lamond and Earnshaw, 1998; Wei *et al.,* 1998; Zeng *et al.,* 1998), the importance of nuclear architecture to intranuclear compartmentalization of regulatory activity is being pursued.

SUBNUCLEAR DOMAINS: NUCLEAR MICROENVIRONMENTS PROVIDE A STRUCTURAL AND FUNCTIONAL BASIS FOR SUBNUCLEAR COMPARTMENTALIZATION OF REGULATORY MACHINERY

An understanding of interrelationships between nuclear structure and gene expression necessitates knowledge of the composition, organization, and regulation of sites within the nucleus that are dedicated to DNA replication, DNA repair, transcription, and processing of gene transcripts. During the past several years there have been development

s in reagents and instrumentation to enhance the resolution of nucleic acid and protein detection by *in situ* hybridization and immunofluorescence analyses. We are beginning to make the transition from descriptive *in situ* mapping of genes, transcripts, and regulatory factors to visualization of gene expression from the three-dimensional perspective of nuclear architecture. Figure 2 displays components of gene regulation that are associated with the nuclear matrix. Initially, *in situ* approaches were utilized primarily for the intracellular localization of nucleic acids. Proteins that contribute to control of gene expression were first identi-fied by biochemical analyses. We are now applying high-resolution *in situ* analyses for the primary identification and characterization of gene regulatory mechanisms under *in vivo* conditions.

We are increasing our understanding of the significance of nuclear domains to the control of gene expression. These local nuclear environments that are generated by the multiple aspects of nuclear structure are tied to the developmental expression of cell growth and tissue-specific genes. Historically, the control of gene expression and characterization of structural features of the nucleus were pursued conceptionally and experimentally as minimally integrated questions. At the same time, however, independent pursuit of nuclear structure and function has occurred in parallel with the appreciation that several components of nuclear architecture are associated with parameters of gene expression or control of specific classes of genes.

For the most part, biochemical parameters of replication and transcription have been studied independently. However, paradoxically, from around the turn of the last century it was recognized that there are microenvironments within the nucleus where regulatory macromolecules are compartmentalized in subnuclear domains. Chromosomes and the nucleolus provided the initial paradigms for the organization of regulatory machinery within the nucleus. Also, during the last several decades, linkages have been established between subtleties of chromosomal anatomy and replication as well as gene expression. Regions of the nucleolus are understood in relation to ribosomal gene expression. The organization of chromosomes and chromatin is well accepted as reflections of functional properties that support competency for transcription and the extent to which genes are transcribed.

It has been only recently that there is an appreciation for the broad-based organization of regulatory macromolecules within discrete nuclear domains (reviewed in Cook, 1999; Kimura *et al.,* 1999; Lamond and Earnshaw, 1998; Leonhardt *et al.,* 1998; Ma *et al.,* 1998, 2000; Misteli, 2000; Misteli and Spector, 1999; Zhong *et al.,* 2000; Scully and Livingston, 2000; Smith *et al.,* 1999; Stein *et al.,* 2000a, b; Stommel *et al.,* 1999; Verschure *et al.,* 1999; Wei *et al.,* 1998; Wu *et al.,* 2000; Zeng *et al.,* 1998; Zhao *et al.,* 2000). Examples of intranuclear compartmentalization now include but by no means are restricted to SC35 RNA processing sites, PML bodies, the structural and regulatory components of nuclear pores that mediate nuclear–cytoplasmic exchange (Moir *et al.,* 2000), coiled (Cajal) bodies, and replication foci, as well as defined sites where steroid hormone receptors and transcription factors reside (Glass and Rosenfeld, 2000; Leonhardt *et al.,* 2000; McNally *et al.,* 2000). The integrity of these subnuclear microenvironments is indicated by structural and functional discrimination between each architecturally defined domain. Corroboration of structural and functional integrity of each domain is provided by the uniqueness of the intranuclear sites with respect to composition, organization, and intranuclear distribution in relation to activity (Hirose and Manley, 2000; Lemon and Tjian, 2000).

We are now going beyond mapping regions of the nucleus that are dedicated to replication and gene expression. We are gaining insight into interrelationships between the subnuclear organization of the regulatory and transcriptional machinery with the dynamic assembly and activity of macromolecular complexes that are required for biological control during development, differentiation, maintenance of cell and tissue specificity, homeostatic control, and tissue remodeling (Javed *et al.,* 2000; Stein *et al.,* 2000b). Equally important, it is becoming evident that the onset and progression of cancer (McNeil *et al.,* 2000; Stein *et al.,* 2000b; Tibbetts *et al.,* 2000) and neurological disorders (Skinner *et al.,* 1997) are associated with and potentially functionally coupled with perturbations in the subnuclear organization of genes and regulatory proteins that relate to aberrant gene replication, repair, and transcription.

Linkage of Nuclear Architecture to Biological Control of Skeletal Gene Expression

Chromatin Remodeling Renders Skeletal Genes Accessible to Regulatory Factors That Control Competency for Transcription

Alterations in the chromatin organization of the osteocalcin gene promoter during osteoblast differentiation provide a paradigm for remodeling of chromatin structure and nucleosome organization that is linked to long-term commitment to phenotype-specific gene expression (Montecino *et al.,* 1994b, 1996). The osteocalcin gene encodes a 10-kDa bone-specific protein that is induced in late-stage osteoblasts at the onset of extracellular matrix mineralization (Aronow *et al.,* 1990; Owen *et al.,* 1990).

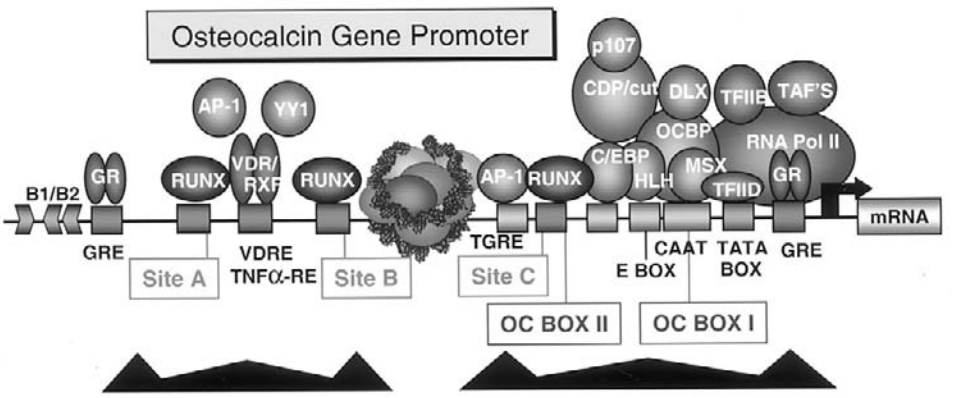

Figure 5 The osteocalcin promoter as a blueprint for responsiveness to osteogenic signals. This schematic representation of promoter elements and cognate factors in the rat osteocalcin promoter depicts three binding sites for Runx2/Cbfa1 (red symbols), steroid hormone responsive sequences and the cognate hormone receptor dimers (blue symbols); a vitamin D responsive element (VDRE); glucocorticoid responsive elements (GRE) and recognition motifs for osteoblast-related transcription factors, e.g., the leucine zipper proteins C/EBP and AP1, homeodomain proteins Dlx5, Msx2, and CDP/cut, helix-loop-helix proteins (yellow symbols), as well as components of the general transcription initation machinery [e.g., the TATA-binding complex (TFIID) and associated factors (TAFs)]. OC boxes I and II represent the principal rate-limiting elements that activate the OC gene in bone cells. The mRNA cap site (hooked arrow) is located at the 3′ end of the promoter, and the regulatory boundary of the promoter at the 5′ end is demarcated by repetitive sequences (e.g., B1 and B2 repeats; horizontal chevrons). A positioned nucleosome resides between distal and proximal promoter domains in the active promoter. Black bars indicate DNase I hypersensitivity observed when the osteocalcin gene is transcribed and which increase in intensity in response to vitamin D. (See also color plate.)

Transcription of the osteocalcin gene is controlled by a modularly organized promoter with proximal basal regulatory sequences and distal hormone responsive enhancer elements (Banerjee *et al.,* 1996; Bortell *et al.,* 1992; Demay *et al.,* 1990; Ducy and Karsenty, 1995; Guo *et al.,* 1995; Hoffmann *et al.,* 1994; Markose *et al.,* 1990; Merriman *et al.,* 1995; Tamura and Noda, 1994; Towler *et al.,* 1994) (Fig. 5 see also color plate). The osteocalcin gene is not expressed in non osseous cells nor is it transcribed in osteoprogenitor cells or early stage proliferating osteoblasts. Following the postproliferative onset of osteoblast differentiation, transcription of the osteocalcin gene is regulated by Runx2 (Fig. 6, see also color plate). Maximal levels of transcription are controlled by the combined activities of the vitamin D response element, C/EBP site, AP-1 regulatory elements, and the OC box. Linear organization of the OC gene promoter reveals proximal regulatory elements that control basal and tissue-specific activity. These include the OC box for homeodomain protein binding and an osteoblast-specific complex, the TATA domain, a Runx site, and a C/EBP site. The distal promoter contains a vitamin D responsive enhancer element (VDRE) that is flanked by Runx sites (Fig. 5). The control of transcription is dependent on protein–DNA interactions in the basal and upstream elements that are in part dependent on the accessibility of cognate regulatory sequences and additionally on the consequences of mutually exclusive protein–DNA interactions.

A relevant example of mutually exclusive occupancy at an OC gene promoter element is competition by YY1 and the vitamin D receptor (VDR) for the VDRE. Such regulatory factor occupancy at the VDRE provides a mechanism for enhancement by VDR/RXR heterodimers and suppression of competency for vitamin D-dependent enhancement by YY1 under appropriate biological conditions (Fig. 7, see also color plate). There is additionally a requirement to account mechanistically for protein–protein interactions between regulatory factors that are components of proximal basal and upstream enhancer complexes. Here mutually exclusive protein–protein interactions of YY1 or VDR (VDRE associated) with TFIIB (TATA associated) occur and must be explained in relation to conformational properties of the OC promoter that can support interactions of these distal and proximal regulatory complexes.

The dynamics of chromatin organization and remodeling permit developmental and steroid hormone responsive changes in the OC gene promoter to accommodate transcriptional requirements. Figures 6 and 7 schematically depict modifications in chromatin structure and nucleosome organization that parallel competency for transcription and the extent to which the osteocalcin gene is transcribed. Changes in chromatin are observed in response to physiological mediators of basal expression and steroid hormone responsiveness. This remodeling of chromatin provides a basis for the involvement of nuclear architecture in growth factor and steroid hormone responsive control of osteocalcin gene expression during osteoblast phenotype development and in differentiated bone cells.

Basal expression and enhancement of osteocalcin gene transcription are accompanied by two changes in the

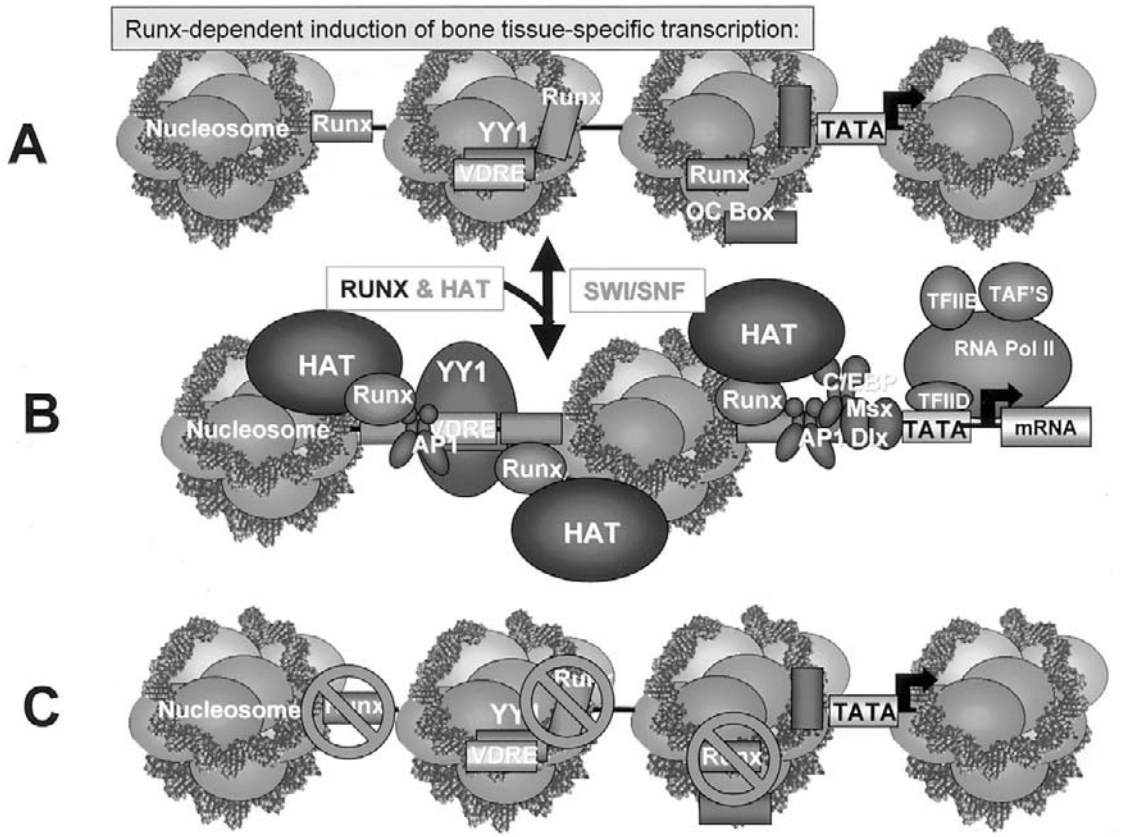

Figure 6 Runx2/Cbfa1-dependent remodeling of the osteocalcin gene promoter. (A) The inactive promoter in nonosseous cells is organized as condensed chromatin in an array of nucleosomes. (B) Induction of osteocalcin gene expression by Runx2/Cbfa1 proteins occurs concomitant with modifications in nucleosomal organization through the recruitment of histone acetyl transferases (HATs) and chromatin remodeling proteins (e.g., SWI/SNF). The interactions of Runx2 proteins with strategically spaced sites in the OC promoter may constrain the position of a nucleosome between proximal and distal promoter domains to facilitate accessibility of the distal vitamin D responsive sequences and proximal bone tissue-specific elements to principal regulatory factors. (C) Mutation of the Runx sites results in the loss of nuclease hypersensitive sites and chromatin remodeling, reflected by an 80% reduction of OC promoter activity in a genomic context. (See also color plate.)

structural properties of chromatin (Fig. 7). DNase I hypersensitivity of sequences flanking the basal, tissue-specific element and the vitamin D enhancer domain is observed (Breen *et al.,* 1994; Montecino *et al.,* 1994a, b). Together with changes in nucleosome placement, a basis for accessibility and *in vivo* occupancy of transactivation factors to basal and steroid hormone-dependent regulatory sequences can be explained. In early stage proliferating normal diploid osteoblasts, when the osteocalcin gene is repressed, nucleosomes are placed in the proximal basal domain and in the vitamin D responsive enhancer promoter sequences. Nuclease hypersensitive sites are not present in the vicinity of these regulatory elements. In contrast, when osteocalcin gene expression is transcriptionally upregulated postproliferatively and vitamin D-mediated enhancement of transcription occurs, the proximal basal and upstream steroid hormone responsive enhancer sequences become nucleosome free and these regulatory domains are flanked by DNase I hypersensitive sites.

Translational positioning of the nucleosomes reflects protein–DNA interactions within the OC promoter that accounts for both formation of the nuclease hypersensitive sites and OC gene transcriptional activity. The Runx2 transcriptional factor is required for developmentally regulated osteocalcin expression (Banerjee *et al.,* 1997; Ducy *et al.,* 1997). Mutations that eliminate Runx-binding sites in the OC promoter prevent chromatin remodeling (Javed *et al.,* 1999) and decrease transcription dramatically. In addition, mutations of the Runx sites that flank the VDRE result in a loss of vitamin D responsiveness, reflecting the requirement of Runx sites for correct promoter architecture (Fig. 6). Runx factors have been shown to form a scaffold for the assembly of multimeric complexes of proteins with histone acetylase and histone deacytelase activities (Westendorf and Hiebert, 1999) that support chromatin remodeling (Figs. 8 and 9, see also color plates). Such complexes are in part influenced by the position of Runx sites in a promoter and protein–protein interactions with factors at nearby

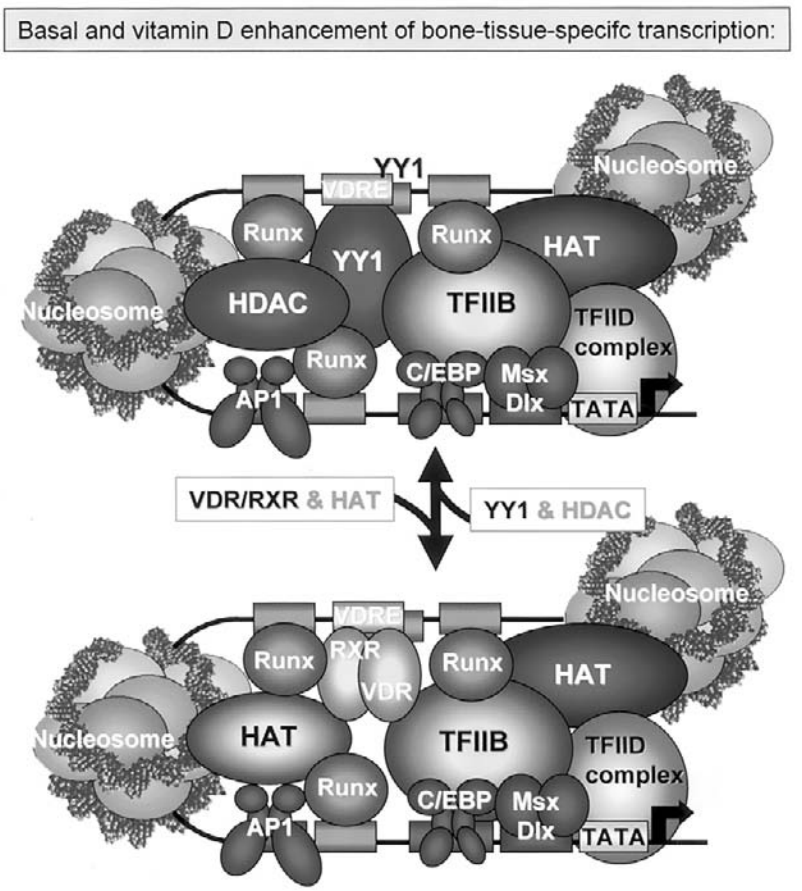

Figure 7 Spatial organization of the osteocalcin promoter during bone tissue-specific basal transcription and vitamin D enhancement. Upon activation of the osteocalcin gene, the promoter adopts a specialized nucleosomal organization involving two accessible regions spanning proximal elements (e.g., AP1, C/EBP, and Msx/Dlx, blue), distal elements (e.g., VDRE, yellow), and a nucleosome (gold) with restricted mobility that is flanked by Runx2 sites (red). In the basal state (top), transcription factors bound to the OC promoter are associated with specific histone acetyl transferases (HATs) that promote an "open" chromatin conformation and protein/protein bridges between distal and proximal elements. Vitamin D-dependent transcription is attenuated in the absence of ligand by the binding of YY1 to a site overlapping with the VDRE that precludes receptor binding and by sequestration of TFIIB, which normally functions as a stimulatory cofactor for the vitamin D response. Upon ligand binding to the vitamin D receptor (VDR) (bottom), VDR displaces YY1 at the distal VDRE, which supports productive interactions with TFIIB at the proximal promoter, as well as recruits additional HATs that further open up the OC gene locus. (See also color plate.)

regulatory elements. Thus, multimerized Runx sites lose the ability to provide an assay for the precise function of gene regulation in the context of a specific promoter. These interactions contribute strong enhancer activity of the osteocalcin gene and downregulation of the bone sialoprotein gene in osteoblasts (Javed *et al.,* 2001), as illustrated in Fig. 9. The developmental and steroid hormone transitions in chromatin are thereby mediated by Runx-dependent protein–DNA and protein–protein interactions. Occupancy of multiple factors at other regulatory elements in the context of the three-dimensional structure of the promoter provides a basis for additional protein–protein interactions that further contribute to the control of transcription.

Intranuclear Trafficking to Subnuclear Destinations: Directing Skeletal Regulatory Factors to the Right Place at the Right Time

The traditional experimental approaches to transcriptional control have been confined to the identification and characterization of gene promoter elements and cognate regulatory factors. However, the combined application of *in situ* immunofluorescence together with molecular, biochemical, and genetic analyses indicates that several classes of transcription factors exhibit a punctate subnuclear distribution. This punctate subnuclear distribution persists after the removal of soluble nuclear proteins and nuclease-digested

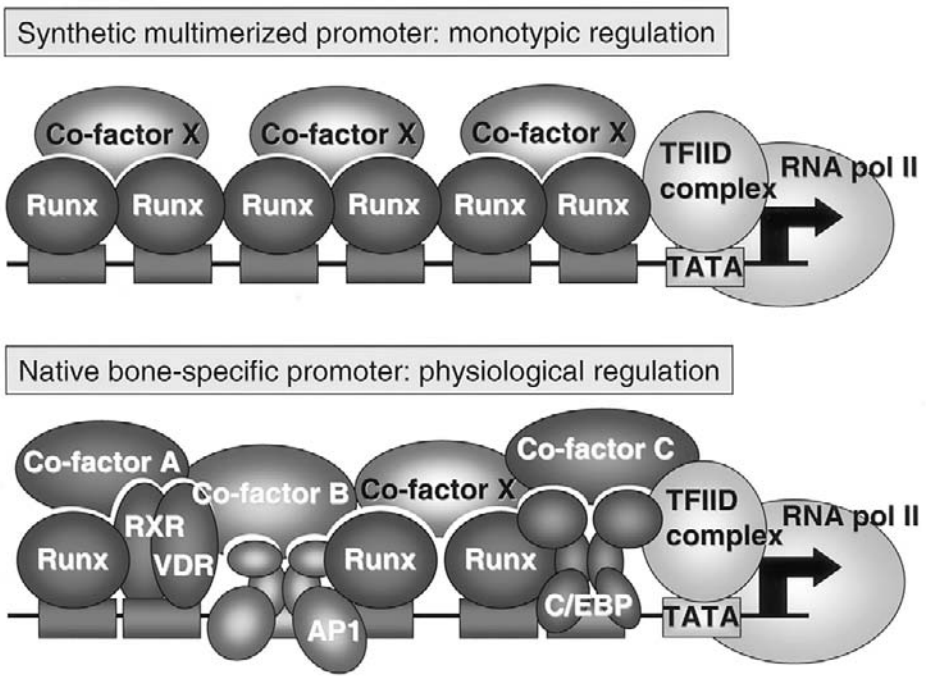

Figure 8 Physiological regulation of gene transcription based on the intricate organization of natural gene promoters. The potential for regulatory interactions of transcription factors and cofactors at synthetic promoters containing multimerized binding sites for Runx/Cbfa (top) and native Runx/Cbfa responsive promoters (bottom) is shown. The natural organization of bone-specific promoters represents a blueprint for physiological control of gene expression and responsiveness to osteogenic factors. Transcriptional control involves irregularly arranged elements (horizontal cylinders) that bind Runx2/Cbfa1 proteins, as well as different classes of transcription factors that synergize with Runx2/Cbfa1 (RXR/VDR, AP1, C/EBP)(oval structures). Synergism involves the association of distinct types of cofactors (e.g., A, B, C, and X) that recognize protein surfaces in Runx2/Cbfa1 and RXR/VDR, AP1, or C/EBP to form heteromeric promoter structures that recruit the TFIID transcriptional initiation complex and RNA polymerase II. The monotypic repetition of Runx elements in synthetic promoters (top) reduces the potential for cofactor interactions and artificially amplifies one component of the transcriptional response. (See also color plate.)

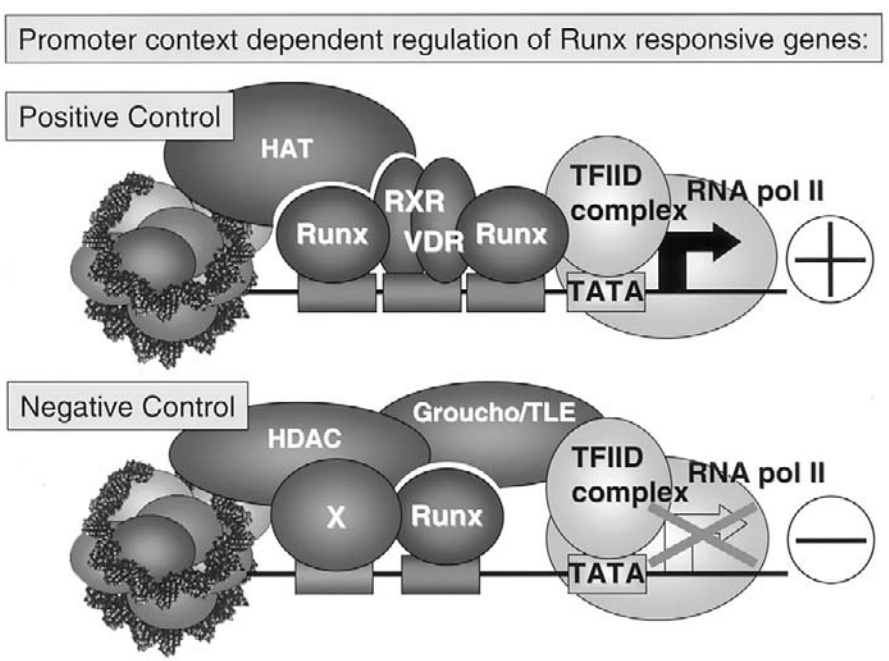

Figure 9 Positive and negative control of Runx2/Cbfa1 responsive genes. Runx2/Cbfa1 is a multifunctional protein that can synergize with different sequence-specific DNA-binding proteins (e.g., AP1, C/EBP) and associate with positive (e.g., Smads, p300) or negative (e.g., groucho/TLE, HDACs) gene regulatory cofactors depending on the promoter context of bone tissue-specific genes. Possible interactions accommodating the positive (top) and negative (bottom) regulation of transcription are shown. Depending on the cell type, coregulatory proteins may modulate transcriptional levels of the gene rather than completely repress the gene as illustrated. (See also color plate.)

chromatin. We propose that the intranuclear organization of regulatory proteins could be linked functionally to their competency to affect gene expression (e.g., Guo *et al.,* 1995; Htun *et al.,* 1996; Nguyen and Karaplis, 1998; Stenoien *et al.,* 1998; Verschure *et al.,* 1999; Zeng *et al.,* 1997). Therefore, one fundamental question is the mechanism by which this compartmentalization of regulatory factors is established within the nucleus. This compartmentalization could be maintained by the nuclear matrix, which provides an underlying macromolecular framework for the organization of regulatory complexes (Berezney and Jeon, 1995; Berezney and Wei, 1998; Penman, 1995). However, one cannot dismiss the possibility that nuclear compartmentalization is activity driven (Misteli, 2000; Pederson, 2000).

Insight into architecture-mediated transcriptional control can be gained by examining the extent to which the subnuclear distribution of gene regulatory proteins affects their activities. We and others observed that members of the Runx/Cbfa family of hematopoietic and bone tissue-specific transcription factors (Banerjee *et al.,* 1997; Chen *et al.,*

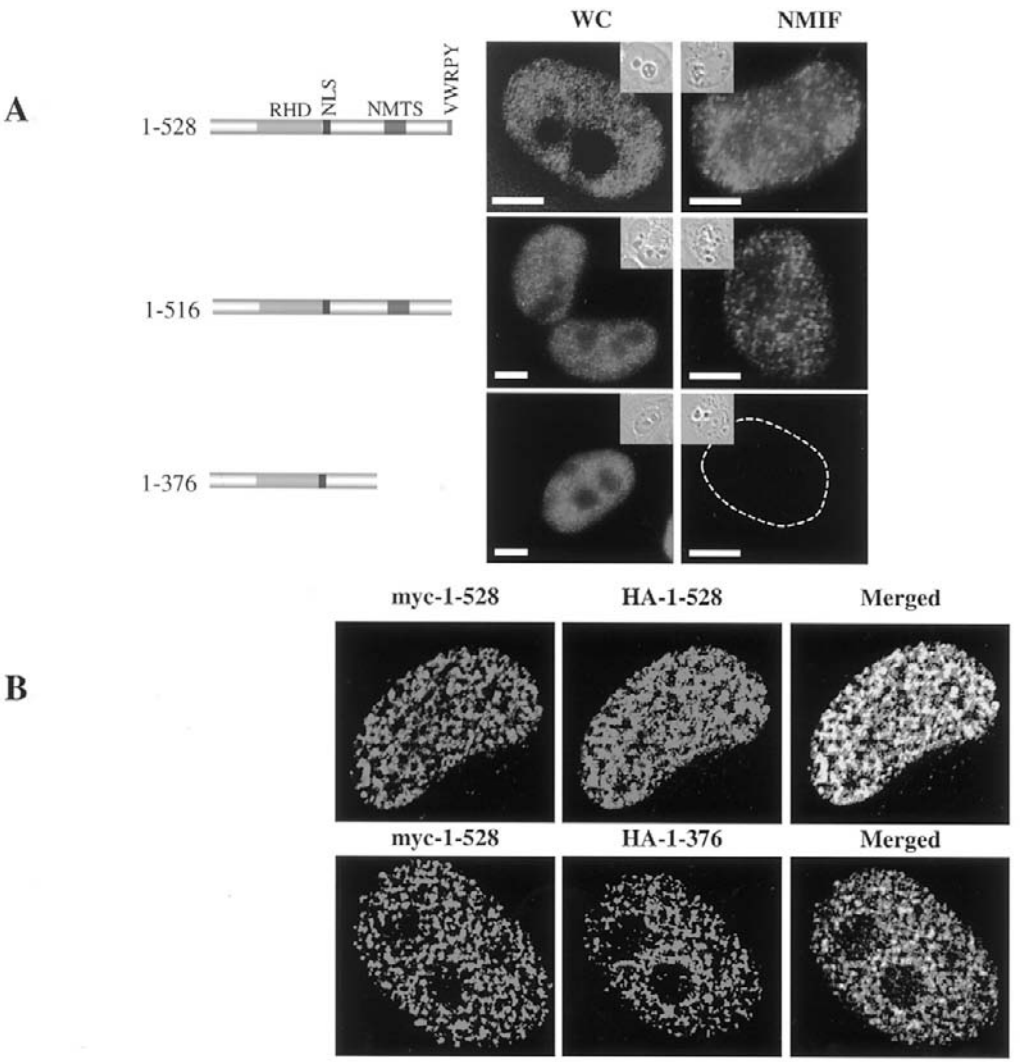

Figure 10 Identification and function of the intranuclear targeting signal (NMTS) that directs Runx factors to nuclear matrix-associated subnuclear domains. (A) Immunofluorescence and differential interference contrast (small inserts) images of whole cell (WC) and nuclear matrix intermediate filament (NMIF) preparations of cells transfected with constructs encoding Runx (Cbfa/AML) related proteins that possess or lack the intranuclear-trafficking signal. The location of the conserved domains, runt DNA-binding domain (RHD), nuclear import signal (NLS), the VWRPY interaction motif for Groucho/TLE proteins, and the intranuclear-trafficking signal (NMTS) that includes a context-dependent transactivation domain in the C-terminal region are indicated. The NMTS was initially defined by deletion mutants of Runx (Cbfa/AML) transcription factors that were assayed for nuclear import and trafficking to punctate nuclear matrix-associated subnuclear sites that support gene expression. Deletion of the NMTS (mutant 1-376) does not compromise nuclear import (WC, whole cells) but subnuclear distribution is abrogated (NMIF). (B) Merged images of Runx2 WT 1-528 (myc tag) and Runx2 mutant protein 1-376 (HA tag) in transfected whole cells show that the absence of NMTS misdirects the factor to domains in the nucleus different from wild type (WT), revealed by distinct red and green foci (bottom). WT proteins, each carrying different fluorescent tags (myc and HA), colocalize to the same sites reflected by a yellow merged image (top). This control demonstrates that fluorescent tags do not influence subnuclear targeting. (See also color plate.)

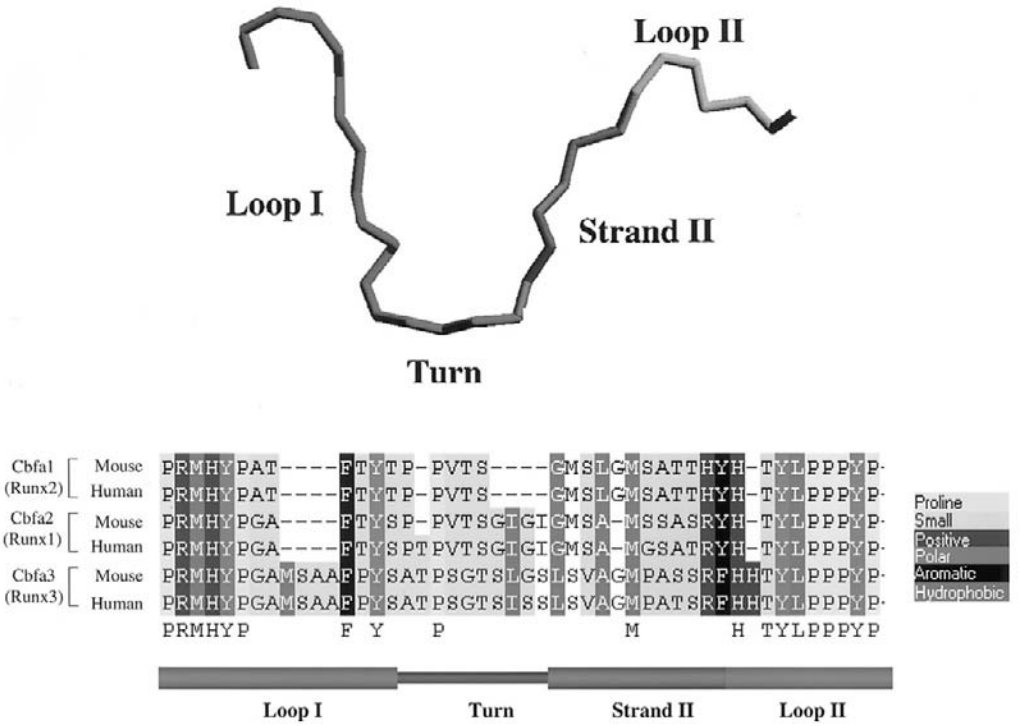

Figure 11 Structure and sequence conservation of the NMTS characterizing Runx1 factors. X-ray diffraction crystallography was carried out on the Runx1 NMTS domain fused to glutathione *S*-transferase at a resolution of –2.7 A. Two loop domains connected by a flexible glycine hinge (turn) are predicted (Tang *et al.,* 1999). Sequence alignment of the segments of the Runx2, Runx1, and Runx3 proteins containing the NMTS. Loop I and loop II are protein-interacting domains for Smad (Zhang *et al.,* 2000) and YAP (Yagi *et al.,* 1999). Comparison of mouse and human sequences shows conservation among the factors and species. The Runx nomenclature for Runt-related transcription factors, recently adopted by the Human Genome Organization, is indicated. (See also color plate.)

1998; Merriman *et al.,* 1995; Zeng *et al.,* 1997, 1998) exhibit a punctate subnuclear distribution and are associated with the nuclear matrix (Zeng *et al.,* 1997). Biochemical and *in situ* immunofluorescence analyses (Fig. 10, see also color plate) established that a 31 residue segment designated the nuclear matrix targeting signal (NMTS) near the C terminus of the Runx factor is necessary and sufficient to mediate association of these regulatory proteins to nuclear matrix-associated subnuclear sites at which transcription occurs (Zeng *et al.,* 1997, 1998). The NMTS functions autonomously and can target a heterologous protein to the nuclear matrix. Furthermore, the NMTS is independent of the DNA-binding domain as well as the nuclear localization signal, both of which are in the N-terminal region of the Runx protein. The unique peptide sequence of the Runx NMTS (Zeng *et al.,* 1997, 1998) and the defined structure obtained by X-ray crystallography (Tang *et al.,* 1998a, 1999) support the specificity of this targeting signal. These data are compatible with a model in which the NMTS functions as a molecular interface for specific interaction with proteins and/or nucleic acids that contribute to the structural (Fig. 11, see also color plate) and functional activities of nuclear domains (Fig. 12). However, at present we cannot formally distinguish whether this interaction between the NMTS and its putative nuclear acceptor strictly reflects targeting or retention.

The idea that specific mechanisms direct regulatory proteins to sites within the nucleus is reinforced by the identification of targeting signals in the glucocorticoid receptor (Htun *et al.,* 1996; Tang *et al.,* 1998b; van Steensel *et al.,* 1995), PTHRP (Nguyen and Karaplis, 1998), the androgen receptor (van Steensel *et al.,* 1995), PIT1 (Stenoien *et al.,* 1998), and YY1 (Guo *et al.,* 1995; McNeil *et al.,* 1998). These targeting signals do not share sequence homology with each other or with the Runx/Cbfa transcription factors. Furthermore, the proteins each exhibit distinct subnuclear distributions. Thus, a series of trafficking signals are responsible for directing regulatory factors to nonoverlapping sites within the cell nucleus. Collectively, the locations of these transcription factors provide coordinates for the activity of gene regulatory complexes.

The importance of architectural organization of regulatory machinery for bone-restricted gene expression is evident from the intranuclear localization of Runx2 coregulatory proteins that control OC gene expression. For example, TLE/Groucho, a suppressor of Runx-mediated transcriptional activation, colocalizes with Runx2 at punctate subnuclear sites (Javed *et al.,* 2000). The Yes-associated protein represents another example of a Runx coregulatory factor that is directed to Runx subnuclear sites when associated with Runx (Zaidi *et al.,* 2000).

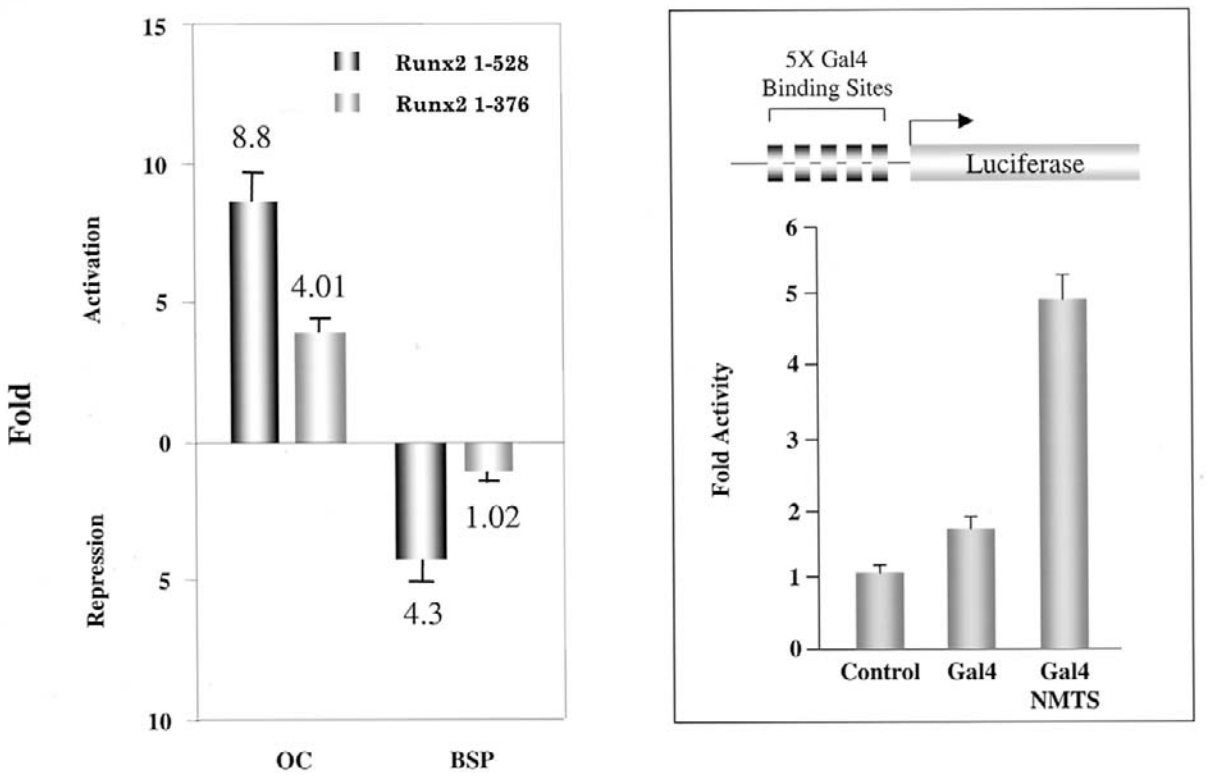

Figure 12 Functional activity of the NMTS. The NMTS supports transcription of promoters as demonstrated by two experimental approaches. (Left) Decreased activation of the osteocalcin promoter (−1.1 kbp OC) and decreased repression of the bone-sialoprotein (−1 kbp BSP) are observed with mutant Cbfa1 lacking the C terminus containing the NMTS (1–376) when compared to WT (1–528). Transient transfections in Hela cells (Javed *et al.*, 2000). (Right) Using the Gal-4 activation system, an NMTS (Runx2)-Gal4 fusion protein increased luciferase activity fourfold over Gal4, reflecting the intrinsic transactivation functions of the NMTS. The control represents empty vector (Zeng *et al.,* 1998).

Intranuclear targeting of regulatory factors is a multistep process, and we are only beginning to understand the complexity of each step. However, biochemical and *in situ* analyses have shown that at least two trafficking signals are required: the first supports nuclear import (the nuclear localization signal) and the second mediates interactions with specific sites associated with the nuclear matrix (the nuclear matrix-targeting signal). Given the multiplicity of determinants for directing proteins to specific destinations within the nucleus, alternative splicing of messenger RNAs might generate different forms of a transcription factor that are targeted to specific intranuclear sites in response to diverse biological conditions. Furthermore, the activities of transcription complexes involve multiple regulatory proteins that could facilitate the recruitment of factors to sites of architecture-associated gene activation and suppression.

Requirements for Understanding Functional Interrelationships between Nuclear Architecture and Skeletal Gene Expression

The regulated and regulatory components that interrelate nuclear structure and function must be established experimentally. A formidable challenge is to define further the control of transcription factor targeting to acceptor sites associated with the nuclear matrix. It will be important to determine whether acceptor proteins are associated with a preexisting core filament structural lattice or whether a compositely organized scaffold of regulatory factors is assembled dynamically.

An inclusive model for all steps in the targeting of proteins to subnuclear sites cannot yet be proposed. However, this model must account for the apparent diversity of intranuclear targeting signals. It is also important to assess the extent to which regulatory discrimination is mediated by subnuclear domain-specific trafficking signals. Furthermore, the checkpoints that monitor the subnuclear distribution of regulatory factors and the sorting steps that ensure both structural and functional fidelity of nuclear domains in which replication and expression of genes occur must be defined biochemically and mechanistically.

There is emerging recognition that the placement of regulatory components of gene expression must be coordinated temporally and spatially to facilitate biological control. The consequences of breaches in nuclear structure–function relationships are observed in an expanding series of diseases that include cancer (McNeil *et al.,* 1999; Rogaia *et al.,* 1997; Rowley, 1998; Tao and Levine, 1999; Weis *et al.,* 1994; Yano *et al.,* 1997; Zeng *et al.,* 1998) and neurological disorders (Skinner *et al.,* 1997). Findings indicate the requirement for the fidelity of Runx/Cbfa/AML subnuclear localization to

support regulatory activity for skeletogenesis *in vivo*. While many of the human mutations in Runx2 associated with cleidocranial dysplasia occur in the DNA-binding domain, several mutations have been identified in the C terminus, which disrupt nuclear matrix association (Zhang *et al.,* 2000). As the repertoire of architecture-associated regulatory factors and cofactors expands, workers in the field are becoming increasingly confident that nuclear organization contributes significantly to the control of transcription. To gain increased appreciation for the complexities of subnuclear organization and gene regulation, we must continue to characterize mechanisms that direct regulatory proteins to specific transcription sites within the nucleus so that these proteins are in the right place at the right time.

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CHAPTER 12

Type I Collagen

Structure, Synthesis, and Regulation

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Introduction

Type I collagen is the most abundant extracellular protein of bones and is essential for bone strength. This chapter first discusses the structure and biosynthesis of type I collagen and how its synthesis is regulated by cytokines, hormones, and growth factors. It then discusses recent results about the organization of regulatory elements in type I collagen genes, many of which are based on studies in transgenic mice.

Collagens can be defined as "structural proteins of the extracellular matrix which contain one or more domains harboring the conformation of a collagen triple helix" (Van der Rest and Garrone, 1991). The triple helix motif is composed of three polypeptide chains whose amino acid sequence consists of Gly-X-Y repeats. Due to this particular peptide sequence, each chain is coiled in a left-handed helix, and the three chains assemble in a right-handed triple helix, where Gly residues are in the center of the triple helix and where the lateral chains of X and Y residues are on the surface of the helix (Van der Rest and Garrone, 1991). In about one-third of the cases X is a proline and Y is an hydroxyproline; the presence of hydroxyproline is essential to stabilize the triple helix and is a unique characteristic of collagen molecules. At the time of this review, 19 different types of collagens have been described, which are grouped in subfamilies depending on their structure and/or their function (for review, see Vuorio and de Crombrugghe, 1990; Van der Rest and Garrone, 1991).

Type I, type II, type III, type V, and type XI collagens form the group of fibrillar collagens. The characteristic feature of fibrillar collagens is that they consist of a long continuous triple helix, which self-assembles into highly organized fibrils. These fibrils have a very high tensile strength and play a key role in providing a structural framework for body structures such as skeleton, skin, blood vessels, intestines, or fibrous capsules of organs. Type I collagen, which is the most abundant protein in vertebrates, is present in many organs and is a major constituent of bone, tendons, ligaments, and skin. Type III collagen is less abundant than type I collagen but its distribution essentially parallels that of type I collagen with the exception of bones and tendons, which contain virtually no type III collagen. Moreover, type III collagen is relatively more abundant in distensible tissues such as blood vessels than in nondistensible tissues. Type V collagen is present in tissues that also contain type I collagen. Type II collagen is a major constituent of cartilage and is also present in the vitreous body. As type II, type XI collagen is present in cartilage.

Type IX, type XII, and type XIV collagens constitute the family of fibril-associated collagens [or fibril-associated collagens with interrupted triple helices (FACIT)]. These collagens are associated with fibrillar collagens and could mediate interactions between fibrillar collagens and other components of the extracellular matrix, or between fibrillar collagens and cells. They are composed of three functional domains (Van der Rest and Garrone, 1991): one that interacts with collagen fibrils, one that projects out of the fibril,

and one that is not a triple helical domain, which interacts with extracellular matrix components or with cells. Type IX collagen, which is the best characterized molecule of this family, is present at the surface of type II collagen fibrils and is covalently cross-linked to these fibrils. Type XII and type XIV collagens are thought to be associated with type I collagen molecules. Type XVI and type XIX collagens, which have been described recently, could also belong to the FACIT family (Myers *et al.,* 1994; Pan *et al.,* 1992).

Collagen-forming sheets are composed of type IV collagen and type VIII collagen. Type IV collagen forms a complex three-dimensional network, which is the major component of basement membranes. Type VIII collagen assembles in hexagonal lattices to form Descemet's membrane and separates corneal endothelial cells from the stroma. Type X collagen, which is synthesized exclusively by hypertrophic chondrocytes, has a high degree of structural similarity with type VIII collagen and could belong to the same family, but its physiological role remains elusive (Rosati *et al.,* 1995).

Structure and Synthesis of Type I Collagen

Structure

Each molecule of type I collagen is typically composed of two α1 chains and one α2 chain [$\alpha1(I)_2-\alpha2(I)$] coiled around each other in a characteristic triple helix, but a very small number of type I collagen molecules can be formed by three α1 chains [$\alpha1(I)_3$]. Both the α1 chain and the α2 chain consist of a long helical domain preceded by a short N-terminal peptide and followed by a short C-terminal peptide (Fig. 1) (for reviews, see Prockop, 1979; Prockop and Kivirikko, 1984; Vuorio and de Crombrugghe, 1990; Van der Rest and Garrone, 1991).

Type I collagen is secreted as a propeptide, but the N telopeptide and the C telopeptide are cleaved rapidly by specific proteases (Fig. 1), and mature molecules assemble to form fibrils. In fibrils, molecules of collagen are parallel to each other; they overlap each other by multiples of 67 nm (distance D), with each molecule being 4.4 D (300 nm) long; there is a 40-nm (0.6 D) gap between the end of a molecule and the beginning of the other (Fig. 2). This quarter-staggered assembly explains the banded aspect displayed by type I collagen fibrils in electron microscopy. In tissues, type I collagen fibrils can be parallel to each other and form bundles (or fibers), as in tendons, or they can be oriented randomly and form a complex network of interlaced fibrils, like in skin. In bone, hydroxyapatite crystals seem to lie in the gaps between collagen molecules.

Synthesis

TRANSCRIPTION

In humans the gene coding for the α1 chain of type I collagen is located on the long arm of chromosome 17 (17q21.3–q22), and the gene coding for the α2 chain is located on the long arm of chromosome 7 (7q21.3–q22). Both genes have a very similar structure (Chu *et al.,* 1984; D′Alessio *et al.,* 1988; de Wet *et al.,* 1987), and this structure is also very similar to that of genes coding for other fibrillar collagens (Vuorio and de Crombrugghe, 1990). The difference in size between the two genes [18 kb for the α1(I) gene and 38 kb for the α2(I) gene] is explained by differences in the size of the introns.

The triple helical domain of the α1 chain is coded by 41 exons, which code for Gly-X-Y repeats, and by two so-called

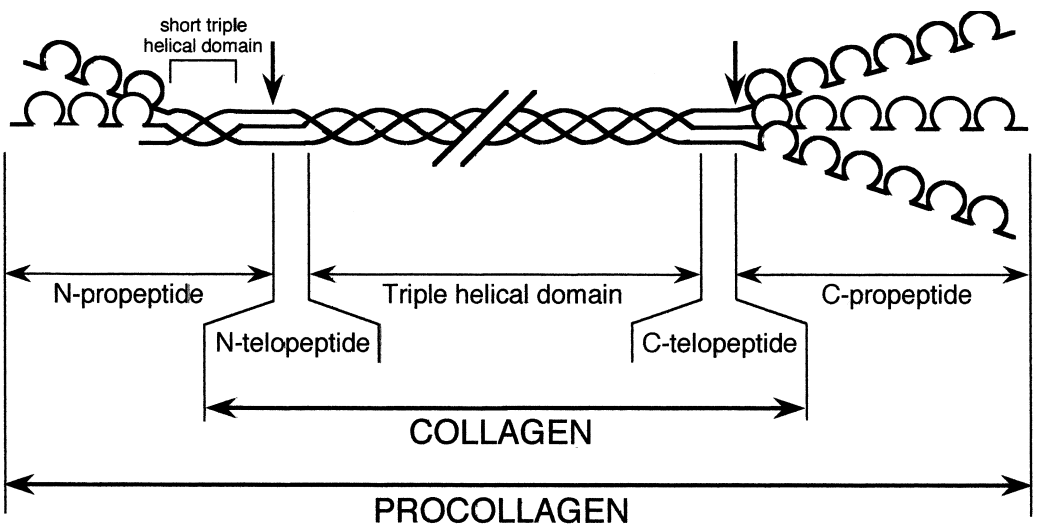

Figure 1 Schematic representation of a type I collagen molecule. Vertical arrows indicate cleavage sites between the mature collagen molecule, the N propeptide and the C propeptide.

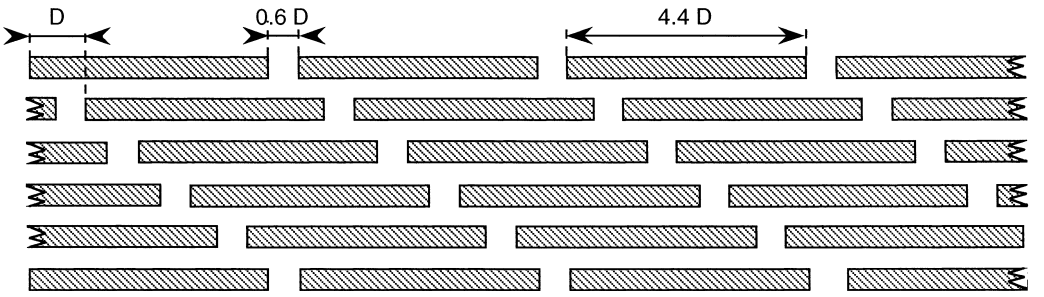

Figure 2 Schematic representation of the quarter-staggered assembly of type I collagen molecules into fibrils. Each collagen molecule is 4.4 D long. Molecules overlap each other by a distance D or multiples of D. There is a 0.6 D gap between the ends of two adjacent nonoverlapping molecules (D = 67 nm).

joining exons. These joining exons code in part for the telopeptides and in part for Gly-X-Y repeats, which are part of the triple helical domain (Fig. 3). The triple helical domain of the α2 chain is coded by 42 exons, plus two joining exons (Fig. 3). Each of the corresponding exons coding for the triple helical domain of the α1 chain and for the triple helical domain of the α2 chain has a similar length (Table I). The only exception is that exons 34 and 35 in the α2 gene, which are 54 bp long each, correspond to a single 108-bp 34/35 exon in the α1 gene. Except for the two joining exons, each exon starts exactly with a G codon and ends precisely with a Y codon, and all the exons are 54, 108 (54 $\times$ 2), 162 (54 $\times$ 3), 45, or 99 bp long (Table I). This organization suggests that exons coding for triple helical domains could have originated from the amplification of a DNA unit containing a 54-bp exon embedded in intron sequences. One hundred and eight- and 162-bp exons would result from a loss of intervening introns. Forty-five- and 99-bp exons would result from recombinations between two 54 exons (Vuorio and de Crombrugghe, 1990; Yamada *et al.,* 1980).

For both the α1 chain and the α2 chain, the C propeptide plus the C telopeptide are coded by 4 exons [exons 48–51 of the α1(I) gene, exons 49–52 of the α2(I) gene]. The first of these exons codes for the end of the triple helical domain, the C-terminal telopeptide, and the beginning of the C-terminal propeptide. The three other exons code for the rest

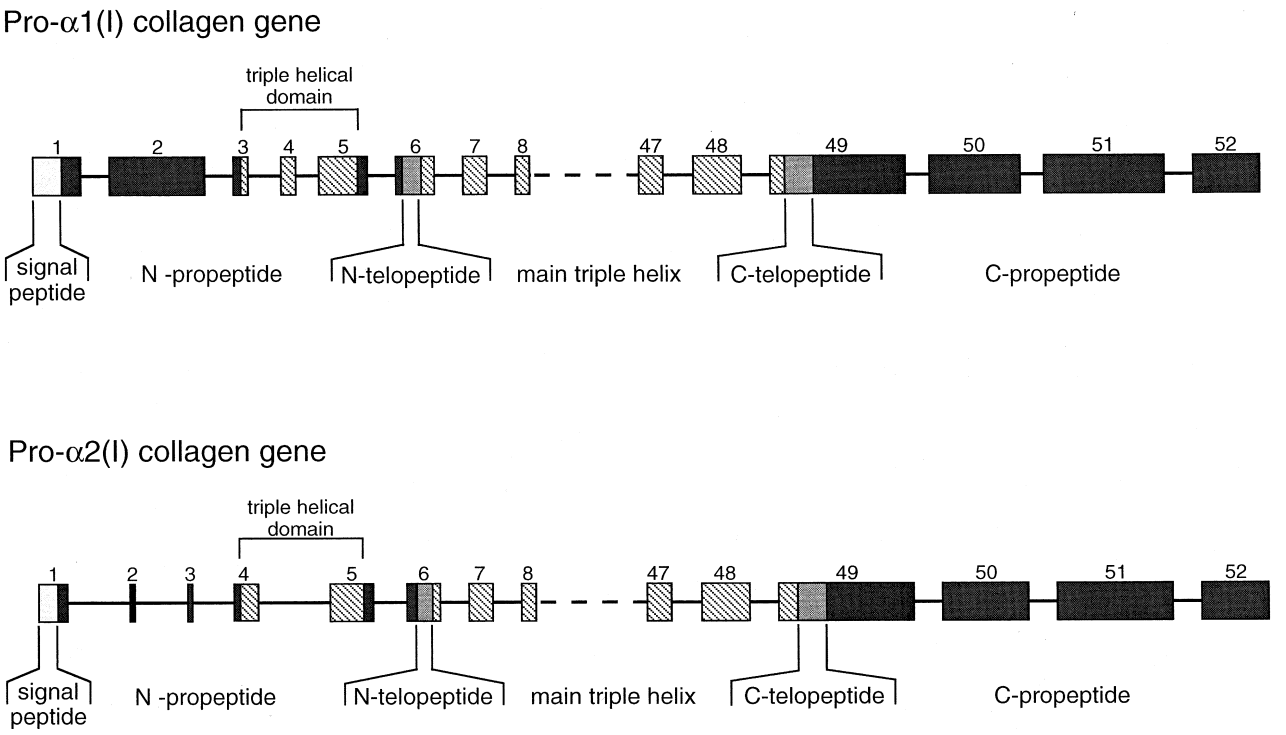

Figure 3 Exon structure of type I procollagen genes. For each chain, the signal peptide is encoded by part of exon 1; the N propeptide is encoded by part of exon 1, by exons 2–5, and by part of exon 6; the N telopeptide is encoded by part of exon 6; the triple helix is encoded by part of exon 6 (joining exon), by exons 7–48, and by part of exon 49 (joining exon); the C telopeptide is encoded by part of exon 49; and the C propeptide is encoded by part of exon 49 and by exons 50–52. Note that N propeptides contain a short helical domain.

Table I Size of Exons Coding for the Triple Helical Domain of Type I Collagen[a]

Exon	Size (bp)	Exon	Size (bp)	Exon	Size (bp)
7	45	21	108	35	54
8	54	22	54	36	54
9	54	23	99	37	108
10	54	24	54	38	54
11	54	25	99	39	54
12	54	26	54	40	162
13	45	27	54	41	108
14	54	28	54	42	108
15	45	29	54	43	54
16	54	30	45	44	108
17	99	31	99	45	54
18	45	32	108	46	108
19	99	33	54	47	54
20	54	34	54	48	108

[a]In the pro-α 1(I) collagen gene, exons 33 (54 bp) and 34 (54 bp) are replaced by a single 108-bp 33/34 exon. The two joining exons (exon 6 and exon 49) are not considered in this table (see text for details).

of the C-terminal propeptide (Fig. 3). The C-terminal propeptide has a globular structure, which is stabilized by two intrachain disulfide bonds (Fig. 1). It contains three (α2 chain) or four (α1 chain) additional cysteine residus that form interchain disulfide bonds. The formation of disulfide bonds precede the triple helix formation and plays an essential role in the intracellular assembly of the three α chains (see Translational and Posttranslational Modifications).

The signal peptide, the N propeptide, and the N telopeptide of the α1 chain, as well as of the α2 chain, are coded by the first six exons (Fig. 3). The N propeptide of the α1 chain contains a cysteine-rich (10 cysteine residue) globular domain, a short triple helical domain, and a short globular domain, which harbors the N-terminal peptidase cleavage site (Fig. 1). The N-terminal propeptide of the α2 chain does not contain a cysteine-rich domain but a short globular domain (Fig. 1). The function of N-terminal propeptides is poorly understood. It has been suggested that they have a role in regulating the diameter of fibrils and/or the rate of transcription of type I collagen chains; moreover, removal of propeptides is a prerequisite for proper fibrillogenesis.

The 3′-untranslated region of both the α1(I) gene and the α2(I) gene contains more than one polyadenylation site, which explains that mRNAs with different sizes will be generated. As in many other genes, the functional role of these different polyadenylation sites are still unknown.

Translation and Posttranslational Modifications

After being transcribed, the pre-mRNA undergoes exon splicing, capping, and addition of a poly(A)tail, which gives rise to a mature mRNA. These mature mRNAs are then translated in polysomes, and the resulting proteins undergo extensive posttranslational modifications before being assembled in a triple helix and released in the extracellular space (for reviews, see Prockop *et al.,* 1979, Prockop and Kivirikko, 1984).

Signal peptides are cleaved from the chains when their N-terminal end enters the cisternae of the rough endoplasmic reticulum.

Both the pro-α1 chain and the pro-α2 chain undergo hydroxylation and glycosylation, and these modifications are essential for the assembly of type I collagen chains in a triple helix. About 100 proline residues in the Y position of the Gly-X-Y repeats, a few proline residues in the X position, and about 10 lysine residues in the Y position undergo hydroxylation, respectively, by a prolyl 4-hydroxylase, a prolyl 3-hydroxylase, and a lysyl hydroxylase. Hydroxylation of proline to hydroxyproline is critical to obtain a stable triple helix, and at 37°C, stable folding in a riple helical conformation cannot be obtained before at least 90 prolyl residues have been hydroxylated. These hydroxylases have different requirements to be active, and in particular they can act only when prolyl or lysyl residues occupy the correct position in the amino acid sequence of the α chain and when peptides are not in a triple helical configuration. Moreover, these enzymes require ferrous ions, molecular oxygen, α-ketoglutarate, and ascorbic acid to be active. This requirement for ascorbic acid could explain some of the consequences of scurvy on wound healing. When lysyl residues become hydroxylated, they serve as a substrate for a glycosyltransferase and for a galactosyltransferase, which add glucose and galactose, respectively, to the ε-OH group. As for hydroxylases, glycosylating enzymes are active only

when the collagen chains are not in a triple helical conformation. Glycosylation interferes with the packaging of mature molecules into fibrils, and increased glycosylation tends to decrease the diameter of fibrils.

While hydroxylations and glycosylations described previously occur, and after a mannose-rich oligosaccharide is added to the C propeptide of each pro- α chain, C propeptides from two α1 chains and one α2 chain associate with the formation of intrachain and interchain disulfide bonds. After prolyl residues have been hydroxylated, and the three C propeptides have associated, a triple helix will form at the C-terminal end of the molecule and then extend toward the N-terminal end; this propagation of the triple helical configuration occurs in a "zipper-like fashion" (Prockop, 1990). If prolyl residues are not hydroxylated or if interchain disulfide bonds are not formed between the C propeptides, the α chain will not fold in a triple helix. Hsp47, which is a collagen-specific molecular chaperone, then stabilizes the triple-helical forms of type I collagen molecules in the endoplasmic reticulum (Tasab *et al.,* 2000; Nagai *et al.,* 2000). As soon as procollagen molecules are in a triple helical conformation, they are transported from the rough endoplasmic reticulum into Golgi vesicles and secreted in the extracellular space. In contrast, in the absence of triple helical folding, collagen molecules will not be secreted.

In the extracellular space, a specific procollagen aminopepeptidase and a specific procollagen carboxypeptidase cleave the propeptides, giving rise to mature collagen molecules (Fig. 1). Cleavage of the propeptide decreases the solubility of collagen molecules dramatically. The free propeptides are believed to be involved in feedback regulation of collagen synthesis.

Fibrillogenesis

In the extracellular space, the molecules of mature collagen assemble spontaneously into quarter-staggered fibrils (Fig. 2); this assembly is directed by the presence of clusters of hydrophobic and of charged amino acids on the surface of the molecules. Fibril formation has been compared to crystallization in that it follows the principle of "nucleated growth" (Prockop, 1990). Once a small number of molecules have formed a nucleus, it grows rapidly to form large fibrils. During fibrillogenesis, some lysyl and hydroxylysyl residus are deaminated by a lysine oxidase, which deaminates the ε-NH_2 group, giving rise to aldehyde derivatives. These aldehydes will associate spontaneously with ε-NH_2 groups from a lysyl or hydroxylysyl residue of adjacent molecules, forming interchain cross-links. These cross-links will increase the tensile strength of the fibrils considerably.

Consequences of Genetic Mutations on Type I Collagen Formation

Osteogenesis imperfecta (also known as "brittle bone disease") is a genetic disease characterized by an extreme fragility of bones. Genetic studies have shown that it is due to a mutation in the coding sequence of either the pro-α1(I) gene or the pro- α2(I) gene, and more than 150 mutations have been identified (for review, see Kuivaniemi *et al.,* 1997). Most severe cases of osteogenesis imperfecta result from mutations that lead to the synthesis of normal amounts of an abnormal chain, which can have three consequences. First, the structural abnormality can prevent the complete folding of the three chains in a triple helix, e.g., if a glycine is substituted by a bulkier amino acid that will not fit in the center of the triple helix. In this case, the incompletely folded triple helical molecules will be degraded intracellularly, resulting in a phenomenon known as "procollagen suicide." Second, some mutations appear not to prevent folding of the three chains in a triple helix, but presumably prevent proper fibril assembly. For example, D. Prockop's group has shown that a mutation of the pro-α1(I) gene that changed the cysteine at position 748 to a glycine produced a kink in the triple helix (Kadler *et al.,* 1991). Finally, some mutations will not prevent triple helical formation or fibrillogenesis but might modify the structural characteristic of the fibrils slightly and thus affect their mechanical properties. In all these cases, the consequence on the mechanical properties of bone is probably similar. Mild forms of osteogenesis imperfecta most often result from a functionally null allele, which decreases the production of normal type I collagen. Null mutations are usually the result of the existence of a premature stop codon or of an abnormality in mRNA splicing. In these cases, the abnormal mRNA appears to be retained in the nucleus (Redford-Badwal *et al.,* 1996; Johnson *et al.,* 2000). A mouse model of *osteogenesis imperfecta* has been obtained by using a knock-in strategy that introduced a $Gly^{349} \rightarrow Cys$ mutation in the pro-α1(I) collagen gene (Forlino *et al.,* 1999). This model faithfully reproduced the human disease.

Ehlers-Danlos syndrome type VIIA and VIIB are two rare dominant genetic diseases characterized mainly by an extreme joint laxity. They result from mutations in the pro-α1(I) collagen gene (Ehlers-Danlos syndrome type VIIA) or in the pro-α2(I) collagen gene (Ehlers-Danlos syndrome type VIIB) that interfere with the normal splicing of exon 6, and a little less than 20 mutations have been described. These mutations can affect the splice donor site of intron 7 or the splice acceptor site of intron 5; in the latter case, there is efficient recognition of a cryptic site in exon 6 (Byers *et al.,* 1997). Thus, these mutations induce a partial or complete excision of exon 6. They do not appear to affect the secretion of the abnormal pro-collagen molecules, but they are responsible for the disappearance of the cleavage site of the N-terminal propeptide and thus for the presence of patially processed collagen molecules in fibrils that fail to provide normal tensile strength to tissues (Byers *et al.,* 1997). Nevertheless, these mutations seem to affect the rate of cleavage of the N-terminal propeptide rather than to completely prevent it, which explains that the phenotype is less severe than for patients who do not have a functional N-proteinase (Ehlers-Danlos syndrome type VIIC).

Regulation of Type I Collagen Synthesis

Different cytokines, hormones, vitamins, and growth factors can modify type I collagen synthesis by osteoblasts and/or fibroblasts (Table II). The effects of these molecules have been studied mainly *in vitro* using either bone organ cultures or cell cultures. In only a few instances, the *in vivo* effects of these factors on type I collagen synthesis have been studied. A degree of complexity is due to the fact that some factors can act directly on type I collagen synthesis but can also act indirectly by modifying the secretion of other factors, which will themselves affect type I collagen synthesis. For example, TNF-α will directly inhibit type I collagen production, but it will also induce the secretion of prostaglandin E_2 (PGE_2) and of IL-1, which will directly affect type I collagen production. Furthermore, PGE_2 will induce the production of IGF-1, which also modifies the rate of type I collagen synthesis.

Growth Factors

Transforming Growth Factor β

In mammals, the transforming growth factor β (TGF-β) family consists of three members (TGF-β1, TGF-β2, and TGF-β3), which have similar biological effects but have different spatial and temporal patterns of expression. These three molecules are part of a large family, the TGF-β superfamily, which also contains proteins such as bone morphogenetic proteins (Kingsley, 1994). TGF-βs are secreted by many cell types, including monocytes/macrophages, lymphocytes, platelets, fibroblasts, osteoblasts, and osteoclasts (Thompson *et al.,* 1989; Thorp *et al.,* 1992). Synthesis by bone cells is quantitatively important because, *in vivo*, the highest levels of TGF-β are found in platelets and bone (Seyedin *et al.,* 1985). Nearly all cells, including osteoblasts and fibroblasts, have TGF-β receptors (Massagué, 1990).

Regulation of type I collagen synthesis by TGF-β has been studied mostly using TGF-β1. It is secreted as pro-TGF-β1, and its propeptide is cleaved in the extracellular space, giving rise to mature TGF-β1. Mature TGF-β1 then associates noncovalently with a dimer of its N-terminal propeptide (also called LAP, for latency-associated peptide), which is itself often disulfide linked to other proteins called LTBPs (for latent TGF-β1 binding proteins). These complexes are devoid of biological activity and can be considered as a storage form of TGF-β1. They increase the stability of TGF-β1 and target it to cell surfaces and extracellular matrix. One of the key steps in regulating the activity of TGF-β1 is the cleavage of latent TGF-β1 from LAP and LTBPs, but the factors responsible for the *in vivo* transformation of latent TGF-β1 into active TGF-β1 are still poorly known. Calpain, cathepsin, or oxygen-free radicals can activate TGF-β1 *in vitro,* but their *in vivo* roles are unknown. Plasmin can activate TGF-β1 *in vitro,* and different groups have postulated that it might play a role *in vivo* (Munger *et al.,* 1997), but plasminogen-null mice did not replicate the phenotype of TGF-β1-null mice, and the pathology of these mice could be alleviated by the removal of fibrinogen (Kulkarni *et al.,* 1993; Bugge *et al.,* 1996). Thrombospondin-1 is also able to transform latent TGF-β1 into active TGF-β1 *in vitro,* and analysis of mice harboring a targeted disruption of the corresponding gene suggests that it probably plays an important role *in vivo* (Crawford *et al.,* 1998). In particular, the phenotype of *thrombospondin-1*-null mice was relatively similar to the one of *TGF-*β1-null mice, and fibroblasts isolated from the former mice had a decreased ability to activate TGF-β1. Nevertheless, thrombospondin-1 is probably not the only molecule that activates TGF-β *in vivo* (Abdelouahed *et al.,*

Table II Schematic Representation of the Effects of Soluble Molecules on Type I Collagen Synthesis by Osteoblastic Cells

	Type I collagen		
Soluble molecule	Synthesis	Transcription	mRNA stability
TGF-β	↗	↗	↗
IGF-I	↗		
IGF-II	↗		
bFGF	↘	↘	→
TNF-α	↘	↘	→
IL-1	↘	↘	→
IFN-γ	↘	↘	↘
PGE_2[a]	↘	↘	
Corticosteroids	↘	↘	↘
PTH	↘	↘	
Vitamin D	↘	↘	→

[a] At high concentrations.

2000). Once active, TGF-β1 binds to specific receptors that belong to the family of serine-threonine kinase receptors (reviewed in Massagué, 1998). It first binds to type II receptors (TBR-II), and then type I receptors (TBR-I) bind to TGF-β1–TBR-II complexes. TGF-β1 molecules, which are present within the extracellular space as homodimers, probably bind two TBR-II and then two TBR-I. When TGF-β1 is bound to its receptors, TBR-II phosphorylates TBR-I, which becomes activated and phosphorylates intracellular proteins called Smad 2 and Smad 3. This phosphorylation modifies the conformation of the Smad proteins and enables them to heterooligomerize with Smad 4, which is another member of the Smad family of proteins. Smad 2–Smad 4 complexes and Smad 3–Smad 4 complexes are then translocated into the nucleus, where they bind to specific DNA sequences called Smad-binding elements, and act as transcription factors (Fig. 4, see also color plate). Several transcription factors and transcriptional coactivators have been shown to cooperate with Smad complexes, such as Sp1, Ap-1, PEBP2/CBF, TFE-3, ATF-2, or CBP/p300 (reviewed in Attisano and Wrana, 2000).

The role of TGF-β on type I collagen synthesis has been demonstrated both *in vivo* and *in vitro. In vivo,* subcutaneous injections of platelet-derived TGF-β in newborn mice increased type I collagen synthesis by dermal fibroblasts with formation of granulation tissue (Roberts *et al.,* 1986). Injections of platelet-derived TGF-β onto the periostea of parietal bone of newborn rats stimulated bone formation, and thus accumulation of extracellular matrix (Noda and Camilliere, 1989). Transgenic mice that overexpressed mature TGF-β1 developed hepatic fibrosis and renal fibrosis (Sanderson *et al.,* 1995). Increased expression of TGF-β2 in osteoblasts in transgenic mice resulted in an osteoporosis-like phenotype with progressive bone loss. The bone loss was associated with an increase in osteoblastic matrix deposition and osteoclastic bone resorption (Erlebacher and Derynck, 1996). Conversely, expression of a dominant-negative TGF-β receptor mutant in osteoblasts led to decreased bone remodeling and increased trabecular bone mass (Filvaroff *et al.,* 1999). In humans, mutations in the latency-associated peptide of TGF-β1 (LAP) causes Camurati–Engelmann disease, a rare sclerosing bone dysplasia inherited in a autosomal-dominant matter. It is unclear whether these mutations impair the ability of the LAP to inhibit TGF-β activity or whether the mutations cause accelerated degradation of TGF-β (Kinoshita *et al.,* 2000; Janssens *et al.,* 2000). Administration of anti-TGF-β antibodies or administration of decorin (which binds to TGF-β and neutralizes its biological activity) downmodulated extracellular matrix accumulation in a model of proliferative glomerulonephritis (Border *et al.,* 1990, 1992; Isaka *et al.,* 1996). *In vitro,* TGF-β stimulates the synthesis

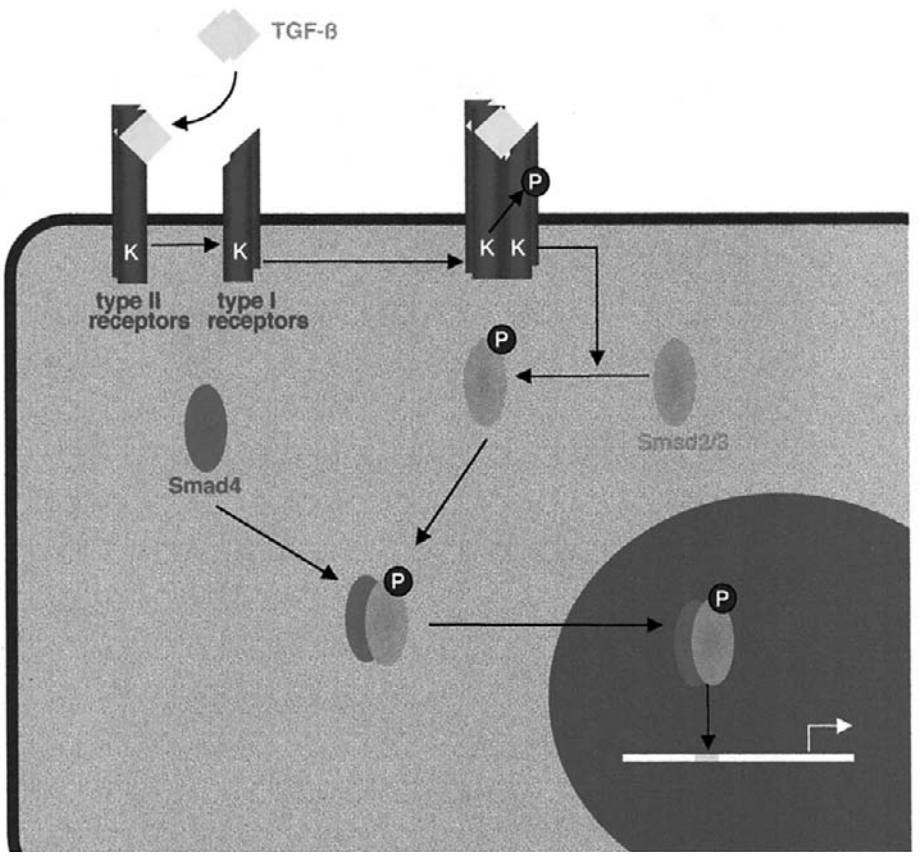

Figure 4 Schematic representation of the Smad signaling pathway mediating transcriptional effects of TGF-β (see text for details). (See also color plate.)

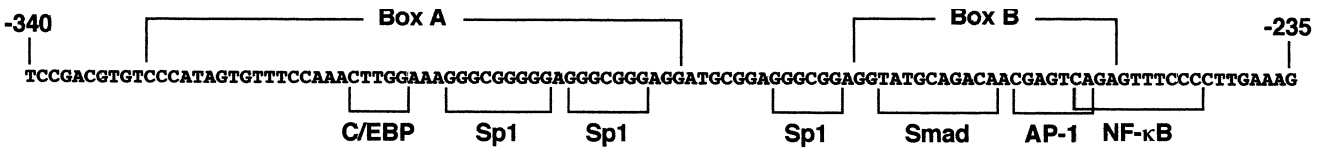

Figure 5 Schematic representation of the cytokine responsive element (CyRC) in the human pro-α2(I) promoter. Sp1, Smad, and AP-1 have been shown to bind to this element and have been implicated in the positive transcriptional effects of TGF-β, whereas C/EBP and NF-κB have been shown to participate in the inhibitory effects of TNF-α.

of most of the structural components of the extracellular matrix by fibroblasts and osteoblasts, including type I collagen (for a review, see Massague, 1990). It also decreases extracellular matrix degradation by repressing the synthesis of collagenases and stromelysins and by increasing the synthesis of tissue inhibitors of metalloproteinases (TIMPs). It increases lysyl-oxidase activity, which may favor interchain cross-linking in collagen fibrils (cf. supra) (Feres-Filho *et al.,* 1995). Finally, it stimulates the proliferation of both fibroblasts and osteoblasts, in contrast to its inhibitory effect on the proliferation of epithelial cells. Moreover, TGF-β may have a role in controlling the lineage-specific expression of type I collagen genes during embryonic development, as there is an excellent temporal and spatial correlation between activation of type I collagen genes and presence of immunoreactive TGF-β in the extracellular environment (Niederreither *et al.,* 1992). Data suggest that part of the profibrotic properties of TGF-β may be indirect, mediated by an increased production of a cysteine-rich protein called connective tissue growth factor (CTGF) (reviewed in Grotendorst, 1997). Expression of the *CTGF* gene in fibroblasts is strongly induced by TGF-β but not by other growth factors, and intradermal injections of TGF-β in neonatal mice induced an overexpression of *CTGF* in skin fibroblasts (Igarashi *et al.,* 1993; Frazier *et al.,* 1996). *In vitro,* CTGF is chemotactic and mitogenic for fibroblasts and it increases the production of type I collagen by these cells, whereas *in vivo* intradermal injections of CTGF induce the formation of a granulation tissue similar to the one induced by injections of TGF-β1 (Frazier *et al.,* 1996; Duncan *et al.,* 1999).

In vitro, TGF-β acts at a pretranslational level, increasing mRNA levels of the pro-α1(I) and pro-α2(I) transcripts. This increase in type I collagen mRNA levels can be due to an increase in the transcription rate of type I collagen genes and/or to an increase in procollagen mRNA stability, with the relative contribution of these two mechanisms depending on cell types and on cultures conditions. For example, mRNA stability was increased in confluent but not in subconfluent cultures of Swiss mouse 3T3 cells (Penttinen *et al.,* 1988).

The effect of TGF-β on transcription of the human pro-α2(I) collagen gene involves Smad complexes (Chen *et al.,* 1999, 2000a). It seems to be mediated through a sequence of the promoter located between 378 and 183 bp upstream of the start site of transcription and is called "TGF-β responsive element," as demonstrated by transfection experiments (Inagaki *et al.,* 1994). Footprinting experiments performed with this sequence revealed two distinct segments interacting with DNA-binding proteins, called box A and box B (Inagaki *et al.,* 1994) (Fig. 5). Box A, which is located between -330 and -286, corresponds to a promoter sequence of the mouse pro-α2(I) collagen gene, which plays an important role in mediating TGF-β effects on transcription (Rossi *et al.,* 1988). It contains two binding sites for Sp1 (Inagaki *et al.,* 1994; Greenwel *et al.,* 1997), as well as a binding site for C/EBP. Box B, which is located between -271 and -250, contains a CAGA box that binds Smad 3/Smad 4 complexes (Zhang *et al.,* 2000), as well as a binding site for AP-1 (Chung *et al.,* 1996) and a potential binding site for NF-kB (Kouba *et al.,* 1999). Data have shown that Smad 3/Smad 4 complexes can bind to box B and mediate TGF-β-induced stimulation of the pro-α2(I) collagen gene, in cooperation with Sp1 proteins that bind to box A (Zhang *et al.,* 2000). The role of AP-1 in mediating the effects of TGF-β has also been suggested, but it is still controversial (Chang and Goldberg, 1995; Chung *et al.,* 1996; Greenwel *et al.,* 1997; Zhang *et al.,* 2000). The transcriptional coactivator CBP/p300, which can bind to Smad complexes, also plays an important role in mediating the effects of TGF-β on the transcriptional activity of the pro-α2(I) collagen gene (Ghosh *et al.,* 2000). Thus, TGF-β probably activates the transcription of the pro-α2(I) collagen gene through the binding of a multimeric complex, which includes Smad 3/Smad 4, Sp1, CBP/p300, and possibly AP-1.

A TGF-β response element has been described about 1.6 kb upstream of the start site of transcription in the rat pro-α1(I) collagen gene (Ritzenthaler *et al.,* 1993) and between-174 and -84 bp in the human pro-α1 collagen gene (Jimenez *et al.,* 1994). The latter sequence contains an Sp1-like binding site and, as boxes A plus B in the human pro-α2(I) promoter, could bind a multimeric complex containing Sp1 or an Sp1-related protein (Jimenez *et al.,* 1994), but none of these two sequences seems to contain a potential Smad-binding site.

INSULIN-LIKE GROWTH FACTORS

Insulin-like growth factor I (IGF-I) is synthesized by many cells, including bone cells, and, unlike other growth factors, circulates in blood. It can stimulate osteoblast and

fibroblast proliferation and increase type I collagen production by these cells. Its effect on type I collagen production by osteoblasts has been demonstrated by using both fetal rat calvariae and osteoblastic cells, and it is related to an increase in corresponding mRNA transcripts (McCarthy *et al.,* 1989a; Thiebaud *et al.,* 1994; Woitge *et al.,* 2000). *In vivo,* administration of IGF-I to hypophysectomized rats increased mRNA transcripts for pro-α1(I) and pro-α2(I) collagen genes in parietal bones (Schmid *et al.,* 1989).

Insulin-like growth factor II (IGF-II), which is produced by bone cells and is one of the most abundant growth factors found in bone extracellular matrix, can also stimulate type I collagen synthesis by osteoblastic cells, with an increase in corresponding mRNA transcripts (McCarthy *et al.,* 1989a; Strong *et al.,* 1991; Thiebaud *et al.,* 1994).

Basic Fibroblast Growth Factor

Basic fibroblast growth factor (bFGF) inhibits type I collagen synthesis by bone organ cultures (Canalis *et al.,* 1988; Hurley *et al.,* 1992), by osteoblastic cells (Hurley *et al.,* 1993; McCarthy *et al.,* 1989b; Rodan *et al.,* 1989), by dermal fibroblasts (Ichiki *et al.,* 1997), and by vascular smooth muscle cells (Kyperos *et al.,* 1998). This inhibitory effect is associated with a decrease in the levels of type I collagen mRNAs (Hurley *et al.,* 1993; Rodan *et al.,* 1989), and the action of bFGF has been shown to be mediated at a transcriptional level (Hurley *et al.,* 1993; Kyperos *et al.,* 1998). Moreover, studies of MC3T3-E1 osteoblastic cells stably transfected with a construct containing either a 2.3-kb segment or a 3.6-kb segment of the rat pro-α1(I) proximal promoter cloned upstream of the CAT reporter gene have shown that the activity of the reporter gene was inhibited by bFGF only in cells harboring the 3.6-kb segment of the promoter, which suggests that a bFGF responsive element is located between 2.3 and 3.6 kb upstream of the start site of transcription (Hurley *et al.,* 1993).

Cytokines

Tumor Necrosis Factor α

Tumor necrosis factor α (TNF-α) is a cytokine secreted mainly by monocytes/macrophages, but osteoblasts seem to be able to produce TNF-α under certain conditions (Gowen *et al.,* 1990). After being cleaved from its propeptide, TNF-α undergoes trimerization and binds to type I receptors, which transduce most of the effects of TNF-α, or to type II receptors. TNF-α stimulates fibroblast and osteoblast proliferation (Gowen *et al.,* 1988), inhibits the production of extracellular matrix components, including type I collagen, and increases collagenase production and thus extracellular matrix degradation. It also counteracts the stimulation of type I collagen production induced by TGF-β. *In vivo,* inoculation of nude mice with TNF-α-producing cells decreased type I collagen production in skin and liver, impaired wound healing, and decreased TGF-β1 synthesis in skin (Buck *et al.,* 1996; Hoglum *et al.,* 1998). In contrast, in two models of pulmonary fibrosis, the administration of anti-TNF-α antibodies decreased collagen production (Piguet *et al.,* 1989, 1990), but this effect may be indirect due to an inhibition of the inflammatory reaction induced by TNF-α. In tissue culture, TNF-α ibited the production of type I collagen by rat fetal calvaria, osteoblastic cells (Bertolini *et al.,* 1986; Canalis, 1987; Centrella *et al.,* 1988; Nannes *et al.,* 1989), and by fibroblastic cells (Diaz *et al.,* 1993; Mauviel *et al.,* 1991; Solis-Herruzo *et al.,* 1988). TNF-α also increases PGE_2 and interleukin 1 (IL-1) production by osteoblasts and fibroblasts, which themselves modulate type I collagen synthesis (cf. infra).

Inhibition of collagen synthesis in fibroblasts by TNF-α is associated with a decrease in mRNA levels for the pro-α1(I) and pro-α2(I) transcripts and in the transcription of type I collagen genes (Solis-Herruzo *et al.,* 1988). In dermal fibroblasts, transfection experiments using the human pro-α2(I) collagen proximal promoter cloned upstream of a reporter gene have shown that the effects of TNF-α on transcription are mediated by the same sequence that mediates the effects of TGF-β (cf. supra), and this sequence has been renamed "cytokine responsive complex" or CyRC (Inagaki *et al.,* 1995; Kouba *et al.,* 1999; Greenwel *et al.,* 2000). TNF-α has been shown to induce the binding of NF-κB and of CCAAT/enhancer-binding proteins (C/EBP) to CyRC (Kouba *et al.,* 1999; Greenwel *et al.,* 2000). These factors may decrease the activity of the pro-α2(I) promoter by interacting with Sp1 proteins bound to CyRC, as TNF-α did not influence the activity of the pro-α2(I) proximal promoter when cells were stably transfected with a cDNA encoding a dominant-negative Sp1 (Zhang *et al.,* 2000). The antagonist activities of TGF-β and TNF-α may be the result of steric interactions between transcription factors binding to CyRC. Furthermore, TNF-α has been shown to activate AP-1, which can interact with Smad proteins and with CBP/p300 "off-DNA" (Verrecchia *et al.,* 2000).

Transfection studies performed using hepatic stellate cells and the rat pro-α1(I) proximal promoter have shown that a sequence located between -378 and -345 bp mediated the inhibitory effects of TNF-α (Iraburu *et al.,* 2000). They could be mediated through the binding of proteins of the C/EBP family, such as C/EBP delta and p20C/EBP beta, which is reminiscent of results obtained with the pro-α2(I) promoter (Iraburu *et al.,* 2000; Greenwel *et al.,* 2000). Other TNF-α response elements have been identified within the pro-α1(I) collagen gene, between -101 and -38 bp and between 68 and 86 bp, using dermal fibroblasts and hepatic stellate cells, respectively (Mori *et al.,* 1996; Hernandez *et al.,* 2000). The latter *cis*-acting element binds proteins of the Sp1 family, while the proteins binding to the former one have not been identified (Mori *et al.,* 1996; Hernandez *et al.,* 2000). Using two lines of transgenic mice harboring the growth hormone reporter gene under the control of either 2.3 kb of the human pro-α1(I) proximal promoter plus the first intron or 440 bp of this promoter plus the first intron, Chojkier's group has reported that different *cis*-acting elements mediate the

inhibitory effects of TNF-α, depending on the tissue (Buck *et al.,* 1996; Hoglum *et al.,* 1998). In skin, the inhibitory effect of TNF-α on the activity of the reporter gene was mediated through a *cis*-acting element located between -2.3 kb and -440 bp (Buck *et al.,* 1996). In contrast, in liver, it was mediated through an element located between -440 and $+$ 1607 bp (Hoglum *et al.,* 1998).

Interleukin 1

Interleukin 1 (IL-1) is a cytokine secreted mainly by monocytes/macrophages, but also by other cells, including fibroblasts, osteoblasts, synoviocytes, and chondrocytes. Two forms of IL-1 have been described, IL-1α and IL-1β, which have little primary structure homology but bind to the same receptor and have similar biological activities.

In vitro, IL-1 has an inhibitory effect on type I collagen production by osteoblasts, which is due to an inhibition of type I collagen gene transcription (Harrison *et al.,* 1990). Nevertheless, it can be masked when low doses of IL-1 are used, as this cytokine stimulates the production of PGE_2 (Smith *et al.,* 1987), which in turn can modulate type I collagen synthesis (cf. arachidonic acid derivatives). Slack *et al.* (1993) have suggested that the inhibitory effects of IL-1 on the transcription of the human pro-α1(I) collagen gene by osteoblasts could be mediated through the binding of AP-1 to the first intron of the gene, but this hypothesis has not been confirmed.

In vitro, the direct effect of recombinant IL-1 on fibroblastic cells is most often an increase in type I collagen production, but sometimes this enhancing effect is apparent only when the IL-1-induced synthesis of PGE_2 is blocked by indomethacin (Diaz *et al.,* 1993; Duncan *et al.,* 1989; Goldring and Krane, 1987). It is mediated through an increase in mRNA levels, but the respective roles of an increase in transcription rate or in mRNA stability are not known.

Interferon γ

Interferon γ (IFN-γ) is a cytokine produced both by monocytes/macrophages and by type I helper T cells. *In vivo,* IFN-γ decreases cutaneous fibrosis after wounding (Granstein *et al.,* 1989) or after insertion of an alloplastic implant (Granstein *et al.,* 1987). *In vitro,* IFN-γ decreases osteoblast and fibroblast proliferation and type I collagen synthesis by these cells (Czaja *et al.,* 1987; Diaz and Jimenez, 1997). This latter effect seems to be due to a decrease in type I collagen mRNA stability (Czaja *et al.,* 1987; Kahari *et al.,* 1990) and in the transcription rate of the pro-α1(I) collagen gene (Rosenbloom *et al.,* 1984; Diaz and Jimenez, 1997; Yuan *et al.,* 1999). Transfection studies using different segments of the human pro-α1(I) proximal promoter have shown the existence of an IFN-γ response element between -129 and -107 bp, which can bind transcription factors of the Sp1 family (Yuan *et al.,* 1999). In the human pro-α2(I) collagen gene, an IFN-γ response element has been identified between -161 and 125 bp using transfection experiments in dermal fibroblasts (Higashi *et al.,* 1998).

Other Cytokines

Interleukin 4 (IL-4) is secreted by type 2 helper T cells and by mastocytes. *In vitro,* IL-4 increases type I collagen production by human fibroblasts by increasing both transcriptional levels of type I collagen genes and stability of the corresponding mRNAs (Postlethwaite *et al.,* 1992; Serpier *et al.,* 1997).

Interleukin 10 (IL-10), which is secreted mainly by monocytes/macrophages, inhibits type I collagen genes transcription and type I collagen production by skin fibroblasts (Reitamo *et al.,* 1994).

Oncostatin M is produced mainly by activated T cells and monocytes/macrophages and belongs to the hematopoietic cytokine family. It is mitogenic for fibroblasts and stimulates type I collagen production by fibroblasts by increasing transcriptional levels of type I collagen genes (Duncan *et al.,* 1995; Ihn *et al.,* 1997). Transfection studies performed using different segments of the human pro-α2(I) collagen gene have shown that a 12-bp segment located between -131 and -120 bp, and that contains a TCCTCC motif, mediated the stimulatory effects of oncostatin M (Ihn *et al.,* 1997).

Arachidonic Acid Derivatives

PGE_2, a product of the cyclooxygenase pathway, is synthesized by various cell types, including endothelial cells, monocytes/macrophages, osteoblasts, and fibroblasts. Its production by these latter cells is increased by IL-1 and TNF-α. PGE_2 has a biphasic effect on type I collagen synthesis by bone organ cultures and by osteoblastic cells. At low concentration, it increases type I collagen synthesis, whereas at higher concentrations it decreases type I collagen synthesis (Raisz and Fall, 1990). PGE_2 induces the production of IGF-I by osteoblastic cells, and part of the stimulatory effect of low doses of PGE_2 on type I collagen production seems to be indirect, mediated by a stimulation of IGF-I production (Raisz *et al.,* 1993). Nevertheless, part of this stimulatory effect is independent of IGF-I production and persists after blocking the effects of IGF-I (Raisz *et al.,* 1993ba). It is of note that when PGE_2 is added to fibroblasts in culture, it inhibits type I collagen synthesis and decreases the levels of the corresponding mRNAs (Fine *et al.,* 1989).

Most of the effects of PGE_2 are mediated through an increase in cAMP levels (Yamamoto *et al.,* 1988), and the activation of collagen synthesis by low doses of PGE_2 could be due to such a mechanism, as cAMP analogs can also increase collagen synthesis in bone (Fall *et al.,* 1994). In contrast, the inhibitory effect of PGE_2, which has been shown to be due to an inhibition of transcription of type I collagen genes, is not mediated through a cAMP-dependent pathway but through a pathway involving the activation of protein kinase C (Fall *et al.,* 1994; Raisz *et al.,* 1993ab). A study using an osteoblastic cell line transfected stably with various segments of the rat pro-α1(I) promoter cloned upstream of a CAT reporter gene has shown that PGE_2 acts

through an element located more than 2.3 kb upstream of the start site of transcription (Raisz *et al.,* 1993a). A more recent study using fibroblasts transfected transiently with a construct containing 220 bp of the mouse pro-α1(I) proximal promoter has shown that PGE_2 can also act through a cis-acting element located within this promoter segment (Riquet *et al.,* 2000).

Hormones and Vitamins

CORTICOSTEROIDS

It has been known for many years that the administration of corticosteroids to patients results in osteoporosis and growth retardation. In mice, corticosteroids have also been shown to decrease collagen production in calvariae (Advani *et al.,* 1997). *In vitro,* incubation of fetal rat calvariae with high doses of corticosteroids, or with lower doses but for a prolonged period of time, decreased the synthesis of type I collagen (Canalis, 1983; Dietrich *et al.,* 1979); this inhibitory effect could also be observed with osteoblastic cell lines (Hodge and Kream, 1988). Nuclear runoff experiments performed using osteoblasts derived from fetal rat calvariae showed that glucocorticoids downregulate transcriptional levels of the pro-α1(I) collagen gene, as well as stability of the corresponding mRNA (Delany *et al.,* 1995). Because corticosteroids inhibit the secretion of IGF-I, part of their inhibitory effect on type I collagen synthesis could be indirect (cf. supra), but calvariae from IGF-I-null mice maintain their responsiveness to glucocorticoids (Woigte *et al.,* 2000). Glucocorticoids can also stimulate type I collagen synthesis by bone organ cultures under certain culture conditions, or by some osteoblastic cell lines, but a study performed with various osteoblastic clones derived from the same cell line has suggested that the stimulation of type I collagen synthesis was indirect, secondary to the induction of differentiation of osteoblastic cells toward a more mature phenotype (Hodge and Kream, 1988).

When added to fibroblasts in culture, corticosteroids usually decrease type I collagen synthesis by acting at a pretranslational level, which is in agreement with their *in vivo* effect on wound healing (Cockayne *et al.,* 1986; Raghow *et al.,* 1986). Stable transfection experiments using the mouse pro-α2(I) proximal promoter fused to a CAT reporter gene and transfected into fibroblasts have shown that sequences located between -2048 and -981 bp and between -506 and -351 bp were important for the corticosteroid-mediated inhibition of transcription, but the *cis*-acting element(s) responsible for this inhibition has not yet be identified (Perez *et al.,* 1992).

PARATHYROID HORMONE

In vitro, parathyroid hormone inhibits type I collagen synthesis by osteoblastic cell lines as well as by bone organotypic cultures (Dietrich *et al.,* 1976; Kream *et al.,* 1986). This inhibitory effect is associated with a decrease in the levels of procollagen mRNAs (Kream *et al.,* 1980, 1986). When calvariae of transgenic mice harboring a 1.7-, 2.3-, or 3.6-kb segment of the rat pro-α1(I) proximal promoter were cultured in the presence of parathyroid hormone, there was a parallel decrease in the incorporation of [^{3}H]proline and in the activity of the reporter gene, suggesting that the pro-α1(I) collagen promoter contains a *cis*-acting element located downstream of -1.7 kb, which mediates the inhibition of the pro-α1(I) collagen gene expression induced by parathyroid hormone (Kream *et al.,* 1993; Bogdanovic *et al.,* 2000). Furthermore, the effect of parathyroid hormone on the levels of expression of the reporter gene were mimicked by cAMP and potentiated by a phosphodiesterase inhibitor, suggesting that the inhibitory effects of parathyroid hormone are mediated mainly by a cAMP-signaling pathway (Bogdanovic *et al.,* 2000).

VITAMIN D

In vitro, the active metabolite of vitamin D_3, $1,25(OH)_2D_3$, has been shown to inhibit type I collagen synthesis by bone organ cultures and by osteoblastic cells, and this inhibitory effect is due to an inhibition of the transcription of type I collagen genes (Bedalov *et al.,* 1998; Harrison *et al.,* 1989; Kim and Chen, 1989; Rowe and Kream, 1982). Transfection studies performed with the rat pro-α1(I) proximal promoter led to the identification of a vitamin D responsive element between -2.3 and -1.6 kb (Pavlin *et al.,* 1994). Nevertheless, when transgenic mice harboring a 1.7-kb segment of the rat pro-α1(I) promoter cloned upstream of a CAT reporter gene were treated with $1,25(OH)_2D_3$, the levels of expression of the CAT reporter gene decreased, which suggests that a vitamin D response element is located downstream of -1.7 kb (Bedalov *et al.,* 1998). Similarly, when calvariae from these mice were cultured in the presence of $1,25(OH)_2D_3$, it inhibited reporter gene expression (Bedalov *et al.,* 1998). It is of note that part of the effects of vitamin D on type I collagen could be mediated through an inhibition of the production of IGF-I, as vitamin D has been shown to inhibit IGF-I production (Scharla *et al.,* 1991).

An increase in type I collagen synthesis after treatment of primary osteoblastic cells or of osteoblastic cell lines with vitamin D has also been reported, and this would also be due to a pretranslational effect of vitamin D (Franceschi *et al.,* 1988; Kurihara *et al.,* 1986). To explain the opposite effects of this hormone, it has been suggested that vitamin D could increase type I collagen synthesis in relatively immature cells, whereas it would inhibit this synthesis in more mature osteoblastic cells (Franceschi *et al.,* 1988). As for glucocorticoids, the stimulatory effect of vitamin D could be indirect, secondary to the induction of differentiation of osteoblastic cells toward a more mature phenotype.

THYROID HORMONES

Thyroid hormones have been shown to inhibit type I collagen production by cardiac fibroblasts, and this effect was associated with a decrease in the levels of pro-α1(I) mRNA (Chen *et al.,* 2000b). Transfection studies have shown that thyroid hormones modulate transcriptional levels of the

pro-α1(I) collagen gene through a *cis*-acting element located between −224 and 115 bp (Chen *et al.,* 2000b).

Transcriptional Regulation of Type I Collagen Genes

Expression of the pro-α1(I) collagen gene and the pro-α2(I) collagen gene is coordinately regulated in a variety of physiological and pathological situations. In many of these instances it is likely that the control of expression of these two genes is mainly exerted at the level of transcription, suggesting that similar transcription factors control the transcription of both genes.

This section considers successively the proximal promoter elements of these genes and then the nature of cell-specific enhancers located in other areas of these genes. Information about the various DNA elements has come from transient expression experiments in tissue culture cells, *in vitro* transcription experiments, and experiments in transgenic mice. *In vitro* transcription experiments and large measure transient expression experiments identify DNA elements that have the potential of activating or inhibiting promoter activity. These DNA elements can be used as probes to detect DNA-binding proteins. However, transient expression and *in vitro* transcription experiments do not take into account the role of the chromatin structure in the control of gene expression. Transgenic mice are clearly the most physiological system to identify tissue-specific elements; the DNAs that are tested are integrated into the mouse genome and their activities are presumably also influenced by their chromatin environment. In transgenic mice experiments, the *E scherichia coli* β-galactosidase reporter gene offers the advantage that its activity can be detected easily by the X-Gal histochemical stain so that the cell types in which the transgene is active can be identified by histology.

Transient transfection experiments using various sequences of either the pro-α1(I) proximal promoter or the pro-α2(I) proximal promoter cloned upstream of a reporter gene and introduced in fibroblasts have delineated positive and negative *cis*-acting regulatory segments in these two promoters. Footprint experiments and gel-shift assays performed using these regulatory elements as DNA templates have also delineated sequences that interact with DNA-binding proteins. However, only a few transcription factors that bind to these promoter regulatory sequences have been identified precisely.

Proximal Promoters of Type I Collagen Genes

CIS-ACTING ELEMENTS AND TRANSCRIPTION FACTORS BINDING TO THE MOUSE PRO-A2(I) PROXIMAL PROMOTER

Several functional *cis*-acting elements have been identified in the 350-bp proximal promoter of the mouse pro-α2(I) collagen gene. One of these is a binding site for the ubiquitous CCAAT-binding protein, CBF. This transcription factor is formed by three separate subunits, named A, B, and C, which have all been cloned and sequenced (Maity *et al.,* 1990; Sinha *et al.,* 1995; Vuorio *et al.,* 1990). All three subunits are needed for CBF to bind to the sequence containing the CCAAT box located between -84 and -80 and activate transcription (Maity *et al.,* 1992). *In vitro* data suggest that the A and C subunits first associate to form an A−C complex and that this complex then forms a heteromeric molecule with the B subunit (Sinha *et al.,* 1995). Mutations in the CCAAT box that prevent the binding of CBF decrease the transcriptional activity of the pro-α2(I) proximal promoter three to five times in transient transfection experiments of fibroblastic cell lines (Karsenty *et al.,* 1988). Purified CBF as well as CBF composed of its three recombinant subunits also activate the pro-α2(I) promoter in cell-free nuclear extracts previously depleted of CBF (Coustry *et al.,* 1995). Two of the three subunits of CBF contain transcriptional activation domains.

In addition to the binding site for CBF, footprinting experiments and gel-shift studies identified other binding sites in the first 350 bp of the mouse pro-α2(I) promoter. Three GC-rich sequences, located at about −160 bp (between −176 and −152 bp), −120 bp (between −131 and −114 bp), and −90 bp (between −98 and −75 bp), have been shown to interact with DNA-binding proteins by footprint experiments and gel-shift assays (Hasegawa *et al.* 1996). The corresponding regions were also protected in *in vivo* and *in vitro* footprinting experiments performed using the human pro-α2(I) promoter (Ihn *et al.,* 1996). A deletion in the mouse promoter encompassing these three footprinted sequences completely abolished the transcriptional activity of the pro-α2(I) proximal promoter in transient transfection experiments using fibroblastic cell lines. Proteins binding to these redundant sites are mainly ubiquitous proteins and include Sp1, proteins different from Sp1 that bind to an Sp1 consensus-binding site, and proteins that bind to a Krox consensus site. Proteins that bind to the two proximal segments also bind to the most upstream GC-rich segment, with the exception of CBF, suggesting a redundancy among functionally active DNA segments of the pro-α2(I) proximal promoter. Transfection experiments and gel-shift assays performed using the human pro-α2(I) promoter have suggested that the *cis*-acting element located at −160 bp also binds a repressor element (Ihn *et al.,* 1996).

The mouse pro-α2(I) proximal promoter has been shown to bind NF1/CTF about 300 bp upstream of the start site of transcription (between −310 and −285 bp), and the binding site for this transcription factor appears to be involved in mediating the effects of TGF-β on transcription of the pro-α2(I) gene (Rossi *et al.,* 1988); however, this site is not present in the human promoter. Other studies identified three short *cis*-acting GC-rich elements in the human pro-α2(I) collagen gene between −330 and −255, which are capable of binding Sp1 (Tamaki *et al.,* 1995), as well as an Ap-1-binding site (Chang and Goldberg, 1995; Chung *et al.,* 1996), a binding site for NF-κB (Kouba *et al.,* 1999), one for C/EBP (Greenwel *et al.,* 2000), and one for Smad

complexes (Zhang *et al.,* 2000) (Fig. 5). Additional studies presented evidence that a protein complex, which includes Sp1 and Smad proteins, binds to this segment of the human promoter and participates in the TGF-β activation of this promoter (Inagaki *et al.,* 1994; Greenwel *et al.,* 1997; Zhang *et al.,* 2000). This *cis*-acting element has also been shown to mediate the inhibitory effects of TNF-α (Inagaki *et al.,* 1995) through the binding of NF-κB (Kouba *et al.,* 1999) and/or C/EBP (Greenwel *et al.,* 2000) (Fig. 5). The antagonist activities of TGF-β and TNF-α may result from sterical interactions between different DNA-binding proteins. Due to the ability of this *cis*-acting element to mediate the effects of both TGF-β and TNF-α, Ramirez' group suggested naming it "cytokine responsive element" (or CyRC).

TRANSCRIPTION FACTORS BINDING TO THE MOUSE PRO-α1(I) PROXIMAL PROMOTER

In the mouse pro-α1(I) collagen gene, the sequence between -220 and the TATA box presents strong homologies with the sequence of the pro-α2(I) gene in the same region. This DNA segment contains binding sites for DNA-binding factors that also bind to the proximal pro-α2(I) promoter (Karsenty and de Crombrugghe, 1990). These DNA elements in the pro-α1(I) proximal promoter include a binding site for CBF between -90 and -115 (Karsenty and de Crombrugghe, 1990). A second CCAAT box located slightly more upstream is, however, unable to bind CBF, suggesting that sequences surrounding the CCAAT motif also have a role in CBF binding. DNA transfection experiments with the pro-α1(I) promoter showed that point mutations in the CBF-binding site decreased promoter activity (Karsenty and de Crombrugghe, 1990). The CBF-binding site is flanked by two identical 12-bp repeat sequences that are binding sites for Sp1 and probably other GC-rich binding proteins (Nehls *et al.,* 1991). In transient transfection experiments, a mutation in the binding site that prevents the binding of Sp1 surprisingly increased the activity of the promoter, and overexpression of Sp1 decreased the activity of the promoter (Nehls *et al.,* 1991). It is possible that several transcription factors with different activating potentials bind to overlapping binding sites and compete with each other for binding to these sites; the overall activity of the promoter could then depend on the relative occupancy of the different factors on the promoter DNA. Two apparently redundant sites between -190 and -170 and between -160 and -130 bind a DNA-binding protein previously designated inhibitory factor 1 (IF-1), as substitution mutations in these sites that abolished DNA binding resulted in an increase in transcription (Karsenty and de Crombrugghe, 1990). Formation of a DNA–protein complex with these two redundant elements in the pro-α1(I) promoter was also shown to be competed by the sequence of the pro-α2(I) promoter between -173 and -143, suggesting that both type I promoters contained binding sites for the same protein (Karsenty and de Crombrugghe, 1991). Experiments have shown that a new member of the Krox family, designated c-Krox, binds to these two sites in the pro-α1(I) promoter (Galéra *et al.,* 1994). In addition, c-Krox binds to a site located near the CCAAT box in the pro-α1(I) promoter and to three GC-rich sequences in the pro-α2(I) proximal promoter, located between -277 and -264 bp, between -175 and -143 bp, and near the CCAAT box, respectively (Galéra *et al.,* 1996). c-Krox appears to be expressed preferentially in skin (Galéra *et al.,* 1994), but data suggest that it is also expressed in chondrocytes and plays a role in regulating the expression of the pro-α1(II) collagen gene (Galéra *et al.,* 2000).

The 220-bp pro-α1(I) proximal promoter is extremely active both in transient transfection experiments and in *in vitro* transcription experiments (Maity *et al.,* 1988; Karsenty and de Crombrugghe, 1990). It is likely that the high transcriptional activity in these systems is due to a combination of active transcription factors that bind to this proximal promoter. Other protected regions have been identified by footprint experiments in the pro-α1(I) proximal promoter upstream of -220 bp, but the transcription factors binding to these protected sequences still remain to be identified (Ravazzolo *et al.,* 1991).

Overall, several DNA-binding proteins bind to the proximal promoters of the two type I collagen genes; these proteins, which include CBF, Sp1, and other GC-rich binding proteins, are mainly ubiquitous proteins. It is likely that their transcriptional function and eventually their DNA-binding properties offer opportunities for regulation by intracellular signaling pathways triggered by a variety of cytokines.

Organization of Upstream Segments of Type I Collagen Genes

ORGANIZATION OF UPSTREAM ELEMENTS IN THE PRO-α1(I) COLLAGEN GENE

In complete contrast with its high level activity in transient expression and *in vitro* transcription experiments, the 220-bp pro-α1(I) proximal promoter is almost completely inactive in stable transfection experiments (J. Rossert *et al.,* unpublished observations) and in transgenic mice (Rossert *et al.,* 1996). Data obtained with transgenic mice harboring various fragments of the mouse pro-α1(I) proximal promoter indicate that upstream elements are needed for the tissue-specific expression of this gene (Rossert *et al.,* 1995). These experiments also suggest a modular arrangement of separate *cis*-acting elements that activate the pro-α1(I) gene in different type I collagen-producing cells (Rossert *et al.,* 1995). Transgenic mice harboring 900 bp of the mouse pro-α1(I) proximal promoter expressed the *lacZ* and luciferase reporter genes almost exclusively in skin. When mice harbored 2.3 kb of the pro-α1(I) proximal promoter, both transgenes were expressed at low levels in skin and at high levels in osteoblasts and odontoblasts, but they were not expressed in other type I collagen-producing cells. Finally, when transgenic mice contained 3.2 kb of the pro-α1(I) proximal promoter cloned upstream of the *lacZ* gene, this reporter gene

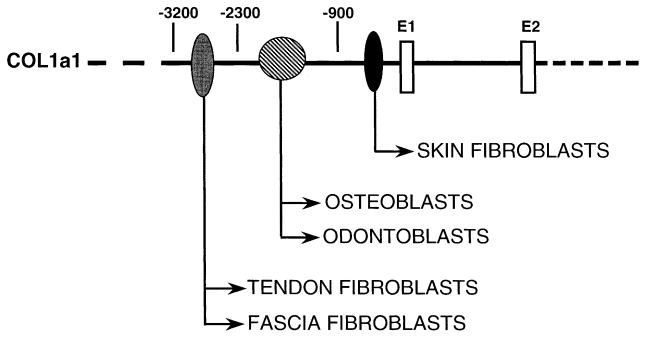

Figure 6 Schematic representation of the modular arrangement of different regulatory domains identified in the mouse pro-α1(I) collagen promoter. We postulate that still other regulatory domains remain to be identified. E1; exon 1; E2; exon 2.

was expressed at high levels in osteoblasts and in odontoblasts, but also in tendon and fascia fibroblasts. These results suggested the hypothesis that separate lineage-specific *cis*-acting elements would activate the pro-α1(I) collagen gene in osteoblasts, in odontoblasts, and in different subpopulations of fibroblasts (Fig. 6). Data obtained for human and rat pro-α1(I) collagen genes are also consistent with such a modular arrangement of different regulatory elements (Dodig *et al.,* 1996; Bogdanovic *et al.,* 1994; Liska *et al.,* 1994; Pavlin *et al.,* 1992). The direct consequence of such a modular arrangement is that it should be possible to selectively modulate the activation of type I collagen genes in well-defined subpopulations of type I collagen-producing cells. Analyses of transgenic mice harboring different segments of the mouse pro-α1(I) promoter located between -3.2 and -19.5 kb only identified a *cis*-acting element located between -7 and -8 kb that specifically enhanced reporter gene expression in uterus (Krempen *et al.,* 1999; Terraz *et al.,* 2001). Thus, *cis*-acting elements that activate the gene in most fibroblastic cells are probably located upstream of -19.5 kb or downstream of the transcription start site.

One *cis*-acting element responsible for the activation of the mouse pro-α1(I) collagen gene in osteoblasts has been identified precisely by generating transgenic mice harboring various segments of the mouse pro-α1(I) proximal promoter cloned upstream of a minimal promoter [first 220 bp of the pro-α1(I) proximal promoter] and of the *lacZ* gene (Rossert *et al.,* 1996). This 117-bp segment, located between -1656 and -1540 bp, is a minimal sequence able to induce high levels of expression of the reporter gene in osteoblasts. In these mice the transgene becomes active at the same time during embryonic development when osteoblasts first appear in the different ossification centers. This so-called "osteoblast-specific element" can be divided into three subsegments that have different functions. The 29-bp A segment, which is located most 5' (-1656 to -1628 bp), is required to activate the gene in osteoblasts. A deletion of the A element or a 4-bp mutation in the TAAT sequence of this segment completely abolished the expression of the reporter gene in osteoblasts of transgenic mice. The C segment, which is located at the 3' end of the 117-bp sequence (-1575 to -1540 bp), is required to obtain consistent high level expression of the reporter gene in transgenic mice. When this C segment was deleted, the *lacZ* gene was expressed at very low levels and only in a small proportion of transgenic mice. The function of the intermediary segment (B segment) is still poorly understood, but it could be to prevent a promiscuous expression of the gene and in particular expression of the gene in the nervous system. When this B element was deleted, the *lacZ* gene was expressed at high levels in osteoblasts, but also in some discrete areas of the nervous system. This 117-bp osteoblast-specific element is very well conserved among species (Fig. 7), and the essential role of the A element has been confirmed using transgenic mice harboring a 3.6-kb segment of the rat pro-α1(I) proximal promoter with a mutation in the TAAT sequence of the A element (Dodig *et al.,* 1996). Footprint experiments and gel-shift assays have identified a DNA-binding protein that is present only in nuclear extracts from osteoblastic cell lines, and which binds to the A element (Rossert *et al.,* 1996; Dodig *et al.,* 1996), but the DNA for this transacting factor remains to be cloned.

ORGANIZATION OF UPSTREAM ELEMENTS OF THE MOUSE PRO-α2(I) PROMOTER

The activity of the mouse 350-bp pro-α2(I) proximal promoter in transgenic mice is very low compared to that of

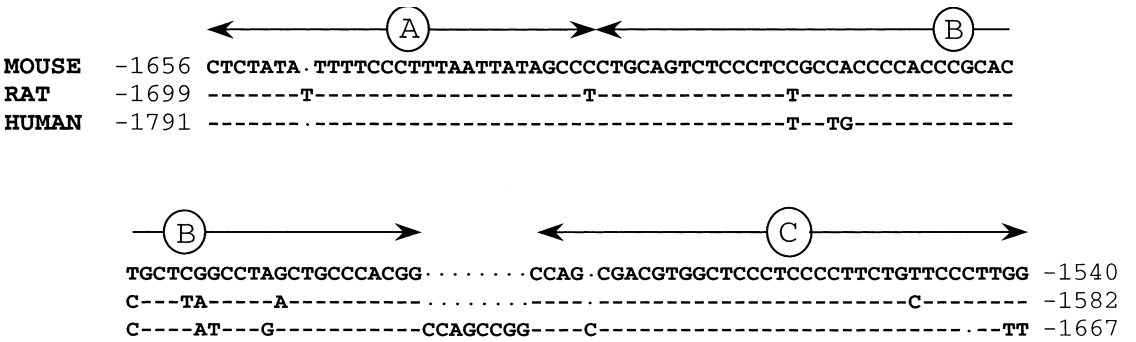

Figure 7 Sequence of the 117-bp "osteoblast-specific element." This element has been identified within the mouse pro-α1(I) collagen promoter, but highly similar sequences exist in the human and in the rat pro-α1(I) collagen promoter. From Rossert *et al.* (1996).

the corresponding endogenous gene. Although this low-level activity appears to be present selectively in fibroblasts and mesenchymal cells (Niederreither *et al.,* 1992), the precise sequences and the factors responsible for this tissue specificity have not yet been identified. Results have shown that upstream elements are also involved in the control of the tissue-specific expression of this gene and are needed for high-level expression (Bou-Gharios *et al.,* 1996). An element located 13 to 17.5 kb upstream of the start site of transcription, and named "far upstream enhancer," increased the levels of expression of the *lacZ* and luciferase reporter genes considerably when it was cloned upstream of the 350-bp mouse pro-α2(I) proximal promoter. Moreover, this element by itself contributed to the tissue-specific expression of a reporter gene. Indeed, when it was cloned upstream of a minimal promoter that has no tissue-specific expression by itself [first 220 bp of the pro-α1(I) proximal promoter], it conferred a tissue-specific expression to the *lacZ* reporter gene in transgenic mice.

Interestingly, in transgenic mice harboring the *lacZ* reporter gene cloned downstream of a pro-α2(I) promoter segment containing the far upstream enhancer, fibroblastic cells expressed the *lacZ* reporter gene at very high levels but only a subset of osteoblastic cells expressed this reporter gene, whereas odontoblasts and fully differentiated tendon fibroblasts did not (Bou-Gharios *et al.,* 1996). In 15.5-day embryos and newborn mice, the reporter gene was expressed selectively in the mandible, in the clavicles, and mainly in the growing parts of the calvaria, whereas other bones, including long bones of the limbs and ribs, essentially did not express the *lacZ* gene. Only occasionally could some expression be seen in regions of periosteal ossification, but areas of endochondral ossification were always negative. Moreover, in addition to this spatially restricted pattern of expression, there was also a temporal restriction in the pattern of expression of the *lacZ* reporter gene in osteoblasts, and the regions that expressed the reporter gene at highest levels correlated with regions of new bone growth. The molecular mechanisms underlying this heterogenous expression of the *lacZ* reporter gene in osteoblasts are still unknown, but two different explanations could account for it. First, the osteoblasts that form intramembranous bone and the ones that form endochondral bone could respond to different genetic programs of development. In addition to the program directing expression of both type I collagen genes in all osteoblasts, illustrated by transgenic mice harboring the osteoblast element present in the pro-α1(I) collagen promoter, a program would exist that would be mainly active in osteoblasts at the growing edges of membranous bones. Second, expression of the *lacZ* reporter gene in newly formed osteoblasts of membranous bones could be due to the fact that the *lacZ* reporter gene is expressed by mesenchymal precursors of fully differentiated osteoblasts and that the *E. coli* β-galactosidase protein can still be detected some time after the gene is no longer transcribed actively. Besides this heterogenous expression of the *lacZ* reporter gene in bone, the lack of expression of the *lacZ* gene in odontoblasts and in fully differentiated tendon fibroblasts suggests that other elements exist that control expression in these cells and strongly supports a modular organization of different regulatory domains in the mouse pro-α2(I) promoter, as described for the pro-α1(I) promoter.

MODE OF ACTION OF TISSUE-SPECIFIC ELEMENTS

The mode of action of the different lineage-specific transcription elements and their postulated cognate-binding proteins is still unknown, but a study of hypersensitive sites (Bou-Gharios *et al.,* 1996; Liau *et al.,* 1986) and *in vivo* footprinting experiments (Chen *et al.,* 1997) strongly suggest that the chromatin structure of discrete areas in the regulatory regions of type I collagen genes is different in cells when these genes are being transcribed actively compared to cells in which they are silent. These experiments suggest that in intact cells expressed ubiquitously, transcription factors such as CBF bind to the proximal promoters of type I collagen genes only in cells in which the genes are transcribed actively. While *in vivo* footprint experiments show a protection of the CCAAT box in different fibroblastic cell lines, such a protection does not exist in cell lines that do not produce type I collagen. Similarly, hypersensitive sites corresponding to the far upstream enhancer of the pro-α2(I) promoter can be detected only in cells that express type I collagen. The importance of chromatin structure is also highlighted by comparison of transient and stable transfection experiments (J. Rossert *et al.,* unpublished observations). When a chimeric construct harboring the pro-α1(I) osteoblast-specific element cloned upstream of a minimal promoter and of the *lacZ* reporter gene was transfected stably in different cell lines, it was expressed in the ROS17/2 osteoblastic cell line, but not in two fibroblastic cell lines or in a cell line that does not produce type I collagen. In contrast, in transient transfection experiments, the same chimeric construction was expressed at high levels by all cell lines. These results suggest a model where the binding of a lineage-specific transcription factor to specific enhancer segments of type I collagen genes would result in opening the chromatin around the promoter and allow ubiquitous transcription factors to bind to the proximal promoter and to activate transcription of the genes.

First Intron Elements

FIRST INTRON OF THE PRO-α1(I) COLLAGEN GENE

Different negative or positive regulatory segments have been identified within the first intron, but most of the transcription factors binding to these regulatory segments are still unknown. A sequence of the first intron of the human pro-a1(I) gene located about 600 bp downstream of the transcription start site binds AP-1, and a mutation that abolished this binding diminished the expression of a reporter gene in transient transfection experiments (Liska *et al.,* 1990). Another segment of the first intron of the human gene, which extends from 820 to 1093 bp, has been shown

to inhibit the activity of a reporter gene in transient transfection experiments (Liska *et al.,* 1992). This sequence contains two binding sites for an Sp1-like transcription factor, and mutations in these two Sp1-binding sites tended to increase the activity of the reporter gene (Liska *et al.,* 1992). An Sp1-binding site is also located at about 1240 bp, in the human gene, and a frequent G→T polymorphism in this Sp1-binding site (G1242T) has been linked with low bone mineral density and increased risk of osteoporotic vertebrate fracture (Grant *et al.,* 1996), which suggests that it may be important for normal levels of type I collagen synthesis by osteoblasts.

The phenotype of Mov 13 mice suggested that the first intron of the pro-α1(I) collagen gene could play a role in the expression of this gene. These mice, which harbor a retrovirus in the first intron of the pro-α1(I) collagen gene (Harbers *et al.,* 1984), express this gene in osteoblasts and odontoblasts, but not in fibroblastic cells (Kratochwil *et al.,* 1989; Löhler *et al.,* 1984; Schwarz *et al.,* 1990). Nevertheless, the presence of tissue-specific regulatory elements in the first intron of the pro-α1(I) collagen gene has long been controversial. Two groups have reported that in transgenic mice harboring the proximal promoter of either the human or the rat pro-α1(I) gene, the pattern of expression of the reporter gene was the same whether or not these mice harbored the first intron of the pro-α1(I) collagen gene (Bedalov *et al.,* 1994; Sokolov *et al.,* 1993). In contrast, data obtained by *in situ* hybridization in transgenic mice harboring 2.3 kb of the human pro-α1(I) proximal promoter suggested that the first intron of this gene was necessary to obtain high-level expressions of the transgene in the dermis of skin (Liska *et al.,* 1994). Only mice harboring the first intron, in addition to the 2.3-kb proximal promoter segment, expressed the human growth hormone reporter gene at high levels in skin. In order to clarify this issue, Bornstein's group generated knock-in mice with a targeted deletion of most of the first intron (Hormuzdi *et al.,* 1998). Mice homozygous for the mutated allele developed normally and showed no apparent abnormalities. Nevertheless, in heterozygous mice, the mutated allele was expressed at normal levels in skin, but at lower levels in lung and muscle, and its levels of expression decreased with age in these two tissues. Thus, the first intron does not play a role in the tissue-specific expression of the pro-α1(I) gene, but it seems to be important for maintaining normal transcriptional levels of this gene in certain tissue.

First Intron of the pro-α2(I) Collagen Gene

The first intron of the mouse pro-α2(I) gene has also been shown to contain a tissue-specific enhancer in transient transfection experiments (Rossi and de Crombrugghe, 1987). In transgenic mice, however, the presence of this tissue-specific enhancer apparently had no effect on the pattern of expression of a CAT reporter gene (Goldberg *et al.,* 1992). Furthermore, the *trans*-acting factors binding to this enhancer are still unknown.

Posttranscriptional Regulation of Type I Collagen

Even if the control of expression of type I collagen genes appears to be mainly exerted at the level of transcription, type I collagen production can also be regulated at a posttranscriptional level. For example, TGF-b and IFN-g modulate not only the levels of transcription of type I collagen genes, but also the stability of the corresponding mRNAs (cf. supra). Similarly, activation of hepatic stellate cells is associated with a dramatic increase in the stability of the pro-α1(I) collagen mRNA. Run-on experiments have shown that the half-life of this mRNA was increased about 15-fold in activated rat hepatic stellate cells when compared with quiescent ones (Stefanovic *et al.* 1997).

Analysis of the 3′-untranslated region of the mouse pro-α1(I) mRNA has led to the identification of a C-rich sequence, located 24 nucleotides downstream of the stop codon, that plays a critical role in the regulation of mRNA stability (Stefanovic *et al.,* 1997) through the binding of a complex containing a protein called αCP2. When a construct containing a mutation in this C-rich sequence was transfected in NIH/3T3 fibroblasts, the half-life of the corresponding mRNA was only 35% of the half-life of the wild-type mRNA (Stefanovic *et al.,* 1997). Furthermore, αCP2 could also enhance the translational rate of the pro-α1(I) gene through interactions with poly(A)-binding proteins. The binding of αCP2 to the pro-α1(I) mRNA could be regulated by posttranlational modifications of this protein, such as phosphorylations (Lindquist *et al.,* 2000). Another protein, α1-RBF67, also binds to the 3′-untranslated region of the pro-α1(I) mRNA and may regulate its stability (Määttä *et al.,* 1994).

The sequence of the mouse pro-α1(I) mRNA surrounding the start codon could also play a role in regulating mRNA stability. This sequence has been shown to form a stem–loop structure, and mutations that prevent its formation decreased the stability of the mRNA dramatically (Stefanovic *et al.,* 1999). In cells that produce high levels of type I collagen, such as fibroblasts and activated hepatic stellate cells, a protein complex can bind to this stem–loop, provided that the mRNA is capped, and this binding probably increases the stability of the mRNA (Stefanovic *et al.,* 1999).

Perspectives

To further understand the activity of type I collagen genes in osteoblasts, the proteins that bind to osteoblast-specific elements will need to be identified and their cDNAs cloned. One will also have to examine the interactions between these and other proteins binding to osteoblast-specific enhancers and the proteins that bind to the proximal promoter elements. Similar questions will also need to be answered for other cell-type-specific enhancers that control expression of the type I collagen genes in other cell types. The mechanisms by which the chromatin of the proximal promoters of the type I collagen genes is selectively disrupted in cells that synthesize the type I collagen

chains and the role played in this disruption by DNA-binding proteins will also need to be addressed. In addition, the transcription factors that mediate the effects of cytokines need to be identified further, and the modulation of their activities in response to intracellular signaling triggered by these cytokines will need to be characterized.

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CHAPTER 13

Collagen Cross-Linking and Metabolism

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Introduction

In constituting about 90% of the matrix protein of bone, collagen clearly play an important role in determining the characteristic of the tissue. Much of the research on collagen has focused on the extensive postribosomal modifications that occur during biosynthesis of the molecule, as these intracellular changes have major influences on the assembly, cross-linking, mineralization, and degradation of collagen fibrils. The aim of this chapter is to bring together current knowledge on the mechanisms of collagen cross-linking and how these are influenced by specific postribosomal modifications. These changes are also viewed in the context of collagen metabolism, with particular reference to the utilization of certain collagen metabolites as markers of bone metabolism.

Although some 20 genetically distinct collagen types are known (von der Mark, 1999), bone contains predominantly the principal a fibrillar form, collagen type I, but with small amounts of collage V and III. Collagen V interacts with type I fibrils (Birk *et al.,* 1988) and may have some regulatory role on fibril diameter and orientation, as has been shown for cornea. Collagen III in bone is generally limited to anatomically distinct regions, such as tendon insertion sites (Keene *et al.,* 1991). Thus, for the purposes of this chapter, the properties of collagen type I will be considered, as these dominate the primarily structural function of collagen in bone.

Cross-Link Formation

As reviewed in Chapter 12, collagen type I fibrils form spontaneously within the extracellular space once the N- and C-terminal propeptides of procollagen have been removed by specific proteases. During fibrillogenesis, the final enzymatic modification of collagen occurs: conversion of lysine or hydroxylysine residues within both N- and C-terminal telopeptides to aldehydes by lysyl oxidase. Subsequently, all collagen cross-linking steps occur spontaneously by virtue of the specific alignment of molecules within the fibrils.

As indicated in an overview of the cross-linking process (Fig. 1), the hydroxylation state of telopeptide lysine residues is crucial in determining the pathway of collagen cross-linking; this step is determined by an intracellular modification during collagen biosynthesis.

Hydroxylation of telopeptide lysine residues is known to be accomplished by a separate enzyme system to that which hydroxylates lysines in the central chain portion destined to become the helix. Indirect evidence for the existence of an enzyme, now referred to as telopeptide lysyl hydroxylase (TLH), was obtained from the lack of effect of purified helical lysyl hydroxylase on isolated telopeptides (Royce and Barnes, 1985). More direct evidence has been obtained from a family with a rare form of osteogenesis imperfecta, Bruck syndrome, characterized by bone fragility in affected individuals: their bone collagen lacks any cross-links derived from the hydroxylysine pathway and contains only immature, telopeptide lysine-derived cross-links (Bank *et al.,* 1999). It has been suggested that of the known variants of helical lysyl hydroxylase (procollagen-lysine, 2-oxoglutarate, 5-dioxygenase 1, 2, and 3: PLOD1–3), it was tissue-specific expression of PLOD2 that largely accounted for telopeptide hydroxylation in osteoblastic cells (Uzawa *et al.,* 1999). PLOD2, which has been localized to chromosome 3, is, however, unlikely to be a candidate for the defect in Bruck syndrome, as the latter was shown to be located on chromosome 17 (Bank *et al.,* 1999). There also appear to be

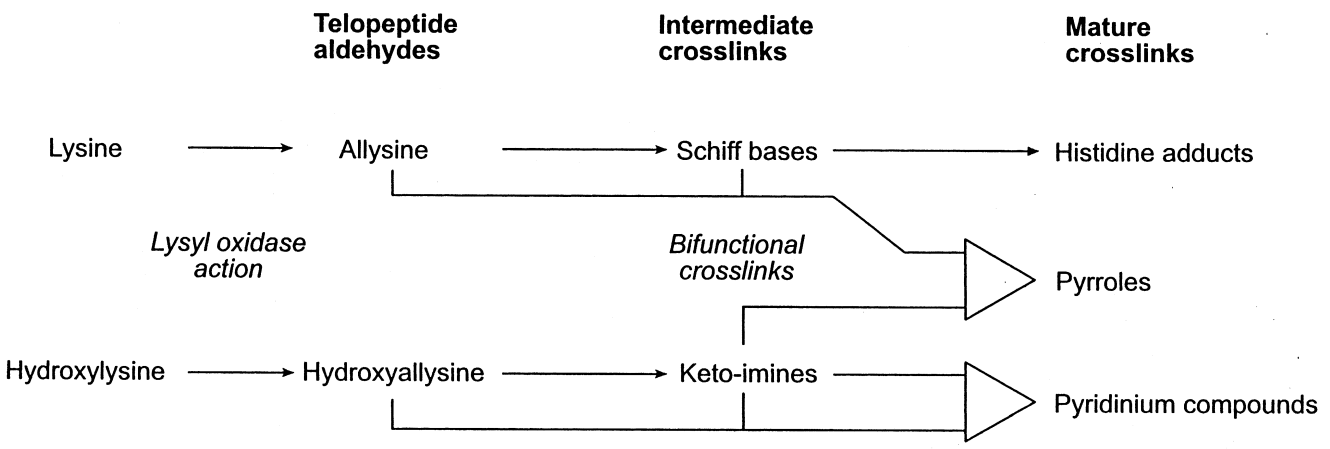

Figure 1 Formation of collagen cross-links from lysine- or hydroxylysine-derived telopeptide aldehydes giving rise to Schiff base or ketoimine difunctional bonds, respectively. On maturation, the Schiff bases are converted primarily to nonreducible, histidine adducts, whereas the ketoimines react with hydroxylysine aldehyde or a second ketoimine to give pyridinium cross-links. Pyrrole cross-link formation requires the presence of both lysine- and hydroxylysine-derived products.

tissue-specific forms of telopeptide lysyl hydroxylase, as indicated by the normal patterns of cross-linking observed in the adjacent ligament (mainly collagen type I) and cartilage (collagen type II) of Bruck syndrome patients (Bank *et al.,* 1999). Further studies of the TLH enzyme systems should provide a better understanding of the tissue-specific control of collagen cross-linking.

Cross-Link Structure

Intermediate Cross-Links

The preponderance of hydroxylysine aldehydes in bone collagen telopeptides ensures that most of the difunctional cross-links initially formed are relatively stable bonds. Thus, in contrast to tissues such as skin, where the telopeptide lysine aldehydes interact with adjacent molecules to give Schiff base (-N = CH-) cross-links, the presence of the hydroxyl group allows an Amadori rearrangement to a more stable, ketoimine form. Both the Schiff base and the ketoimine forms of cross-link are reducible by borohydride, a technique that enabled the Schiff base compounds to be stabilized for identification (Bailey *et al.,* 1974). Although the ketoimine bonds are sufficiently stable to allow isolation of peptides containing these bonds, the cross-links are quantified after reduction with borohydride to the well-characterized compounds dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL).

The reducible, bifunctional cross-links are referred to as intermediates because of their conversion during maturation of the tissue to nonreducible compounds, which are generally trivalent. Such a process can therefore be considered to provide additional stability to the fibrillar network, although, because of some ambiguities in the mechanisms involved, this has not been demonstrated directly.

Pyridinium Cross-Links

One of the first maturation products of the intermediate cross-links to be identified was pyridinoline (PYD) or hydroxylysyl pyridinoline (HP), a trifunctional 3-hydroxypyridinium compound (Fujimoto *et al.,* 1978). An analogue, deoxypyridinoline (DPD) or lysyl pyridinoline (LP), has also been identified in bone (Ogawa *et al.,* 1982). Both of these compounds (Fig. 2) are derived from intermediate ketoimines by reaction either with another difunctional cross-link (Eyre and Oguchi, 1980) or with a free hydroxylysine aldehyde group (Robins and Duncan, 1983). The chemistry of these two proposed mechanisms are very similar, but there are implications in terms of structural function of the cross-links. The involvement of two difunctional compounds results in a cross-link between three collagen molecules, whereas the alternative mechanism is more likely to link only two molecules (see Fig. 2).

Pyrroles

The notion that collagen contained pyrrolic cross-links was developed by Scott and colleagues (1981) based on the observation that tissues solubilized by enzyme treatment gave a characteristic pink color with *p*-dimethylaminobenzaldehyde. These compounds were termed Ehrlich chromogens (EC) and, in later experiments, diazo-affinity columns were used to bind covalently the pyrrole-containing peptides from enzyme digests of bone (Scott *et al.,* 1983) and skin (Kemp and Scott, 1988); these were partially characterized by amino acid analysis. A similar affinity chromatography approach was used to demonstrate that Ehrlich chromogen cross-links were present at the same loci as the pyridinium cross-links in bovine tendon (Kuypers *et al.,* 1992). This work culminated in a proposed structure and mechanism of formation for pyrroles analogous to that for pyridinium cross-link formation: this mechanism involves

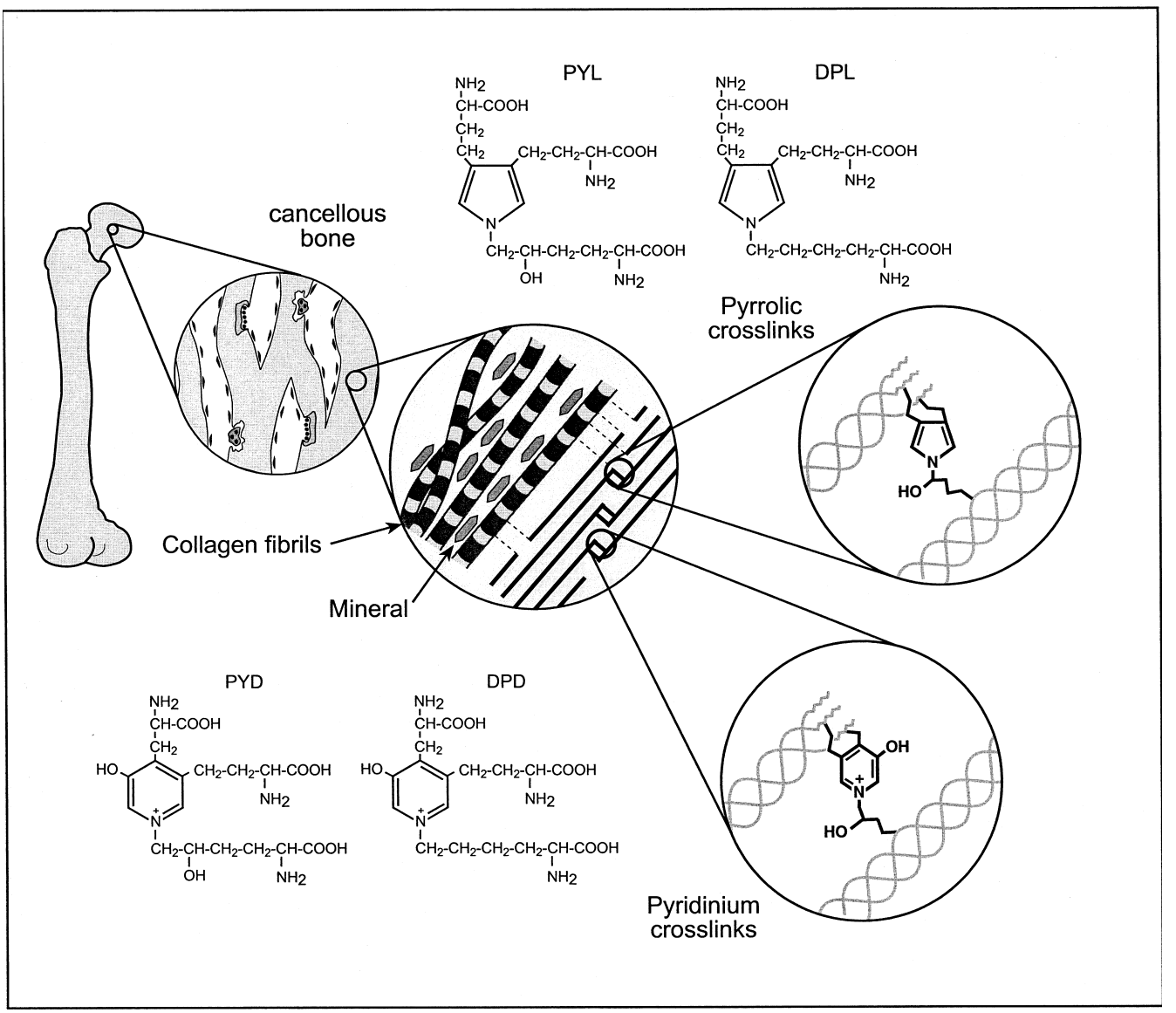

Figure 2 Stabilization of bone matrix by pyridinium and pyrrolic cross-links. Slightly higher concentrations of the mature cross-links are present in cortical compared with remodeling cancellous bone (inset) showing schematically mineralized collagen fibrils having a banded appearance arising from the precise alignment of collagen molecules in a quarter-staggered array. The overlap is stabilized by cross-links at both N- and C-terminal ends. (Insets) Pyridinium and pyrrole compounds linking N-terminal telopeptides to an adjoining helix: cross-linking may also involve telopeptides from two different molecules in register. Pyridinium cross-links are present at both N- and C-terminal sites, but pyrroles are located predominantly at the N terminus. Depending on the degree of hydroxylation of the helical lysine residue, two analogues of both the pyridinium and the pyrrolic cross-links are formed.

reaction of a difunctional, ketoimine cross-link with a lysyl aldehyde- rather than hydroxylysyl aldehyde-derived component (Kuypers *et al.,* 1992), where the latter may be a second difunctional cross-link (Hanson and Eyre, 1996).

Isolation and characterization of the pyrrolic cross-link(s) have been hampered by the instability of the pyrrole to acid or alkali hydrolysis. The use of repeated enzyme digestion of decalcified bone matrix to isolate pyrrole-containing peptides was not possible because, as these peptides were reduced in size and enriched, the pyrrole tended to oxidize or polymerize. By synthesizing new Ehrlich reagents, however, it has been possible to both stabilize the pyrrolic cross-links and facilitate their isolation and characterization by mass spectrometry (Brady and Robins, 2001). Both predicted analogues of the pyrrole (Fig. 2) were identified as the derivatized cross-link. Consistent with previous nomenclature, the trivial names pyrrololine (PYL) and deoxypyrrololine (DPL) have been proposed for the underivatized cross-links, which have been synthesized chemically (Adamczyk *et al.,* 1999).

Location of Cross-Links

Within the quarter-staggered, fibrillar array of collagen molecules, almost all cross-links have been shown to be

located at the 4D overlap position (see Fig. 2). Thus, N-telopeptide-derived cross-links are linked to the C-terminal part of the helix [residue 930 in the α1(I) chain], whereas C-telopeptide-derived cross-links are adjacent to the N-terminal end of the helix at residue 87. Because there is no oxidizable lysine in the C-telopeptide of the α2(I) chain, a more restricted number of cross-links is possible at this site compared to the N-terminal end. It has been established that the pyridinium and pyrrole cross-links are both located at these sites but that there are differences in their relative amounts. Thus, in bone collagen, the pyrrolic cross-links involve predominantly the N-terminal telopeptide (Hanson and Eyre, 1996), although there is some evidence for their location at the C-terminal end (Brady and Robins, 2001). Pyridinium cross-links are present at both ends of the molecule (Hanson and Eyre, 1996; Robins and Duncan, 1987) but, in human tissue, there is more DPD relative to PYD at the N terminus compared with the C-telopeptide-derived cross-linking region (Hanson and Eyre, 1996). The helical Hyl residue toward the N-terminal end (residue 87) is much more likely to be glycosylated than its C-terminal counterpart so that glycosylated pyridinium cross-links are relatively common, whereas glycosylated pyrrolic cross-links have not yet been detected.

Spectrum of Cross-Linking

The variations in telopeptide lysine hydroxylation give rise to a spectrum of different cross-linking patterns (Fig. 3). Bone collagen occupies a central position in this spectrum by virtue of the partial hydroxylation within the telopeptides, resulting in the formation of both pyridinium and pyrrolic cross-links. In cartilage, where telopeptide hydroxylation is essentially complete, only pyridinium cross-links are present with no pyrrolic forms. At the opposite end of the spectrum, skin also has no pyrrolic cross-links because of the absence of any telopeptide lysyl hydroxylase activity in this tissue. Helical lysyl hydroxylase activity has a much less profound effect on cross-link composition (Fig. 3) but does control the relative proportions of PYD:DPD and PYL:DPL.

Age-Related Changes in Lysine-Derived Cross-Links

The conversion of intermediate, borohydride-reducible cross-links to pyridinium compounds is well documented, but the stoichiometry is less clear. Studies of the aging *in vitro* of bone indicated a 2:1 molar ratio of the ketoimine precursor to the pyridinium cross-link (Eyre, 1981), an observation that is consistent with the proposed mechanism of formation of the trivalent cross-link (Eyre and Oguchi, 1980). Because of the difficulties in measuring specifically pyrrole cross-links, there is little information to date on their changes during maturation and with age. As relatively reactive species, pyrrolic cross-links have the potential to undergo further interactions within the fibril during the aging process. Preliminary analyses using specialized reagents (J. Brady, unpublished results) suggest

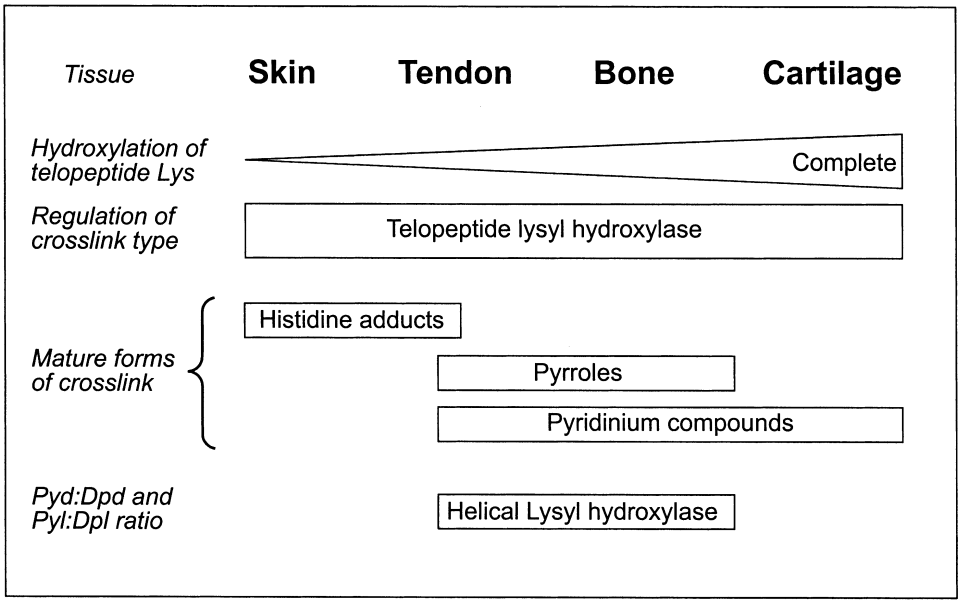

Figure 3 Spectrum of tissue-specific cross-linking resulting from the activity of telopeptide lysyl hydroxylase to give complete hydroxylation in cartilage but no significant hydroxylation of telopeptide lysine in skin collagen. The action of this intracellular enzyme regulator leads to the differences shown in mature cross-links. Pyrrolic cross-links are absent at the extremes of activity but are major components of bone and some tendons. The action of the intracellular enzyme, helical lysyl hydroxylase, regulates the relative proportions of mature pyridinium and pyrrolic cross-links in bone and tendon.

that, although the concentrations of pyrrole cross-links in adult bone collagen remain relatively constant during adult life, much higher concentrations are present in bone from adolescent and younger age groups. In this respect, therefore, pyrroles differ markedly from pyridinium cross-links, where the concentrations in bone increase in the first two decades of life and remain constant thereafter (Eyre *et al.,* 1988).

In most soft tissues, the content of intermediate, reducible bonds is very low after the cessation of growth (Robins *et al.,* 1973), but bone is unusual in retaining a relatively large proportion of reducible bonds. One possible reason for this is the continual turnover through the remodeling of bone, resulting in a higher proportion of recently formed fibrils compared with other tissues. In support of this view, low bone turnover in osteopetrotic rats was found to be associated with high concentrations of pyridinium cross-links in cancellous and compact bone, which were partially normalized by the restoration of osteoclast formation with colony-stimulating factor 1 treatment (Wojtowicz *et al.,* 1997). Another possible explanation for relatively low concentrations of mature cross-links in bone is that the mineralization process itself inhibits the maturation of reducible bonds to mature cross-links. Support for the latter has been obtained from *in vitro* experiments, which showed that the rate of conversion for demineralized bone was much higher than for bone without demineralization (Eyre, 1981). The ultimate concentrations of pyridinium cross-links attained in these experiment were, however, not markedly different and it is unclear whether the observed differences in kinetics play an important part *in vivo*. Other studies have indicated that the mineralization process causes alterations in the molecular packing of bone collagen fibrils, resulting in the cleavage of intermediate cross-links (Otsubo *et al.,* 1992). An alternative view is that the patterns of collagen cross-links produced, and by implication the structure attained, are instrumental in regulating mineralization. These conclusions derive from experiments using model systems where changes were observed in the total amounts of pyridinium cross-links and, more importantly, the Pyd/Dpd ratio in mineralizing turkey tendon (Knott *et al.,* 1997) and canine fracture callus (Wassen *et al.,* 2000).

Other Age-Related Changes

Changes in protein structure due to age-related modifications such as progressive deamidation, racemization, or nonenzymatic glycosylation of specific amino acid residues are well recognized. These changes have profound effects on the functional properties of the matrix and may alter interactions with cells and other matrix constituents, thus affecting the metabolism of the protein. Although a detailed discussion of the many protein modifications that occur during aging is beyond the scope of this chapter, specific changes due to isomerization and racemization of aspartyl residues in collagen telopeptides are discussed because of its implications for the measurement of collagen metabolites as bone resorption markers.

Isomerization and Racemization of Asp in Telopeptides

The racemization of amino acids in proteins has long been used as a means of assessing the "age" of proteins (Helfman and Bada, 1975). Different amino acids racemize at different rates, but aspartyl (or asparaginyl) residues racemize particularly rapidly because of the association with isomerization events. Conversion to a D-aspartyl or -asparaginyl residue occurs more readily when this residue is adjacent to a glycine, thus allowing the formation of a succinimide intermediate, which leads to L- and D- isomers of both α and β forms (Fig. 4). Early studies of collagen structure utilized the presence of susceptible aspartyl- or asparaginyl-glycyl bonds to affect specific cleavage at that site with hydroxylamine (Bornstein, 1970). The presence of isomeric forms of -Asp-Gly- bonds in collagen telopeptides was recognized by Fledelius and colleagues (1997a), who showed that the proportion of β-aspartyl residues within the C-terminal telopeptide of α1(I) increased with age in human and animal tissues. Measurements in urine reflected similar age changes, with higher α/β ratios detected in children compared with adults (Fledelius *et al.,* 1997a). Later studies of the Asp-Gly bond in the N-telopeptide of the α2(I) chain revealed that isomerization also occurs at this end of the molecule (Brady and Robins, 1999), although there were some differences between N- and C-terminal telopeptides in the relative α/β ratios in bone and urine.

The isomerization and racemization of aspartyl residues in telopeptides potentially have applications in monitoring the relative rates of metabolism of different pools of bone. A systematic study of the kinetics of isomerization and racemization of C-telopeptide aspartyl residues using synthetic peptides aged *in vitro* indicated that the ratio most discriminatory in terms of indicating biological age was αL/αD (Cloos and Fledelius, 2000). Analysis of these ratios in bone samples using immunoassays specific for each form of CTx indicated that children and Paget's patients had a turnover time of 2–3 months, whereas those from healthy adults and patients with osteoporosis were longer lived. In an extension to these studies, analyses of the relative rates of turnover of a wide range of human tissues using the specific CTx immunoassays (Gineyts *et al.,* 2000) suggested that collagen turnover in most of the soft tissues examined, including arteries, heart, lung, and skeletal muscle, was much higher than that in bone. These rather surprising results probably arise because of the limited solubilization of the tissue with trypsin, although the amounts solubilized were not reported (Gineyts *et al.,* 2000). Without heat denaturation before trypsinization, however, only younger, less cross-linked tissue will be extracted, leading to an overestimate of the turnover rate. Reexamination of this topic with appropriate methodology is certainly warranted, but caution still needs to be exercised in quantifying enzyme digests of tissues where the immunoassays used have specific structural requirements.

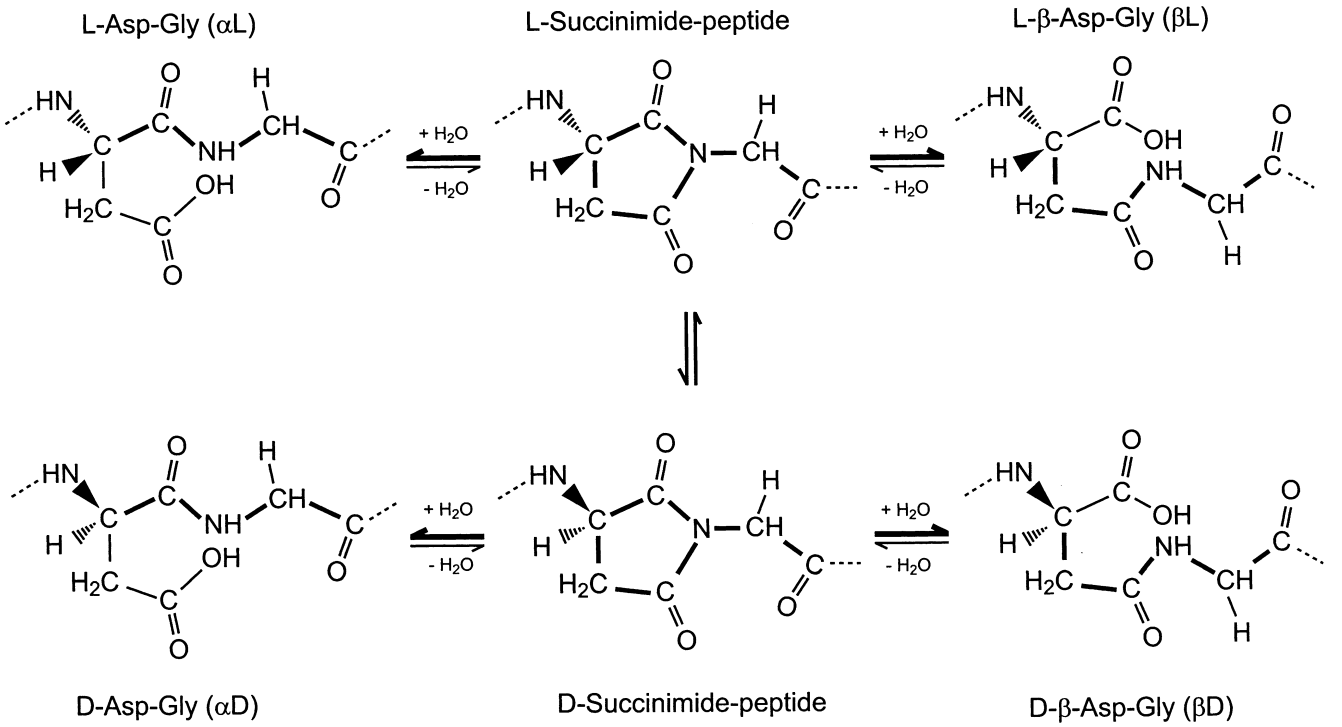

Figure 4 Isomerization and racemization of Asp-Gly peptides through succinimide intermediates giving rise to the αL, αD, βL, and βD forms detected within the C-terminal telopeptide of collagen type I in bone (Cloos and Fidelius, 2000). Interchange is predominantly through the succinimide, but some direct racemization of the peptides may also occur.

Degradative Pathways

Action of Osteoclastic Cells

The major role of osteoclasts in bone resorption has been discussed extensively in this volume and will not, therefore, be described here in detail. Of the many proteases expressed by osteoclasts, current evidence indicates that the cysteine proteinase cathepsin K plays a predominant part in degrading bone matrix. Initially cloned from rabbit osteoclasts (Tezuka *et al.,* 1997), cathepsin K was subsequently shown to be expressed predominantly in this cell type for other mammalian species. Unlike other cathepsins, this enzyme is capable of solubilizing demineralized, fibrillar bone collagen, and recombinant cathepsin K was shown to be more effective than the matrix metalloproteinases(MMPs) in this respect *in vitro* (Garnero *et al.,* 1998a). The importance of cathepsin K for bone metabolism *in vivo* was demonstrated by the discovery that pycnodysostosis, an autosomal recessive skeletal dysplasia, resulted from mutations in this enzyme (Gelb *et al.,* 1996). The lack of cathepsin K was later shown to give characteristic changes in the patterns of urinary bone markers (Nishi *et al.,* 1999), and confirmation that cathepsin K was important in determining the fragments produced from both N-terminal (Atley *et al.,* 2000) and C-terminal (Sassi *et al.,* 2000) sites of collagen type I was obtained from studies *in vitro*.

Although cathepsin K clearly has an important role in osteoclastic bone resorption, there are many other enzymes that may play a role. MMPs, which are abundant in bone (Knott *et al.,* 1997), include collagenases with the ability to cleave native collagen fibrils and gelatinases able to degrade further the denatured chain fragments produced.

Extraskeletal Processing of Collagen Fragments

Hepatic and Renal Influences

There is currently little evidence on whether the liver plays a significant role in the further processing of collagen fragments. Early studies showed that ^{125}I-labeled monomeric α1(I) chains injected into rats were taken up rapidly by liver endothelial and Kupffer cells (Smedsrod *et al.,* 1985). For the endothelial cells at least, this process was receptor mediated and was accompanied by lysosomal degradation of the denatured collagen chains. Whether the relatively small fragments of collagen that emanate from bone will be similarly sequestered and metabolized by the liver is unknown and further experimental evidence is needed.

In contrast, there is good evidence that the kidney has an important role in controlling the patterns of collagen degradation products from bone and other tissues. Initially, evidence was again obtained from animal experiments in which immunostaining of rat kidney sections with antibodies recognizing only denatured collagen showed large accumulations of collagen fragments in proximal renal tubules (Rucklidge *et al.,* 1986). Subsequent studies following the

fate of injected ^{3}H-labeled collagen fragments by autoradiography showed rapid uptake by proximal tubule epithelial cells and vacuolar transport to lysosomes (Rucklidge *et al.,* 1988) where antibody reactivity was lost, presumably through degradation of the peptides.

Analyses of serum and urinary concentrations of pyridinium cross-link components in children provided evidence that free pyridinium cross-links were in part produced in the kidney (Colwell and Eastell, 1996). This study showed that the proportion of free Dpd in serum was about half that in urine. Analysis of free Dpd in serum for older children revealed a negative correlation with the total cross-link output (Colwell and Eastell, 1996) and a similar correlation was noted in urine for a group of pre- and postmenopausal women (Garnero *et al.,* 1995). These data led to the hypothesis that the renal processing of collagen fragments was a saturable process whereby increased collagen turnover resulted in a progressive decrease in the proportion of free cross-links and a corresponding increase in their peptide forms (Colwell and Eastell, 1996; Garnero *et al.,* 1995; Randall *et al.,* 1996). This hypothesis is probably an oversimplification, however, as an analysis of the results for a wide range of healthy individuals and patients with metabolic bone diseases indicated only a weak correlation between the proportion of free Dpd and total cross-link output (Robins, 1998). Comparisons of serum and urinary immunoassays for telopeptide markers indicated a greater degree of renal processing of the N-terminal relative to C-terminal components (Fall *et al.,* 2000), although it is unclear whether this is related to the increased protease resistance of C-telopeptides imparted by the presence of isoaspartyl residues. Several studies have established that the patterns of collagen cross-link-containing components can be affected by various treatments for disease (Garnero *et al.,* 1995; Robins, 1995; Kamel *et al.,* 1995), and the effects of amino-bisphosphonates have received most attention in this respect.

Effects of Bisphosphonates

Much interest in this aspect was created by a report that measurements of free and peptide-bound cross-links in patients receiving acute, intravenous treatment with pamidronate for 3 days showed essentially no changes in free pyridinium cross-link concentrations, whereas there were large decreases in telopeptide-based assays and, to a lesser extent, in HPLC measurements of total cross-links (Garnero *et al.,* 1995). Although these finding appeared to over-estimate the bisphosphonate effects compared with another similar study (Delmas, 1993), subsequent investigations of the effects of longer term bisphosphonate treatment have confirmed that there are changes in the patterns of collagen degradation components. Treatment of postmenopausal women with the amino-bisphosphonate neridronate over a 4-week period resulted in a significant increase in the proportion of free Dpd (Fig. 5), with an apparently greater response to therapy in the peptide-bound fraction (Tobias *et al.,* 1996). This study also showed that there were no changes in the proportion of free Pyd, leading to an increased Pyd/Dpd ratio in the peptide fraction (Fig. 5). The changes in cross-link ratio were initially thought to represent altered tissue contributions to the cross-links, but, as Dpd is more prevalent at the N-terminal portion of collagen (Hanson and Eyre, 1996), these changes probably indicate differential effects on proteolytic degradation of the N- and C-telopeptide cross-linked regions.

Bisphosphonates appear to inhibit bone resorption through several mechanisms involving direct effects on osteoclasts and their precursors (Flanagan and Chambers, 1991; Murakami *et al.,*1995; Hughes *et al.,* 1995) or indirectly through effects on osteoblasts (Sahni *et al.,* 1993). Two classes of these compounds may be distinguished pharmacologically, with the more potent, nitrogen-containing bisphosphonates acting primarily through inhibition of protein prenylation (Benford *et al.,* 1999). Specifically, amino-bisphosphonates have been shown to activate caspase-3-like enzymes, the cysteine proteinases that act as the main executioner enzymes during apoptosis. It is conceivable, therefore, that these compounds may also affect the activity of enzymes involved in the degradation of collagen fragments. Whether this occurs in bone, which seems likely in view of the accumulation of bisphosphonates in this tissue, or in other organs involved in peptide processing is at present unknown. Thus, in addition to inhibiting bone resorption, bisphosphonates may also alter the patterns of collagen degradation products, a fact that is crucial in interpreting biochemical monitoring of these processes (see later).

Release of Cross-Linked Components from Bone *in Vitro*

The use of osteoclastic cells cultured on dentine slices or with bone particles has given information on the mechanisms and extent of collagen degradation in bone. Cross-linked N-telopeptide fragments (NTx) were shown to be released into medium from human bone, whereas no free pyridinium cross-links could be detected by HPLC (Apone *et al.,* 1997). Confocal microscopy of labeled bone surfaces has revealed the intracellular pathway of proteins, including degraded collagen type I, through osteoclasts (Nesbitt and Horton, 1997), and preliminary studies show that cathepsin K colocalized with the degrading collagen (Nesbitt *et al.,* 1999). These types of study, combining immunolocalization of collagen fragments with the response to enzyme inhibitors, provide a powerful technique to address the cellular mechanism of bone collagen resorption. Cathepsin K was shown to solubilize demineralized bone *in vitro* through cleavage at sites in both the telopeptides and within the collagen helix (Garnero *et al.,* 1998a). Size-exclusion chromatography of cathepsin K-digested bone has confirmed the extensive degradation of collagen, with immunodetection telopeptide fragments in the range of 2–12 kDa for NTx and 2–4 kDa for CTx (J. Brady and S. Robins, unpublished results).

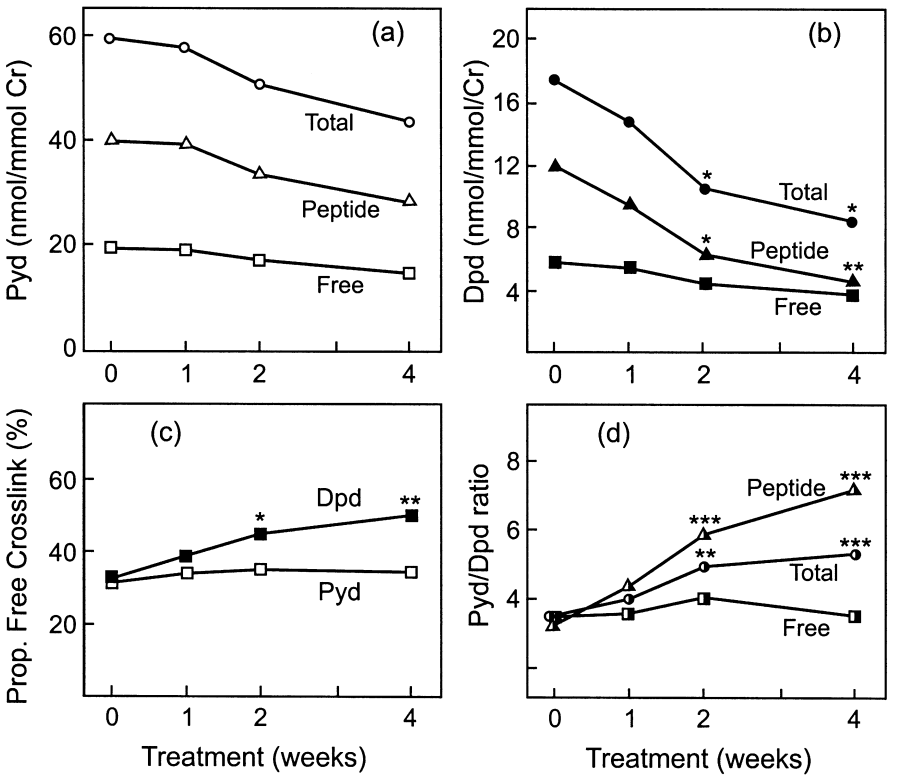

Figure 5 Effects on urinary pyridinium cross-link excretion of treating postmenopausal women with the amino-bisphosphonate neridronate for 4 weeks. In collections made at baseline, 1, 2, and 4 weeks, the concentrations of total (circles) and free (squares) Pyd (open symbols) and Dpd (closed symbols) were measured by HPLC allowing calculation of the peptide forms (triangles). Data are shown for Pyd (a) and Dpd (b), as well as the proportions of free cross-link (c) and the Pyd/Dpd ratio (d). Bisphosphonate treatment was accompanied by a significant rise in the proportion of free Dpd in urine (c) and consequent increases in Pyd/Dpd for the total and peptide forms (* $p \leq 0.01$; ** $p \leq 0.001$; *** $p < 0.001$, versus baseline). Results are the means for six individuals; error bars are omitted for clarity. Data are from Tobias *et al.* (1996).

Collagen Metabolites as Markers of Bone Metabolism

N- and C-Terminal Propeptides as Formation Markers

Procollagen type I, the initially synthesized product, is about 50% larger than the collagen molecule in fibrils, having large extension peptides at both N- and C-terminal ends. These propeptides are removed *en bloc* by separate proteases at or near the cell surface during secretion of the molecule. The intact C-terminal propeptide (PICP) containing intermolecular disulfide bonds can be detected in the blood as a 100- kDa component (Melkko *et al.,* 1990) and several commercial assays are now available. The assay has been used successfully to assess growth (Trivedi *et al.,* 1991), but its sensitivity to relatively small changes in bone formation, such as those accompanying menopause, has been rather limited.

Immunoassays for the N-terminal propeptide of procollagen I (PINP) have received renewed interest. A component isolated from amniotic fluid referred to as fetal antigen 2 was shown to be the N-propeptide (Teisner *et al.,* 1992). In serum, there are components related to the N-propeptide having apparent molecular masses of about 100 and 30 kDa, but different assays react differently with these components (Orum *et al.,* 1996; Melkko *et al.,* 1996). It has been suggested (Risteli *et al.,* 1995) that the smaller component is a degradation product related to the short helical domain, but there is currently some doubt about this interpretation (Jensen *et al.,* 1998). Although preliminary clinical data using PINP assays are encouraging, the fact the PINP and PICP assays can, in certain clinical applications, give different results emphasizes the importance of gaining further knowledge about the degradative pathways and clearance of these molecules.

Strategies for the Development of Bone Resorption Markers

Because of the importance of collagen type I in the properties of bone, it is perhaps not surprising that most bone resorption markers are based on components or fragments of this protein. Until the mid-1980s, urinary hydroxyproline was the main bone resorption assay available but, in the intervening years, advances in the knowledge of collagen structure and metabolism have not only led to the development of new markers, but also a greater appreciation of the

drawbacks inherent in hydroxyproline measurements. It is, however, important to keep in mind the difficulties with hydroxyproline measurements, as it is these criteria that form the basis for judging the improvements of the new markers.

URINARY HYDROXYPROLINE

The fact that hydroxyproline is present in all genetically distinct types of collagen (van der Mark, 1999) and in many other proteins with collagenous domains, such as the complement component C1q, lung surfactant protein, and acetylcholinesterase, creates a major drawback in terms of tissue specificity. In certain diseases, C1q turnover may be particularly elevated (Krane *et al.,* 1977), making significant contributions to hydroxyproline excretion. Nevertheless, because of continual remodeling, bone always represents a major contributor to any resorption marker. It has been estimated that in normal adults, bone degradation constitutes about 50% urinary hydroxyproline excretion, but this figure is much higher when bone turnover is elevated, as in Paget's disease (Deacon *et al.,* 1987). Collagen synthesis may also give rise to hydroxyproline excretion in two ways. The first arises because the N-propeptide of procollagen type I contains a short helical segment with hydroxyproline residues, which comprise just under 10% of the total. Release and subsequent degradation of the N-propeptide during fibril formation therefore contribute to the hydroxyproline pool. The second, perhaps more important, contributor of hydroxyproline from collagen synthesis is from the degradation of newly synthesized procollagen before secretion of the molecule. Experiments *in vitro* have shown that intracellular degradation appears to comprise a basal, lysosome-mediated level of about 15% of the total procollagen synthesized, but this can be augmented by other mechanisms to give up to 40% degradation of newly synthesized, hydroxyproline-containing protein (Bienkowski, 1984). Even taking into account the various contributors to the hydroxyproline pool, an added difficulty in using this marker is the fact that about 90% of released hydroxyproline is metabolized in the liver, a proportion that is assumed to be constant but there is little confirmatory experimental evidence. Two other problems with urinary hydroxyproline affect the applicability of the marker. The first is the well-documented contribution from dietary sources of hydroxyproline, thus necessitating the imposition of gelatin-free diets for at least 1 day before collection of the sample. The second is the practical difficulty in performing the assay, which requires hydrolysis of the sample and often an extraction with organic solvents.

Thus, criteria to be considered in judging improved bone resorption markers include specificity for bone, degradation only of mature tissue, lack of metabolism of marker, lack of necessity for dietary precautions, and ease of measurement.

URINARY HYDROXYLYSINE GLYCOSIDES

This assay was originally introduced primarily as a means to overcome the need for dietary restrictions before sampling, as the urinary glycosides were shown to be almost independent of dietary intake (Segrest and Cunningham, 1970). Collagen contains both mono- and di-saccharide derivatives O-linked to hydroxylysine, but the former, galactosyl-hydroxylysine (Gal-Hyl), predominates in bone and the urinary concentrations provide reasonable measures of resorption rate (Krane *et al.,* 1977; Bettica *et al.,* 1992). This marker may, however, be derived from all collagen precursors, and the values obtained will potentially be affected considerably by the intracellular degradation of procollagen. Renewed interest in the urinary assay was generated by the development of HPLC assay methods for urine (Moro *et al.,* 1984; Yoshihara *et al.,* 1993) and serum (Al-Dehaimi *et al.,* 1999), but measurement of Gal-Hyl remains labor-intensive and the few immunoassays that have been described (Leigh *et al.,* 1998) appear not to have been widely used.

PYRIDINIUM CROSS-LINKS

As discussed earlier, pyridinium cross-links are maturation products of lysyl oxidase-mediated cross-linking and their concentrations in urine therefore reflect only the degradation of insoluble collagen fibers and not of any precursors. The ratio Pyd:Dpd in urine is similar to the ratio of these two cross-links in bone, suggesting that both of the cross-links are likely to be derived predominantly from bone. Because of its more restricted tissue distribution, generally to mineralized tissues (Eyre *et al.,* 1984; Seibel *et al.,* 1992), Dpd is often described as a more bone-specific marker: this notion was reenforced by the close correlation between Dpd excretion and an independent, stable isotope method for determining bone turnover rate (Eastell *et al.,* 1997). Initially, the assays for pyridinium cross-links were HPLC methods with a hydrolysis and prefractionation step (Black *et al.,* 1988); despite later automation of the procedure (Pratt *et al.,* 1992), these procedures are time-consuming. The observation that the ratio of free to peptide-bound cross-links was similar in urine from healthy individuals and from patients with a range of metabolic bone disorders (Robins *et al.,* 1990; Abbiati *et al.,* 1993) opened the way for direct analysis of urine samples without the need for the hydrolysis step. This in turn led to the development of specific immunoassays for Dpd (Robins *et al.,* 1994) or for both pyridinium cross-links (Gomez *et al.,* 1996), and some of these immunoassays are now more widely available on multiple clinical analysers. The excretion of pyridinium cross-links has been shown to be independent of dietary ingestion of these compounds (Colwell *et al.,* 1993). Overall, therefore, these markers satisfy most of the criteria for bone markers outlined previously. Changes in the metabolism of the pyridinium components can, however, give rise to problems, particularly where this leads to alterations in the proportions of free to bound cross-links. Treatment with amino-bisphosphonates appears to give particular problems in this respect, as discussed later.

PEPTIDE ASSAYS

Instead of using cross-links themselves as markers, several groups have developed assays based on specific

antibodies raised against isolated collagen peptides containing the cross-links. The NTx and ICTP assays exemplify this type of development.

NTx Assay The antigen for the cross-linked N-telopeptide assay was isolated from the urine of a patient with Paget's disease of bone, and an immunoassay based on a monoclonal antibody was developed (Hanson *et al.,* 1992). This assay showed detectable reaction with urine from normal individuals, as well as large increases associated with elevated turnover. Although the antibody recognizes components in urine containing pyridinium cross-links (Hanson *et al.,* 1992), this type of cross-link is not essential and peptides containing pyrrolic cross-links may also be detected (Hanson and Eyre, 1996). Some form of cross-link must, however, be present for antibody recognition, thus ensuring that only degradation products of mature tissue are detected.

ICTP Assay This assay detects fragments from the C-telopeptide region of collagen type I. The antigen was a partially purified, cross-linked peptide from a bacterial collagenase digest of human bone collagen (Risteli *et al.,* 1993). Again, the isolated peptide contained pyridinium cross-links, but this type of bond was not essential for reactivity with the rabbit antiserum used in the assay. The ICTP assay was designed as a serum assay, which distinguishes it from most other bone resorption markers that were originally intended for urinary measurements. Although the ICTP assay fulfills many of the marker criteria discussed previously, metabolism of the analyte has proved to be an important factor limiting its application. The observation that cathepsin K cleaves within the epitope for the ICTP antibody (Sassi *et al.,* 2000) appears to explain why this assay is relatively insensitive to changes in bone remodeling mediated by normal osteoclastic activity. In contrast, pathological increases in bone degradation, such as those occurring in myeloma (Elomaa *et al.,* 1992) or metastatic bone disease (Aruga *et al.,* 1997), are well detected by the assay, as other enzyme systems, probably including MMPs, seem to be involved.

CTx Assay Development of the CTx assay involved an alternative strategy. Instead of using isolated cross-linking components, the antigen used initially was a synthetic octapeptide corresponding to the C-terminal telopeptide sequence containing the lysine residue involved in cross-linking (Bonde *et al.,* 1994). The aim of this procedure was to detect all types of cross-linking moieties and not be restricted by isolating specific components. Such a strategy was valid in detecting all collagen fragments derived from the C terminus but was less secure in avoiding the measurement of all collagen precursors. In fact, the synthetic peptide used contained a proportion of the more antigenic isoaspartyl residues, giving rise to the assay now referred to as β-CTx. The presence of the time-dependent modification in the analyte was beneficial in terms of the applicability of the assay, as this ensured that all components detected would be from mature collagen and not from any precursors. Later development of an assay specific for α-CTx (Fledelius *et al.,* 1997b) raised the possibility of utilizing the α/β ratio as an additional index of bone metabolism by indicating the "age" of the bone being resorbed. This approach proved to be valid for high turnover states such as Paget's disease (Garnero *et al.,* 1998b) but of limited value in other situations. The main reason for this is that the rate of isomerization of aspartyl residues is relatively rapid compared with the average time collagen remains in bone between deposition and resorption. The latter is probably of the order of 5 years (Eriksen, 1986), whereas the half-time of C-terminal β-Asp formation in bone is about 6 months (Cloos and Fledelius, 2000). Thus, full equilibrium of the different aspartyl isomers is generally achieved before the bone is resorbed. In a preliminary report, however, measurement of α/β ratios, including racemic variants of CTx in a large prospective study, was shown to be related to the risk of fracture in a 5-year follow-up (Garnero *et al.,* 2000). The group who fractured appeared to have less mature bone as indicated by a relative increase in α components; whether these changes resulted from an increased turnover of collagen at preexisting microfracture sites is as yet unclear.

With two α1(I) chain telopeptides in each collagen molecule, isomerization of aspartyl residues can give rise to cross-linked components containing α-α, α-β, or β-β forms (Fledelius *et al.,* 1997a), which can lead to uncertainties for data interpretation. An assay format involving two antibodies each recognizing a β-form C-telopeptide ensures that only fully mature molecules are detected (Rosenguist *et al.,* 1998; Christgau *et al.,* 1998). This form of assay, which is applicable to both urine and serum, represents a significant advance and is also available on multiple clinical analyzers.

Disturbances of Degradative Metabolism

As discussed previously, treatment with amino-bisphosphonates represents a major area of uncertainty in the application of bone resorption markers. Because the proportion of free pyridinium cross-links is increased by the treatment, the apparent decrease in bone resorption indicated by these markers is less than the true value. This change is measurable and has been well documented. The pools of peptides undergoing further degradation to give free cross-links are, however, those being measured by the NTx and CTx assays. Consequently, the changes in degradative metabolism caused by bisphosphonates will result in decreased concentrations of these peptides larger than those warranted by the decrease in true bone resorption: the extent of these overestimates of bone resorption rate cannot be ascertained easily. In practical terms, these considerations have a limited impact on the applications of these markers to monitor treatment. Where more precise indications of the true changes in bone resorption rate are required, however, the use of total (hydrolyzed) pyridinium cross-links gives results less susceptible to changes in degradative metabolism.

Concluding Remarks

In the past decade, major advances have been made in understanding the structure and metabolism of bone collagen. In terms of cross-linking, most of the structural components have been identified, but more information is needed on the functional significance of the different cross-links. This is particularly true for the pyrroles, which correlative studies have suggested exert a relatively more important effect on biomechanical properties (Knott and Bailey, 1998). The way that collagen cross-linking affects the mineralization of bone is an intriguing question, but further studies are required to establish whether the patterns of cross-links formed play a causal or merely a permissive role.

The new biochemical markers of bone metabolism are providing an additional tool for the clinical management of patients. The bone resorption markers in particular represent a significant improvement over previously available methods. Whether based on specific cross-link components or on cross-linked, collagen type I telopeptides, the methods have good specificity for bone, provided that no abnormal fibrotic pathologies, such as liver cirrhosis, are present. Most of the markers are, however, susceptible to changes in degradative metabolism, and the development of simple, direct assays that overcome these problems provides ample challenges for future research.

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CHAPTER 14

Bone Matrix Proteoglycans and Glycoproteins

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Introduction

While the organic matrix of bone is composed primarily of collagen(s) (as reviewed in a previous chapter), the existence of other noncollagenous components was first postulated by Herring and co-workers in the 1960s. Using degradative techniques, a variety of carbohydrate-containing moieties were extracted and partially characterized (Herring *et al.,* 1974). The major breakthrough in the chemical isolation and characterization of noncollagenous bone matrix proteins came with the development of techniques whereby proteins could be extracted in an intact form (Termine *et al.,* 1980; Termine *et al.,* 1981). While these procedures were suitable for the isolation of the more abundant bone matrix proteins, the advent of osteoblastic cultures that faithfully retain phenotypic traits of cells in this lineage allowed for the discovery of other proteins that end up in the matrix. While they are not as abundant as the so-called structural elements, their importance in bone physiology cannot be underestimated. This has been underscored by the identification of mutations in a number of these proteins that result in abnormal bone. Many of these low-abundance proteins are discussed in subsequent chapters.

Collagen(s) is by far and away the major organic constituent of bone matrix (Table I). However, collagen may not be the direct nucleator of hydroxyapatite deposition. Physicochemical studies based on predictions of the surface topography of the hydroxyapatite unit cell predict that such a nucleator would have a β-pleated sheet structure, a feature that is not found in the predicted structure of the collagen molecule (Addadi *et al.,* 1985). In addition, collagen is not present in the extracellular environment in an unbound form. In other words, there are a large number of matrix proteins that have been found to bind to collagen, thereby forming fibrils, and it is probable that collagen serves as a scaffolding upon which nucleators are oriented. Consequently, the nucleators of hydroxyapatite deposition are most likely members of the noncollagenous components of the organic bone matrix. This chapter discusses the major structural proteins (proteoglycans and glycoproteins) found in bone matrix. These proteins have been reviewed extensively (Gokhale *et al.,* 2001). This is an area that is expanding rapidly due to the generation of better tools, such as antibodies, cDNA probes, and genomic constructs. These reagents have been quite useful in determining the pattern and regulation of expression. Furthermore, the development of transgenic animals that either overexpress or are deficient in these proteins has also provided insight into their potential function.

Proteoglycans

This class of molecules is characterized by the covalent attachment of long chain polysaccharides (glycosaminoglycans, GAGs) to core protein molecules. GAGs are composed of repeating carbohydrate units that are sulfated to varying degrees and include chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), and heparan sulfate (HS). Different subclasses of proteoglycan are generally characterized by the structure of the core protein and by the nature of the GAG (Table II). Although other

Table I Characteristics of Collagen-Related Genes and Proteins Found in Bone Matrix

Collagens	Gene	Protein	Function
Type I	COL1A1 17q21.3-22 18 kb, 51 exons 7.2 and 5.9 kb mRNA COL1A2 7q21.3-22 35 kb, 52 exons 6.5 and 5.5 kb mRNA	$[\alpha1(I)_2\alpha2(I)]$ $[\alpha1(I)_3]$	Most abundant protein in bone matrix (90% of organic matrix), serves as scaffolding, binds and orients other proteins that nucleate hydroxyapatite deposition
Type X	COL10A1	$[\alpha1(x)_3]$	Present in hypertrophic cartilage but does not appear to regulate matrix mineralization
Others: Type III Type V	COL3A1 2q24.3-q31 COL5A1 COL5A2 2q24.3-q31 COL5A3	$[\alpha1(III)]_3$ $[\alpha1(V)_2\alpha2(V)]$ $[\alpha1(V)\alpha2(V)\alpha3(V)]$	Present in bone in trace amounts, may regulate collagen fibril diameter, their paucity in bone may explain the large diameter size of bone collagen fibrils
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types of molecules can be sulfated, proteoglycans bear greater than 95% of the sulfate groups within any organic matrix (Schwartz, 2000).

Aggrecan and Versican (PG-100)

There are two large chondroitin sulfate proteoglycans associated with skeletal tissue that are characterized by core proteins with globular domains at the amino and carboxy termini and by binding to hyaluronan to form large aggregates. Aggrecan is virtually cartilage specific, but mRNA levels have been detected in developing bone (Wong *et al.,* 1992). In the nanomelic chick, there is a mutation in the aggrecan core protein such that it is not expressed in cartilage (Primorac *et al.,* 1999). However, there is a slight effect on bones that form via the intramembranous pathway, an unexpected finding as these bones would not be expected to be affected by abnormal cartilage development.

Closely related, but not identical, is a soft connective tissue-enriched proteoglycan termed versican, which is most localized to loose, interstitial mesenchyme in developing bone. It has been hypothesized that it captures space that will ultimately become bone (Fisher *et al.,* 1985). It is this proteoglycan that is being destroyed as osteogenesis progresses. It is noteworthy that the core protein of versican contains EGF-like sequences (Zimmermann *et al.,* 1989), and release of these sequences may influence the metabolism of cells in the osteoblastic lineage. As osteogenesis progresses, versican is replaced by two members of another class of proteoglycans that contain core proteins of a different chemical nature (Fisher *et al.,* 1985).

Decorin (PG-II) and Biglycan (PG-I)

The two small proteoglycans that are heavily enriched in bone matrix are decorin and biglycan, both which contain chondroitin sulfate chains in bone, but bear dermatan sulfate in soft connective tissues. They are characterized by core proteins that contain a leucine-rich repeat sequence, a property shared with proteins that are associated with morphogenesis such as *Drosophila* toll protein and chaoptin, the leucine-rich protein of serum and adenylate cyclase (Fisher *et al.,* 1989). The three-dimensional structure of another protein containing this repeat sequence, ribonuclease inhibitor protein, has been determined by physicochemical methods, and the structure predicts a highly interactive surface for protein binding (Kobe *et al.,* 1995).

While decorin and biglycan share many properties due to the large degree of homology of their core proteins, they are also quite distinct, as best demonstrated by their pattern of expression (Bianco *et al.,* 1990). In cartilage, decorin is found in the interterritorial matrix away from the chondrocytes, whereas biglycan is in the intraterritorial matrix. In keeping with this pattern, during endochondral bone formation, decorin is widely distributed in a pattern that is virtually indistinguishable from that of type I collagen. It first appears in preosteoblasts, is maintained in fully mature osteoblasts, and is subsequently downregulated as cells become buried in the extracellular matrix to become osteocytes. However, biglycan exhibits a much more distinctive pattern of distribution. It is found in a pericellular location in distinct areas undergoing morphological delineation. It is upregulated in osteoblasts and, interestingly, it is maintained in osteocytic lacunae. It is speculated that osteocytes act as mechanoreceptors within the bone matrix (Burger *et al.,* 1999) and that proteoglycans, possibly biglycan or cell

Table II Gene and Protein Characteristics of Proteoglycans, Leucine-Rich Repeat Proteins, and Glycosaminoglycans in Bone Matrix

	Gene	Protein	Function
Versican (PG-100)	5q12-14 90 kb, 15 exons one splice variant 10, 9, 8 kb mRNAs	1×10^6 intact protein, ~360 kDa core, ~12 CS chains of 45 kDa, G1 and G3 globular domains with hyaluronan-binding sites, EGF and CRP-like sequences	May "capture" space that is destined to become bone
Decorin	12q21-23 >45 kb, 9 exons alternative promoters, 1.6 and 1.9 kb mRNA	~130 kDa intact protein, ~38-45 kDa core with 10 leucine-rich repeat sequences, 1 CS chain of 40 kDa	Binds to collagen and may regulate fibril diameter, binds to TGF-β and may modulate activity, inhibits cell attachment to fibronectin
Biglycan	Xq27 7 kb, 8 exons 2.1 and 2.6 kb mRNA	~270 kDa intact protein, ~38-45 kDa core protein with 12 leucine-rich repeat sequences, exons, 2 CS chains of 40 kDa	May bind to collagen, may bind to TGF-β, pericellular environment, a genetic determinant of peak bone mass
Fibromodulin	1q32 8.5 kb, 3 exons	59 kDa intact protein, 42 kDa core protein with leucine-rich repeat sequences, one N-linked KS chain	Binds to collagen, may regulate fibril formation, binds to TGF-β
Osteoglycin (Mimecan)	9q21,3-22 33kb, 8 exons 3.7 kb mRNA	299 aa precursor, 105 aa mature protein leucine-rich repeat sequences	Binds to TGF-β, no GAG in bone, keratan sulfate in other tissues
Osteoadherin	9q21.3-22 4.5 kb mRNA	85 kDa intact protein, 47 kDa core protein, 11 leucine-rich repeat sequences, RGD sequence	May mediate cell attachment
Hyaluronan	Multigene complex	Multiple proteins associated outside of the cell, structure unknown	May work with versican-like molecule to capture space destined to become bone

surface-associated molecules (such as heparan sulfate proteoglycans), may act as transducers of sheer forces within canaliculi. Transgenic mice that are deficient in decorin have primarily thin skin (Danielson *et al.,* 1997), whereas mice deficient in biglycan fail to achieve peak bone mass and develop osteopenia (Xu *et al.,* 1998).

Although decorin and biglycan are found in soft connective tissues that do not mineralize, their presence in osteoid makes them potential candidates as nucleators of hydroxyapatite precipitation. Decorin does not appear to be a direct nucleator, as it has no effect on hydroxyapatite precipitation or crystal growth in solution assays, and it has a low affinity for calcium. In similar assays, biglycan has varying effects depending on concentration. While biglycan has a low affinity for calcium, at low concentrations it facilitates hydroxyapatite precipitation but inhibits precipitation at high concentration. It is thought that sulfate-containing molecules must be removed prior to matrix mineralization and that they may mask sites that will ultimately act as nucleators. Consequently, it is unlikely that decorin or biglycan are initiators of matrix mineralization (reviewed in Gokhale *et al.,* 2001).

Both decorin and biglycan have been found to bind to transforming growth factor (TGF)-β and to regulate its availability and activity (Schonherr *et al.,* 2000). Decorin binds to collagen (decorating collagen fibrils), as does biglycan. Another activity has been demonstrated by *in vitro* cell attachment assays where decorin and biglycan were both found to inhibit bone cell attachment, presumably by binding to fibronectin and inhibiting its cell–matrix-binding capabilities (Grzesik *et al.,* 1994). It is not clear how this *in vitro* phenomenon relates to normal bone cell physiology, but it points to a role for these proteoglycans in modulating cell–matrix interactions.

Other Leucine-Rich Repeat Sequence Proteins and Proteoglycans

Interestingly, there are at least 60 proteins that have been found to contain the leucine-rich repeat sequence (LRR), and many of them are also proteoglycans (small leucine-rich proteoglycans, SLRPs) (Matsushima *et al.,* 2000). One LRR found in bone is osteoglycin, previously termed osteoinductive factor and later found to be a protein bound to TGF-β (Ujita *et al.,* 1995). This molecule is similar but not identical to the proteoglycan PG-Lb, which has now been found to be epiphycan, localized primarily in epiphyseal cartilage. More recently, another LRR has been localized to developing bone. Unlike other LRRs, it has an aspartic acid-rich amino sequence and, for this reason, has been named asporin (Henry *et al.,* 2001; Lorenzo *et al.,* 2001). Other members of the SLRP family found in bone include fibromodulin, which contains keratan sulfate and binds to collagen fibrils in regions distinctly different from those of decorin (Hedbom *et al.,* 1993), and osteoadherin, which also contains the cell attachment sequence RGD (Sommarin *et al.,* 1998). While these proteins appear to be "born to bind," definitive functions are not known. Other proteoglycans have been isolated from a variety of animal species by using varying techniques such as HAPGIII (so named for its ability to bind to hydroxyapatite) and PG-100, which has been shown subsequently to be homologous to versican as reviewed previously (Gokhale *et al.,* 2001).

Although not generally found in the extracellular matrix, heparan sulfate proteoglycans found associated with, or intercalated into, cell membranes may be very influential in regulating bone cell metabolism. The receptors for several growth factors (TGF-β and FGFs, to name two) have been found to associate with heparan sulfate (either bound covalently to core proteins or as free glycosaminoglycans). These associations are now known to modulate growth factor and receptor activity (Schonherr *et al.,* 2000). One class of heparan sulfate proteoglycans is linked to cell membranes by phosphoinositol linkages that are cleavable by phospholipase C (glypicans). Consequently, their activity may be in the pericellular environment or in the extracellular matrix. Intercalated heparan sulfate proteoglycans (the syndecan family) have been postulated to regulate cell growth, perhaps through association with various factors (Vlodavsky *et al.,* 1995). The complete cast of heparan sulfate proteoglycans present in the cellular and pericellular environment is not yet complete.

Hyaluronan

This unsulfated glycosaminoglycan is not attached to a protein core and is synthesized by a completely different pathway (Table II). While other glycosaminoglycans are formed by the transfer of growing glycosaminoglycan chains from a lipid carrier (dolichol phosphate) to a protein carrier, hyaluronan is synthesized in the extracellular environment by a group of enzymes that are localized on the outer cell membrane. Large amounts of hyaluronan are synthesized during early stages of bone formation and may associate with versican to form high molecular weight aggregates, although this association has not been demonstrated to occur in developing bone. Very little is known about the potential function of hyaluronan in bone formation, but in other tissues it is speculated to participate in cell migration and differentiation (Fedarko *et al.,* 1992).

Glycoproteins

Virtually all of the bone matrix proteins are modified posttranslationally to contain either N- or O-linked oligosaccharides, many of which can be modified further by the addition of phosphate and/or sulfate (Table III). In general, compared to their soft connective tissue counterparts, bone matrix proteins are modified more extensively and in a different pattern. In some cases, differences in posttranslational modifications result from differential splicing of heterogeneous nuclear RNA, but in general, it results from differences in the activities of enzymes located along the intracellular pathway of secretion. The pattern of posttranslational modifications may be cell type specific and consequently may be of use in distinguishing protein metabolism from one tissue type versus another. The development of probes and antibodies against these types of tissue-specific determinants may be of great diagnostic value.

The number of glycoproteins that have been identified in bone matrix grows by leaps and bounds every year. This is due in part to the explosion of sequence information from cDNA libraries, where one has the ability to pick up even the scarcest of clones. What follows next is a brief description of the more abundant bone matrix glycoproteins that most likely play major structural as well as metabolic roles. Other glycoprotein species have been identified primarily as growth factors, produced both endogenously and exogenously, and will be covered in more detail elsewhere in this volume.

Osteonectin (SPARC, Culture Shock Protein, and BM40)

With the development of procedures to demineralize and extract bone matrix proteins without the use of degradative enzymes, osteonectin was one of the first proteins isolated in intact form. This molecule was so named due to its ability to bind to Ca^{2+}, hydroxyapatite, and collagen and to nucleate hydroxyapatite deposition (Termine *et al.,* 1981). The osteonectin molecule contains several different structural features, the most notable of which is the presence of two EF hand high-affinity calcium-binding sites. These structures are usually found in intracellular proteins, such as calmodulin, that function in calcium metabolism (reviewed in Yan *et al.,* 1999).

Table III Gene and Protein Characteristics of Glycoproteins in Bone Matrix

	Gene	Protein	Function
Alkaline phosphatase	1 50 kb, 12 exons alternative promoters, one RFLP 2.5, 4.1 4.7 kb mRNA	Two identical subunits of ~80 kDa, disulfide bonded, tissue specific post-translational modifications	Potential Ca^{2+} carrier, hydrolyzes inhibitors of mineral deposition such as pyrophosphates
Osteonectin	5q31-33 20 kb, 10 exons, one RFLP, 2.2, 3.0 kb mRNA	~35–45 kDa, intra molecular disulfide bonds, α helical amino terminus with multiple low affinity Ca^{2+} binding sites, two EF hand high affinity Ca^{2+} sites, ovomucoid homology, glycosylated, phosphorylated, tissue-specific modifications	May mediate deposition of hydroxyapatite, binds to growth factors, may influence cell cycle, positive regulator of bone formation
Tetranectin	Two genes 12 kb, 3 exons 1 kb mRNA	21 kDa protein composed of four identical subunits of 5.8 kDa, sequence homologies with asialoprotein receptor and G3 domain of aggrecan	Binds to plasminogen, may regulate matrix mineralization

Although osteonectin is highly enriched in bone, it is also expressed in a variety of other connective tissues as specific points during development, maturation or repair processes *in vivo.* SPARC (secreted protein, acidic, rich in cysteine) was identified after induction by cAMP in teratocarcinoma cells and was found to be produced at very early stages of embryogenesis. Interestingly, if osteonectin is inactivated by the use of blocking antibodies during tadpole development, there is a disruption of somite formation and subsequent malformations in the head and trunk (Purcell *et al.,* 1993). Mice that are deficient of osteonectin present with severe cataracts (Bassuk *et al.,* 1999) and develop osteoporosis (Delany *et al.,* 2000).

Constitutive expression in the adult tissue is limited to cells associated intimately with mineralized tissues, such as hypertrophic chondrocytes, osteoblasts, and odontoblasts, and ion-transporting cells, such as mammary epithelium, distal tubule epithelium in the kidney, and salivary epithelium (cells associated with basement membrane, hence the name BM-40). Transient expression has been noted in other cell types, such as decidual cells in the uterus and in testis when cells are undergoing a maturation event. *In vitro,* expression appears to be deregulated rapidly, resulting in expression by cells that would not be expressing high levels *in situ,* hence its designation as a culture shock protein.

There have been numerous studies using both intact molecule and peptides derived from different regions. Many of these structure–function studies have been performed in endothelial cell cultures, from which culture shock protein was originally isolated. From these studies, osteonectin has been implicated in regulating the progression of the cell through the cell cycle, cell shape, cell–matrix interactions, binding to metal ions, binding to growth factors, and modulating enzymatic activities (Yan *et al.,* 1999). However, many of these activities have not been found or have not been tested in osteoblastic cultures. It should also be recognized that the activity of a peptide might not occur *in vivo* when it is taken out of context of the intact protein or naturally occurring degradative products.

Tetranectin

This tetrameric protein has been identified in woven bone and in tumors undergoing mineralization (Wewer *et al.,* 1994). This protein shares sequence homologies with globular domains of aggrecan and asialoprotein receptor, but it is not known what function it plays in bone metabolism to date.

RGD-Containing Glycoproteins

Some of the major glycoproteins in bone matrix also contain the amino acid sequence Arg-Gly-Asp (RGD), which conveys the ability of the extracellular matrix protein to bind to the integrin class of cell surface receptors (Ruoslahti, 1996) (Table IV). This binding is the basis of many cell attachment activities that have been identified by *in vitro* analysis; however, it should be noted that it is not yet clear how this *in vitro* activity translates into *in vivo* physiology. The bone matrix contains at least eight RGD-containing glycoproteins [collagen(s), thrombospondin, fibronectin, vitronectin, fibrillin, osteoadherin, osteopontin, and bone sialoprotein]. While this

Table IV Gene and Protein Characteristics of Glycoproteins in Bone Matrix - Continued RGD-Containing Glycoproteins

RGD-containing glycoproteins	Gene	Protein	Function
Thrombospondins	TSP-1 - 15q15 TSP-2 - 6q27 TSP-3 - 1q21-24 TSP-4 - 5q13 COMP - 19p13.1 4.5–16 kb, 22 exons 4.5–6.1 kb mRNA	~450 kDa molecule, three identical disulfide-linked subunits of ~150–180 kDa, homologies to fibrinogen, properdin, EGF, collagen, von Willebrand, *P. falciparum* and calmodulin, RGD at the C terminal globular domain	Cell attachment (but usually not spreading), binds to heparin, platelets, type I and V collagens, throm fibrinogen, laminin, bin, plasminogen and plasminogen activator inhibitor, histidine-rich glycoprotein, TSP-2 is a negative regulator of bone formation
Fibronectin	2p14-16, 1q34-36 50 kb in chicken, 50 exons, multiple splice forms, 6 RFLPs, 7.5 kb mRNA	~400 kDa with two non-identical subunits of ~200 kDa, composed of type I, II, and III repeats, RGD in the 11th type III repeat 2/3 from N terminus	Binds to cells, fibrin heparin, gelatin, collagen
Vitronectin	17q ~70 kDa, RGD close to N 4.5 kb, 8 exons, 1.7 kb mRNA	Cell attachment protein, terminus, homology to somatomedin B, rich in cysteines, sulfated, phosphorylated	binds to collagen, plasminogen and plasminogen activator inhibitor, and to heparin
Fibrillin	15q15-23, 5 (two different genes), 110 kb, 65 exons, 10 kb mRNA	350 kDa, EGF-like domains, RGD, cysteine motifs	May regulate elastic fiber formation
Osteopontin	4q13-21 8.2 kb, 7 exons, multiple alleles, one RFLP, one splice variant, several alleles 1.6 kb mRNA	~44-75 kDa, polyaspartyl stretches, no disulfide bonds, glycosylated, phosphorylated, RGD located 2/3 from the N-terminal	Binds to cells, may regulate mineralization, may regulate proliferation, inhibits nitric oxide synthase, may regulate resistance to viral infection, a regulator of bone resorption
Bone sialoprotein	4q13-21 15 kb, 7 exons, 2.0 mRNA	~46-75 kDa, polyglutamyl stretches, no disulfide bonds, 50% carbohydrate, tyrosine-sulfated, RGD near the C terminus	Binds to cells, may initiate mineralization
BAG-75	Gene not yet isolated, mRNA not yet cloned	~75 kDa, sequence homologies to phosphophoryn, osteopontin and bone sialoprotein, 7% sialic acid, 8% phosphate	Binds to Ca^{2+}, may act as a cell attachment protein (RGD sequence not yet confirmed), may regulate bone resorption

would appear to be a case of extreme redundancy, both *in vivo* and *in vitro* analysis indicates that the proteins are not equivalent in their abundance or pattern of expression during bone formation and in other tissues or in their *in vitro* activities (Grzesik *et al.,* 1994).

Thrombospondins

Thrombospondins are a family of multifunctional proteins. Thrombospondin-1 was first identified as the most abundant protein in platelet α granules, but is found in many tissues during development, including bone (Robey *et al.,* 1989). Subsequently, four other members have been described, including the identification of COMP (cartilage oligomeric matrix protein) as thrombospondin-5 (Adolph *et al.,* 1999; Newton *et al.,* 1999). In bone, all forms are present, synthesized by different cell types at different stages of maturation and development (Carron *et al.,* 1999). Thrombospondins have many proposed activities, including binding to a large number of matrix proteins and cell surface proteins. *In vitro,* it mediates bone cell adhesion in an RGD-independent fashion, indicating the presence of other sequences in the molecule that

are required. Furthermore, cell spreading requires the synthesis of other proteins. The thrombospondin-2-deficient mouse has been found to have increased cortical thickness compared to normal littermates (Hankenson *et al.,* 2000), perhaps due to the fact that it appears to be a stimulator of bone resorption (Carron *et al.,* 1995)

Fibronectin

Fibronectin is synthesized by many connective tissue cells and is a major component of serum. There are a large number of different mRNA splice variants such that the number of potential forms is quite high. Consequently, bone matrix could contain fibronectin that originates from exogenous as well as endogenous sources (reviewed in Romberger, 1997). The precise form that is present in cells in the osteoblastic lineage is unknown. Fibronectin is produced during early stages of bone formation and has been found to be highly upregulated in the osteoblastic cell layer. Interestingly, bone cell attachment to fibronectin *in vitro* is in an RGD-independent fashion (Grzesik *et al.,* 1994). However, this correlates well with the expression of the fibronectin receptor, $\alpha_4\beta_1$, which binds to a sequence other than RGD in the fibronectin molecule and is also expressed by some osteoblastic cells. Cell–matrix interactions mediated by fibronectin–$\alpha_4\beta_1$ binding may play a role in the maturation sequence of cells in the osteoblastic lineage.

Vitronectin

This serum protein, first identified as S-protein due to its cell-spreading activity, is found at low levels in mineralized matrix (Grzesik *et al.,* 1994). Its cell surface receptor, $\alpha_v\beta_3$, is distributed broadly throughout bone tissue. There may also be endogenous synthesis of a related form (Seiffert, 1996). In addition to cell attachment activity, it also binds to and affects the activity of the plasminogen activator inhibitor (Schvartz *et al.,* 1999).

Fibrillins

In addition to the RGD sequence, fibrillin-1 and fibrillin-2 are glycoproteins that also contain multiple EGF-like repeats. They are major components of microfibrils, and mutations in these genes lead to Marfan's syndrome, which exhibits abnormalities in bone growth (Ramirez *et al.,* 1999). It is not yet known if it is produced at a specific stage of bone formation, remodeling, or turnover; however, it is known that they associate with LTBP (latent TGF-β-binding protein) in microfibrils. (Dallas *et al.,* 2000).

Small Integrin-Binding Ligands with N-linked Glycosylation (SIBLINGs)

Several bone matrix proteins are characterized by the presence of relatively large amounts of sialic acid. Interestingly, they are clustered at 4q21-23 and appear to have arisen by gene duplication. The two best characterized, osteopontin and bone sialoprotein, also contain the RGD sequence, as does another one of the family members, dentin matrix protein-1 (DMP-1). For this reason, the family has been termed SIBLINGs (Fisher *et al.,* 2001). Other SIBLINGs include dentin sialoprotein, DSP, and dentin phosphoprotein, DPP, which are coded for by the same gene, now termed dentin sialophosphoprotein, DSPP (Butler *et al.,* 1995). The latest member of the family, matrix extracellular glycoprotein, MEPE, was isolated from ongogenic osteomalacic tumors and may contribute to the renal phosphate exhibited by these patients (Rowe *et al.,* 2000). There may also be another member of the family, BAG-75 (Gorski *et al.,* 1997); however, primary sequence information and chromosomal localization are unavailable at this time.

Osteopontin (Spp, BSP-I)

This sialoprotein was first identified in bone matrix extracts, however, it was also identified as the primary protein induced by cellular transformation. In bone, it is produced at late stages of osteoblastic maturation corresponding to stages of matrix formation just prior to mineralization. *In vitro,* it mediates the attachment of many cell types, including osteoclasts. In osteoclasts, it has also been reported to induce intracellular signaling pathways as well. In addition to the RGD sequence, it also contains stretches of polyaspartic acid and it has a fairly high affinity for Ca^{2+}; however, it does not appear to nucleate hydroxyapatite formation in a number of different assays. Osteopontin has been reviewed by Sodek *et al.* (2000) and is covered in greater detail in another chapter in this volume.

Bone Sialoprotein (BSP-II)

The other major sialoprotein is bone sialoprotein, composed of 50% carbohydrate (12% is sialic acid) and stretches of polyglutamic acid (as opposed to polyaspartic acid in osteopontin). The RGD sequence is located at the carboxy terminus of the molecule, whereas it is located centrally in osteopontin. The sequence is also characterized by multiple tyrosine sulfation consensus sequences found throughout the molecule, in particular in regions flanking the RGD (Fisher *et al.,* 1990). Sulfated BSP has been isolated in a number of animal species, however, the levels appear to be variable.

Bone sialoprotein exhibits a more limited pattern of expression than osteopontin. In general, its expression is tightly associated to mineralization phenomena (although there are exceptions). In the skeleton, it is found at low levels in chondrocytes, in hypertrophic cartilage, in a subset of osteoblasts at the onset of matrix mineralization, and in osteoclasts (Bianco *et al.,* 1991). Consequently, BSP expression marks a late stage of osteoblastic differentiation and an early stage of matrix mineralization. Outside of the skeleton, BSP is found in trophoblasts in placental membranes, which

in late stages of gestation fuse and form mineralized foci. A BSP-deficient mouse has been generated, but reportedly does not exhibit a skeletal phenotype, possibly due to compensation of BSP function by other SIBLINGs.

BSP may be multifunctional in osteoblastic metabolism. It is very clear that it plays a role in matrix mineralization as supported by the timing of its appearance in relationship to the appearance of mineral and its Ca^{2+} binding properties. BSP has a very high affinity for calcium. The polyglutamyl stretches were thought to be solely responsible for this high affinity, however, studies using recombinant peptides suggest that while the polyglutamyl stretches are required, they are not the sole determinants (Stubbs *et al.,* 1997). Unlike osteopontin, BSP does nucleate hydroxyapatite deposition in a variety of assays.

It is also clear from *in vitro* assays that BSP is capable of mediating cell attachment, most likely through interaction with the somewhat ubiquitous $\alpha_v\beta_3$ (vitronectin) receptor. Bone cells attach to the intact molecule in an RGD-dependent fashion. However, when BSP is fragmented, either endogenously by cells or using commercially available enzymes, the fragment most active in cell attachment does not contain the RGD sequence (Mintz *et al.,* 1993). Studies indicate that the sequence upstream from the RGD mediates attachment (in an RGD independent fashion) and suggest that the integrin-binding site is more extended than had been envisioned previously (Stubbs, 1996). Sequences flanking the RGD site are often tyrosine sulfated. However, it is not known how sulfation influences BSP activity, as *in vitro,* unsulfated BSP appears to be equivalent in its activity. Once again, it is not clear if currently available *in vitro* assays are sufficiently sophisticated to determine what influence posttranslational modifications, such as sulfation, have on the biological activity. In addition to sulfation, conformation of the RGD site may also influence the activity of the protein. While the RGD region in fibronectin is found in a looped-out region that is stabilized by disulfide bonding, there are no disulfide bonds in BSP. However, the flanking sequences most likely influence the conformation of the region. It also appears the cyclic conformations have a higher affinity for cell surface receptors than linear sequences (van der Pluijm *et al.,* 1996).

Dentin Matrix Protein-1 (DMP-1)

Although DMP-1 was originally thought to be specific to dentin, it was subsequently found to be synthesized by osteoblasts as well (D'Souza *et al.,* 1997). However, its function in bone metabolism is not presently known.

Serum Proteins

The presence of hydroxyapatite in the bone matrix accounts for the adsorption of a large number of proteins that are synthesized elsewhere and brought into the vicinity via the circulation (Delmas *et al.,* 1984). Most of these proteins are synthesized in the liver and hematopoietic tissue and represent classes of immunoglobulins, carrier proteins, cytokines, chemokines, and growth factors. Interestingly, some of these proteins are also synthesized endogenously by cells in the osteoblastic lineage. It is not known if the origin of a particular factor (and hence proteins with potentially different posttranslational modifications) affects biological activity or not.

Although serum proteins are not synthesized locally, they may have a significant impact on bone metabolism (Table V). Albumin, which is synthesized by the liver, is concentrated in bone severalfold above levels found in the circulation. It is not known whether it plays a structural role in bone matrix formation; however, it does have an influence on hydroxyapatite formation. In *in vitro* assays, albumin inhibits hydroxyapatite growth by binding to several faces of the seed crystal (Garnett *et al.,* 1990).

Table V Gene and Protein Characteristics of Serum Proteins Found in Bone Matrix

Serum proteins	Gene	Protein	Function
Albumin	4q11-22 17kb, 15 exons	69 kDa, nonglycosylated, one sulfhydryl, 17 disulfide bonds, high-affinity hydrophobic-binding pocket	Inhibits hydroxyapatite crystal growth
α_2HS glycoprotein	3 two RFLP 1.5 kb mRNA	Precursor protein of fetuin, cleaved to form A and B chains that are disulfide linked, Ala-Ala and Pro-Pro repeat sequences, N-linked oligosaccharides, cystatin-like domains	Promotes endocytosis, has opsonic properties, chemoattractant for monocytic cells, bovine analog (fetuin) is a growth factor

In addition to this inhibitory activity, it also inhibits crystal aggregation.

Another serum protein, α_2-HS-glycoprotein, is even more highly concentrated in bone than albumin (up to 100× more concentrated). It is known that α_2-HS-glycoprotein is the human analog of bovine fetuin (Ohnishi *et al.,* 1993). This protein is synthesized as a precursor that contains a disulfide bond linking the amino and carboxy-terminal regions. Subsequently, the midregion is cleaved and removed from the molecule, yielding the A and B peptides (much in the same way that insulin is processed). In rat, the midregion is not removed and the molecule consists of a single polypeptide. This protein also contains cystatin-like domains (disulfide-linked loop regions), and another member of this family has been identified in bone matrix extracts.

α_2-HS-glycoprotein has many proposed functions that may also be operative in bone cell metabolism. In other cell culture systems, it has been proposed to promote endocytosis and to have opsonic properties. It is also a chemoattractant for monocytic cells, and consequently, it may influence the influx of osteoclastic precursor cells into a particular area (Nakamura *et al.,* 1999). Furthermore, it is a transforming growth factor-β type II receptor mimic and cytokine antagonist (Demetriou *et al.,* 1996). Fetuin, the bovine homologue has been found to be a major growth-promoting factor in serum. Consequently, this protein may play a very important role in bone cell metabolism irrespective of whether it is synthesized locally or not.

Other Proteins

γ-carboxy glutamic acid-containing proteins are also major constituents of bone matrix (Table VI) and are reviewed in another chapter. In addition to the proteins described earlier there are representatives of many other classes of proteins in the bone matrix, including proteolipids, enzymes and their inhibitors (including metalloproteinases and TIMPs, plasminogen activator and plasminogen activator inhibitor, matrix phosphoprotein kinases, lysosomal enzymes), morphogenetic proteins, and growth factors (reviewed in Gokhale *et al.,* 2001). While their influence on bone cell metabolism is highly significant and they may cause significant alterations of the major structural elements of bone matrix, they are not necessarily part of the structural scaffold of bone matrix (with the possible exception of proteolipids). Important aspects of many of these classes of proteins are reviewed elsewhere.

Control of Gene Expression

In vivo and *in vitro* analysis clearly indicates that the timing and location of bone matrix protein expression are controlled by cells in the osteoblastic lineage as they progress toward maturation. The sequence of molecular events that regulate this progression is mediated by *cis*- and *trans*-acting factors present in the nucleus. *cis*-acting factors (also known as response elements) are present in the promoter region of the gene (the sequence upstream from the gene transcription start site). *cis*-acting factors can be roughly separated into two types: those that serve as binding sites for DNA polymerases (TATA, CAAT) and those that serve as binding sites for *trans*-acting (nuclear binding) factors. The interaction of *cis*-acting sequences within the promoter and *trans*-acting factors thereby modulates the activity of DNA polymerases, resulting in either activation or suppression of gene activity.

Utilizing both *in vitro* and *in vivo* analysis, virtually all of the promoters of the bone matrix protein genes have been characterized. Numerous *cis*-acting elements have been identified in all of the genes by direct sequence analysis, and their activity in serving as binding sites for *trans*-acting nuclear factors has been tested in DNA footprint and mobility assays.

Table VI Gene and Protein Characteristics of γ Carboxy Glutamic Acid-Containing Proteins in Bone Matrix

Gla-containing proteins	Gene	Protein	Function
Matrix Gla protein	12p 3.9 kb, 4 exons	~15 kDa, five Gla residues, one disulfide bridge, phosphoserine residues	May function in cartilage metabolism, a negative regulator of mineralization
Osteocalcin	1 1.2 kb, 4 exons	~5 kDa, one disulfide bridge, Gla residues located in α helical region	May regulate activity of osteoclasts and their precursors, may mark the turning point between bone formation and resorption
Protein S			May be made primarily in the liver, protein S deficiency may result in osteopenia

The most reliable information appears to be derived from studies utilizing transgenic animals that have been engineered to contain parts of the promoter, either wild type or mutated, linked to a reporter molecule, such as chloramphenicol transferase, β-galactosidase, or luciferase. This stance stems from studies of type I collagen, and alkaline phosphatase gene expression whereby sequences identified as active by *in vitro* analysis were not active when placed in the animal. Transgenic animals of this sort have been generated for many of the bone matrix protein promoters and have provided a great deal of information on what factors are controlling both the timing and the location of bone matrix protein expression during bone formation. Salient features of the bone matrix protein promoters are listed in TableVII.

Although not complete, the mechanism by which the pattern of gene expression is controlled during osteogenesis is becoming clearer. This is due in large part to the identification of CBFA1, a transcription factor that is required for bone formation during development and for modeling and remodeling after birth (Ducy, 2000). Deletion of this gene resulted in the generation of mice that were completely devoid of bone (Komori *et al.,* 1997). The promoters of bone matrix proteins expressed at late stages of osteoblastic maturation, osteopontin, bone sialoprotein, and osteocalcin, all have CBFA1-binding sites (Ducy *et al.,* 1997), although it appears that CBFA1 represses bone sialoprotein expression (Javed *et al.,* 2001). After osteogenic commitment by CBFA1 expression, other factors clearly play a role in fine tuning the timing and location of bone matrix protein expression, such as steroid nuclear hormone receptors (Kraichely *et al.,* 1998), members of the Ets1 family (Trojanowska, 2000), and nuclear matrix proteins (Thunyakitpisal *et al.,* 2001), to name just a few. Much has been learned, but there is still much to be discovered.

Table VII Promoter Characteristics of Bone-Related Genes

Protein	Polymerase-binding sites	*cis*-acting factors	*trans*-acting factors
Collagens			
alpha1(I)	TATA, CCAAT, CT rich, AG rich	SP1, VDRE, NF1	CAAT-binding protein, two silencers, VDR
alpha2(I)	CCAAT, CT rich	NF1	CAAT-binding protein, CTF/NF1
Proteoglycans			
Versican	TAATA, CCAAT	+/− elements, XRE, SP1, CRE	CAAT-binding factor
Decorin	Two promoters 1a- GC rich 1b - two TATA, one CAAT	AP1, AP5, NF-κb, Pu/Py mirror repeat	
Biglycan	GC rich	SP1, AP1, AP2, NF1, NF-κb	
Fibromodulin	Analysis unavailable		
Glycoproteins			
Alkaline phosphatase	Two promoters, GC rich, TATA	3 SP1s	VDR, RAR
Osteonectin	GA repeats, S1 sensitve	SP1, AP1, CRE, GHE, HSE, MRE, 1st intron, four CCTG repeats	GGA-binding protein
Tetranectin	Analysis unavailable		
Thrombospondin	Three genes, TATA, GC rich, inverted CAAT	AP1, AP2, SP1, NFY, SRE, Egr1 Egr1	c-Jun
Fibronectin	TATA, CAAT, GC rich	CRE, SP1	ATF2
Vitronectin	Analysis not available		
Fibrillin	TATA, CCAAT, GC rich		
Osteopontin	Inverted CCAAT, TATA, GC box	+/− elements, SP1, AP, AP4, AP5, RAE, TPA, PEA3, THR, GHV, VDRE	VDR, CBFA1-binding site
Bone sialoprotein	Inverted TATA, inverted CCAAT	AP1, CRE, Homeobox, RARE, p53, GRE, VDRE, 1st intron, poly Py, poly AC, YY-1, supressor?	VDR, CBRA1-binding sites
BAG-75	Not yet cloned		
Gla-proteins			
Matrix Gla protein	TATA, CAAT	RARE, VDRE	VDR, RA
Osteocalcin	TATA, CCAAT, overlaps with other elements	OC Box, AP1, AP2, VCE, VDRE, CRE, GRE, NF1, MSX, VA	VDR, c-Fos, CBFA1-binding site
Protein S	Analysis not available		
Serum proteins			
Albumin	TATA, CCAAT	PGRBS, GRE	C/EBP, NHF1, FTF, NF1
α_2HS glycoprotein	Analysis unavailable		

Bone Matrix Glycoproteins and Ectopic Calcifications

The development of sensitive radiographic techniques, in addition to histological observations, has lead to the description of ectopic calcifications in many different pathological disorders. While dystrophic mineralization has long been noted, it was not thought that bone matrix proteins played a role in generating this type of mineralization. Dystrophic mineralization is brought about by cell death (perhaps in the form of apoptosis) and not by the physiological pathways mediated by collagen or matrix vesicles. It may be a real phenomenon in some cases (in particular, in muscle trauma), but bone matrix proteins are now being identified in mineralized foci in several different pathological states. Osteonectin, osteopontin, and bone sialoprotein have been found in mineralized foci in primary breast cancer (Bellahcene *et al.,* 1997). Bone sialoprotein has also been found in other cancers, such as prostate, thyroid, and lung (Bellahcene and Castronovo, 1997; Bellahcene *et al.,* 1997, 1998; Waltregny *et al.,* 1998).

There are several ways in which these proteins can be localized to these foci. It is possible that in some cases, the area mineralizes dystrophically and the bone matrix proteins are adsorbed from the circulation due to their affinity to hydroxyapatite. In some cases, however, mRNAs for bone matrix proteins have been identified and it appears that the proteins are actually synthesized by resident cells that have been triggered (by factors that have yet to be identified). Mammacarcinoma cells are an example of this type of change in phenotypic expression (Bellahcene *et al.,* 1997).

Given the fact that bone matrix proteins are present in these mineralized foci, the next question is why? One clue may be provided by the fact that several of the tumors that contain these mineralized foci, such as breast and prostate cancer, show a propensity to metastasize to bone. However, it is not known how bone matrix protein expression influences the metastatic process. It is possible that the transformation event that caused the expression of the bone matrix proteins to begin with also caused a change in cell surface receptor expression such that if the cells are able to traverse the circulatory system (i.e., get into and out of blood vessels), they are able to attach to and associate with marrow stromal, as would circulating hematopoietic cells. However, it is possible that the expression of bone matrix proteins such as bone sialoprotein and osteopontin allows the cells to lodge in bone matrix due to their high affinity for hydroxyapatite that may be transiently available due to a resorption event.

Another example of ectopic calcification is seen in atherosclerosis, again, associated with the production of bone matrix proteins (Bini *et al.,* 1999). However, in this case, it may be that a population of stem cells exists that are normally quiescent, but then are induced to become osteogenic again by factors that are not known. The aorta has its own vasculature, which may harbor these stem cells. Supporting this hypothesis, pericytes from the retinal vasculature have been shown to undergo bone formation in vitro and *in vivo* (Doherty *et al.,* 1998).

Summary

Bone matrix proteoglycans and glycoproteins are proportionally the most abundant constituents of the noncollagenous proteins in bone matrix. Proteoglycans with protein cores composed of the leucine-rich repeat sequences (decorin, biglycan, fibromodulin, osteoadherin) are the predominant form found in mineralized matrix, although hyaluronan-binding forms (in particular, versican) are present during early stages of osteogenesis. They participate in matrix organization and in regulating growth factor activity. Glycoproteins such as alkaline phosphatase, osteonectin, RGD-containing proteins (osteoadherin, thrombospondin, fibronectin, vitronectin, osteopontin, bone sialoprotein), fibrillin, and tetranectin are produced at different stages of osteoblastic maturation. They exhibit a broad array of functions ranging from control of cell proliferation, cell–matrix interactions, and mediation of hydroxyapatite deposition. The ectopic expression of bone matrix proteins may also play a significant role in pathological states such as bone metastasis in certain forms of cancer and atherosclerosis.

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CHAPTER 15

Osteopontin

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Osteopontin (OPN) is one of the noncollagenous proteins present in bone matrix (Mark *et al.,* 1987). Independently, it was found to be present in the plasma of patients bearing highly metastatic tumors (Brown *et al.,* 1994). Another line of study revealed that the same molecule was expressed at high levels by activated T cells (Singh *et al.,* 1990). OPN was also characterized as a molecule that regulates the calcification of urinary stones because an antibody raised against it was able to block the formation of calcium oxalate-based stones (Shiraga *et al.,* 1992). The presence of OPN in various types of organs, including those with and without matrix, and also in plasma, suggests that this molecule could act both as a structural molecule and as a humoral factor, or cytokine (Nanci, 1999; Rittling and Denhardt, 1999; Denhardt *et al.,* 2001). The recent accumulation of a body of new data has opened a new era of studies on OPN function. This chapter focuses on these novel features of OPN.

Structure of Osteopontin

The amino acid sequence of OPN has been determined for a number of species (Sodek *et al.,* 2000a). The conservation of much of the sequence among these species suggests that this molecule has had a fundamental role in biological systems during evolution. OPN consists of about 300 amino acids. Importantly, an RGDS (arginine, glycine, aspartate, serine) motif is located in the midportion of the molecule. A thrombin cleavage site is located just carboxyl-terminal to the RGDS motif (Senger *et al.,* 1994; Bautista *et al.,* 1994); the products of thrombin cleavage can be observed when serum preparations are analyzed by gel electrophoresis. However, intact OPN is more abundant than the cleavage product in blood (Kon *et al.,* 2000). Therefore, to assay the intact OPN concentration in human blood, plasma rather than serum should be prepared to avoid the effect of thrombin, which is activated in the process of serum preparation. Consideration has been given to measuring circulating OPN levels by ELISA to identify people with high risk for diseases such as osteoporosis or to evaluate the response of patients to particular clinical treatments. However, the fact that OPN is sequestered by factor H may be a complication (Fedarko *et al.,* 2000).

Clinical measurements of OPN in the circulatory system are not restricted only to patients with involutional bone diseases; such measurements may also be relevant to the evaluation of patients with metastatic tumors, with certain kinds of immunodeficiencies, with neuronal diseases, and with urinary stones. At this point, however, it is not certain what would be the contribution of intact OPN to such a diagnosis; also the significance of the levels of its cleavage products, or the function of the cleavage products, in each situation is unclear.

OPN is modified posttranslationally by phosphorylation, the addition of sugars, such as sialic acid, and sulfation (Nagata *et al.,* 1989; Beninati *et al.,* 1994; Sørensen *et al.,* 1995; Neame and Butler, 1998; Zhu *et al.,* 1997; Safran *et al.,* 1998). The levels of glycosylation, sulfation, and phosphorylation vary depending on the organs and the time after synthesis when OPN modification is assessed. Phosphorylation modulates osteoblastic and osteoclastic functions and has been suggested to affect the efficiency of binding to various cell types (Saavedra, 1994; Lasa *et al.,* 1997; Katayama *et al.,* 1998; Ashkar *et al.,* 2000). Sulfation can affect the formation of mineralized bone nodules

in culture (Nagata, 1989). So far, these data are mostly *in vitro,* and therefore, the significance of such posttranslational modifications in the physiological environment, i.e., *in vivo,* has not been elucidated. Transgenic ("knock-in") mice having mutations in sites of posttranslational modifications may help elucidate the specific function of each of the modifications.

OPN is encoded in seven exons (Craig and Denhardt, 1991; Hijiya *et al.,* 1994; Crosby *et al.,* 1995); however, additional, or alternative, exons are sometimes expressed because more than one mRNA species has been observed in Northern analyses. In the case of human OPN, alternative splicing may produce isoforms (Young *et al.,* 1990; Crivello and Delvin, 1992; Parrish and Ramos, 1997). Functional difference among the isoforms as well as the difference in the expression patterns in various tissues, has not yet been clearly documented. As mentioned earler, OPN has been identified in many tissues, and in these tissues this molecule could mediate communication between cells. Thus, OPN could be regarded as a cytokine. Modification and/or splicing would allow more opportunities for this molecule to function differently under particular conditions, thus contributing to the specificity of the signaling between cells.

OPN interacts with the molecules constituting bone matrix. Proteins in the bone matrix are 90% type I collagen and 10% a variety of noncollagenous proteins. OPN is known to bind covalently to fibronectin via transglutamination, and transglutamination of OPN increases its binding to collagen (Beninati *et al.,* 1994; Kaartinen *et al.,* 1999). Other molecules, such as bone sialoprotein (BSP), also bind covalently to type I collagen. Osteocalcin suppresses transglutaminase-catalyzed cross-linking of OPN (Kaartinen *et al.,* 1997). Such a network and mutual regulation among matrix proteins in bone may facilitate conformational changes of the molecules and hence could add additional functions or activation/inactivation switches to the molecules depending on the sites and composi-tion of the interaction between the molecules. So far, however, whether such covalent bonding between matrix proteins and OPN or other noncollagenous molecules plays any role in the physiological maintenance of the bone during the remodeling cycle or in pathological situations such as osteoporosis or osteopenia requires further elucidation of the functional aspects of this interaction.

Another structural uniqueness of OPN is a run of 10–12 aspartic acid residues. This motif gives rise to a localized high negative charge that may be important for the binding of OPN to bone mineral. OPN has a strong affinity to calcified matrix, such as bone, and also to pathological calcifications, such as those seen in sclerotic glomeruli and atherosclerosis. The high affinity of OPN to calcium has been suggested to modulate the nucleation of calcium phosphate during mineralization (Boskey, 1995; Contri *et al.,* 1996; Srivatsa *et al.,* 1997; Sodek *et al.,* 2000b); however, initial studies on the OPN-deficient mouse failed to indicate the presence of any major defect in mineralization (Rittling *et al.,* 1998). Possibly, the role of OPN in bone mineralization is compensated for by other regulatory systems for mineralization.

The molecular conformation of OPN may be altered by the binding of calcium in a manner dependent on the concentration of the Ca^{2+} ion. It has been proposed that depending on such calcium ion-dependent conformational changes, OPN may reveal binding motifs such as the RGD sequence to its cognate receptors or to any other interactive extracellular matrix protein (Singh *et al.,* 1993; Bennett *et al.,* 1997). The dependence of the structure of OPN on the calcium concentration is an attractive feature of this molecule with regard to modulation of its function, e.g., during bone resorption by osteoclasts. For instance, when osteoclasts resorb bone, there are significant changes in the calcium concentration in the secondary lysosome-like closed space underneath the resorbing osteoclast. This calcium concentration may render signals through the calcium sensing receptor (CsR), a seven membrane-spanning type receptor (Kameda *et al.,* 1998; Kanatani *et al.,* 1999). In addition to such calcium signaling, OPN may change its conformation depending on the calcium levels, thereby affecting cell function through its binding to the receptors expressed on the surface of the osteoclasts, such as $\alpha_v\beta_3$. However, it remains to be seen whether there is any functional significance of putative calcium-dependent structural changes in the OPN molecule.

OPN was first found as a secreted protein, and consequently one of the many names that have been given to it is secreted phosphoprotein (SPP) (Denhardt and Guo, 1994). In fact, when osteoblast-like cells such as ROS17/2.8 cells were stained for OPN protein, no major signal can be observed inside the cells. However, OPN mRNA expression in these cells and protein expression in the medium were easily detectable. Therefore, a major part of the OPN protein moves out of the cell immediately after its synthesis. However, the presence of an intracellular form of OPN has been shown (Zohar *et al.,* 1998, 2000).

Whether the intracellular form of OPN is different from other forms of OPN with regard to alternative splicing or posttranslational modifications is not known. The intracellular form of OPN was colocalized with CD44 in extensions of the osteoclasts known as podosomes, but not in the perinuclear regions where BSP has been observed (Suzuki *et al.,* 2000). Therefore, CD44 and OPN as well as the colocalized $\alpha_v\beta_3$ receptors probably form a complex that facilitates osteoclast movement. As osteoblasts produce OPN and $\alpha_v\beta_3$, migration of osteoblasts may also be dependent on the intracellular form of OPN, CD44, and/or $\alpha_v\beta_3$ integrins (Suzuki, 2000). Like CD44, OPN may promote the multinucleation of osteoclast precursors, as it was observed that in OPN-null mice mononuclear cells are more abundant than multinucleated cells, similar to the situation in the CD44-deficient mouse. Migration experiments conducted *in vitro* using the Boyden chamber system indicated that the presence of OPN is required for efficient migration through the membrane pores. Further, this migration was dependent on the presence of ezrin and hyaluronan. OPN also acts after its binding to

$\alpha_v\beta_3$ integrins through Rho to stimulate gelsolin-associated phosphatidylinositol 3-kinase activity, podosome assembly, stress fiber formation, osteoclast motility, and bone resorption (Chellaiah *et al.,* 2000c).

Receptors for OPN

OPN binds to $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_9\beta_1$ integrins (Denhardt and Noda, 1998; Duong *et al.,* 2000; Zheng *et al.,* 2000; Barry *et al.,* 2000). With regard to CD44 binding to OPN, the domain in OPN that interacts with CD44 is not the glycine, arginine, aspartic acid, serine (GRGDS) motif (Katagiri *et al.,* 1999). It has been found that some melanoma cells bind in a non-RGD dependent manner to the v6/v7 isoform of CD44. During the interaction, CD44 may bind to another cell surface molecule, integrins β, and that association may in turn provide the optimal interaction for CD44 to OPN (Katagiri *et al.,* 1999). Details of the interaction of CD44 with OPN remain to be worked out. The presence of additional cell receptors, the various isoforms of CD44, and variable posttranslational modifications (phosphorylation and glycosylation) of OPN and CD44 are all complicating factors.

As OPN binds to the $\alpha_v\beta_3$ integrin, this integrin is considered to be responsible for major signals in response to the binding of OPN (Miyauchi *et al.,* 1991; Zimolo *et al.,* 1994). Postreceptor signaling via the $\alpha_v\beta_3$ integrin is dependent on the cellular background (Zheng *et al.,* 2000). In addition to regulating osteoclastic activity, OPN binding to the $\alpha_v\beta_3$ integrin activates osteoprotegerin expression and protects endothelial cells from apoptosis (Malyankar *et al.,* 2000).

Studies on β_3-deficient mice are relevant to understanding at least part of the function of OPN. Cells prepared from the bone marrow of β_3 knockout mice were able to differentiate into osteoclasts with efficiencies similar to the wildtype (McHugh *et al.,* 2000). This observation indicates that $\alpha_v\beta_3$ is not required for osteoclast formation. The β_3 knockout mice were relatively normal while they were young but they revealed osteosclerosis radiographically by 4 months, suggesting that aging is one of the factors that reveals a phenotype in these mice, again, somewhat similar to OPN-deficient mice. Also, similar to OPN-deficient mice, there was a 3.5-fold increase in osteoclast number, which would appear to compensate for the mild hypocalcemia in the mice. Osteoclasts developed from the bone marrow cells of these mice were less efficient than wild-type cells in excavating pits on dentin slices, showing some inability to resorb bone, again similar to osteoclasts derived from OPN-deficient mice. The difference appears to reside in the cytoskeleton, which is abnormal in the β_3 knockout mice, suggesting a defect in intracellular signaling compared to osteoclasts derived from OPN-deficient mice where formation of the cytoskeleton and actin rings appeared to be normal when the osteoclasts were developed by culturing in the presence of RANKL and M-CSF (Ihara *et al.,* 2001; McHugh *et al.,* 2000). Overall, these findings on β_3 knockout mice further support the notion that the OPN signaling integrin through β_3 integrin pathway is important in regulation of osteoclastic activities.

Osteopontin and Cell Attachment

OPN promotes the attachment of fibroblasts to plastic or glass substrates (Somerman *et al.,* 1988; Reinholt *et al.,* 1990; Helfrich *et al.,* 1992). In bone, OPN is expressed in osteoblasts and its expression is enhanced by vitamin D (Prince *et al.,* 1987). Osteoclasts also express OPN when they are vigorously resorbing bone in human osteoarthritis specimens (Merry *et al.,* 1993; Dodds *et al.,* 1995; Connor *et al.,* 1995). Osteoclasts express $\alpha_v\beta_3$ integrin at high levels (Horton *et al.,* 1995; Duong *et al.,* 2000). Although the $\alpha_v\beta_3$ integrin is not a specific marker of osteoclasts, monoclonal antibodies raised against osteoclasts appear to specifically visualize osteoclasts in bones due to its high abundance. Therefore, $\alpha_v\beta_3$ integrin can be used as one of the markers of osteoclasts.

Immunoelectron microscopic examination using colloidal gold particles indicated that OPN was observed underneath the clear zone of osteoclasts (Reinholt *et al.,* 1990). As clear zones are involved in the attachment of osteoclasts to the bone matrix, the location of OPN appeared to fit its hypothesized function. However, later experiments indicated that OPN may bind to $\alpha_v\beta_3$ integrin expressed on the basolateral surface of the osteoclasts and this binding also generates signals to modulate osteoclastic functions (Zimolo *et al.,* 1994; Zheng *et al.,* 2000). Therefore, including the intracellular form of OPN, osteoclasts could be regulated through more than one pathway of OPN signaling. However, it is not clear whether the intracellular form of OPN is the same in terms of its posttranslational modification as the form released into the extracellular environment that binds to the cells in an autocrine or paracrine manner. With regard to osteoblasts, some reports indicated the presence of the $\alpha_v\beta_3$ integrin on the surface of osteoblasts (Gronthos *et al.,* 1997) and, therefore, there could be a certain commonality in OPN function in osteoblasts and osteoclasts.

OPN is deposited along the cement line and lamina limitans after the cessation of bone resorption by osteoclasts (McKee and Nanci, 1996). This OPN may provide a signal to the osteoblasts that are attracted to the bone resorption sites to deposit bone matrix to fill the cavity. Although OPN-deficient mice do not contain OPN in the cement lines, the bone architecture by itself was not largely different from that of wild-type mice (Rittling *et al.,* 1998; Rittling and Denhardt, 1999). Therefore, significance of the signaling by OPN deposited at the cement line or lamina limitans may be minor *in vivo.* However, when the osteoclasts were cultured individually in an *in vitro* system, the absence of OPN resulted in reduced osteoclast function, indicating a role for this molecule, at least in these cells (Chellaiah *et al.,* 2000b; Ihara *et al.,* 2001).

Osteopontin-Dependent Intracellular Signaling

RGD-containing molecules, such as OPN, bind to integrins on the surface of osteoclasts and induce integrin clustering (Hruska *et al.,* 1995; Rodan and Rodan, 1997; Chellaiah *et al.,* 2000a,c; Duong *et al.,* 2000). This binding initiates intracellular signaling by the phosphorylation of tyrosine residues, including tyrosine 402 on PYK2. The phosphorylation of tyrosine residues leads to binding of Src via its SH2 domain, which then further increases phosphorylation of PYK2 at other sites (Duong *et al.,* 1998, 2000; Duong and Rodan, 1999). Such phosphorylation amplifies the signal, attracting other adaptor molecules to bind PYK2, thereby eliciting signals that activate cellular functions, including adhesion and cytoskeletal structure formation needed for osteoclastic actions, such as sealing zone formation and intracellular trafficking (Nakamura *et al.,* 1999). PYK2 also binds to CAS, however, this interaction is independent of tyrosine phosphorylation (on both of the two molecules).

In addition to interactions between kinases and adaptor molecules at focal adhesion sites, p21GTPase activity is also important in OPN-dependent signaling. One of the targets of rho, mDia1, which appears to be involved in the formation of the actin ring, associates with gelsolin located in the podosomes of osteoclasts (Chellaiah *et al.,* 2000b). In osteoclasts isolated from OPN-deficient mice, podosome structures were similar to those in wild-type mice; however, the mDia1 and gelsolin association was not observed and there was a reduction in osteoclast motility in response to vitronectin (Chellaiah *et al.,* 2000b). Relative to wild-type osteoclasts, OPN-deficient osteoclasts exhibited a decrease in CD44 expression on the cell surface. This defect in the surface expression of CD44 and dissociation between mDia1 and gelsolin was reversed by the addition of exogenous OPN. Thus, it was suggested that OPN-deficiency induces an impairment in the motility of osteoclasts by the suppression of CD44 expression on the cell surface as well as a disruption of the association between mDia1 and gelsolin, thereby suppressing podosome assembly. However, exogenously added OPN only stimulated the motility of osteoclasts, without correcting the depth of the pits formed on the dentin slices. Thus, the two phenomena of reduced CD44 expression and suppression of the formation of podosomes could be causing the osteoclastic cells to be hypomotile (Chellaiah *et al.,* 2000b). This may be the explanation for the inefficiency of osteoclasts in OPN-deficient mice.

Phenotype of Osteopontin-Deficient Mice

Because OPN-deficient mice produced independently in two laboratories do not show any structural alterations in bones at birth and during their subsequent growth period, there does not appear to be a requirement for OPN for normal development (Rittling *et al.,* 1998; Liaw *et al.,* 1998). In these OPN-deficient mouse strains, skeletal defects were not observed, whereas altered wound healing was noted by Liaw *et al.* (1998). Several possibilities have been proposed to explain the apparent lack of major bone phenotype. The first one was that in the absence of OPN, other related molecules, such as those containing RGDS, can compensate for the missing OPN. However, in OPN/vitronectin double knockout mice, no bone abnormalities were noted (Liaw *et al.,* 1998). Another possibility could be that the T-cell-based type I immune response deficiency that results from the absence of OPN (Ashkar *et al.,* 2000) could modify the response of bone due to the alteration in the cytokine network that is involved in the maintenance of both cellular immune responses and the mineralized skeleton.

Osteopontin Plays a Role in Estrogen Depletion-Induced Bone Loss

Although compensation may account for the normal development and normal maintenance of bone in OPN-deficient mice, the difference between the absence and the presence of OPN could be overt in circumstances of accelerated bone turnover, such as osteoporosis. A mouse osteoporosis model made by the ovariectomy-induced depletion of estrogen provided clues to answer the question on the role of OPN in the regulation of bone metabolism (Yoshitake *et al.,* 1999). After ovariectomy, both wild-type mice and OPN-deficient mice exhibited a similar reduction in uterine weight within 4 weeks, suggesting that the hormonal system in OPN-deficient mice was similar to that in wild-type mice, at least in terms of the uterine response to estrogens. That the estrogen system in OPN-deficient mice is normal is suggested by the normal rate of sexual maturation and growth, as well as the normal fertility and littermate size in OPN-deficient mice.

Micro-CT analysis indicated that trabecular bone was lost and that the porosity in the epiphyseal portion of the long bones was decreased by about 60% in ovariectomized wild-type mice (Yoshitake *et al.,* 1999). Micro-CT analysis of the epiphyseal region of the long bones in sham-operated OPN-deficient mice revealed a slight increase in the trabecular bone volume compared to sham-operated wild-type mice. Figure 1 shows that in contrast to the clear reduction in trabecular bone volume seen in wild-type mice after ovariectomy, no major reduction is observed in OPN-deficient mice. The preservation in the levels of bone volume even after ovariectomy could be due to the increase in bone formation, decrease in bone resorption, or both. Dynamic parameters for bone formation in these mice, such as bone formation rate/bone volume (BFR/BV), were increased in ovariectomized wild-type mice as shown previously. In the case of OPN-deficient sham-operated mice, the value was similar to sham-operated wild-type mice. However, no significant increase in BFR was observed in OPN-deficient mice, indicating the absence of high turnover status in bone metabolism even after ovariectomy. Similar basal levels of BFR suggest that bone formation activity in OPN-deficient mice is basically normal.

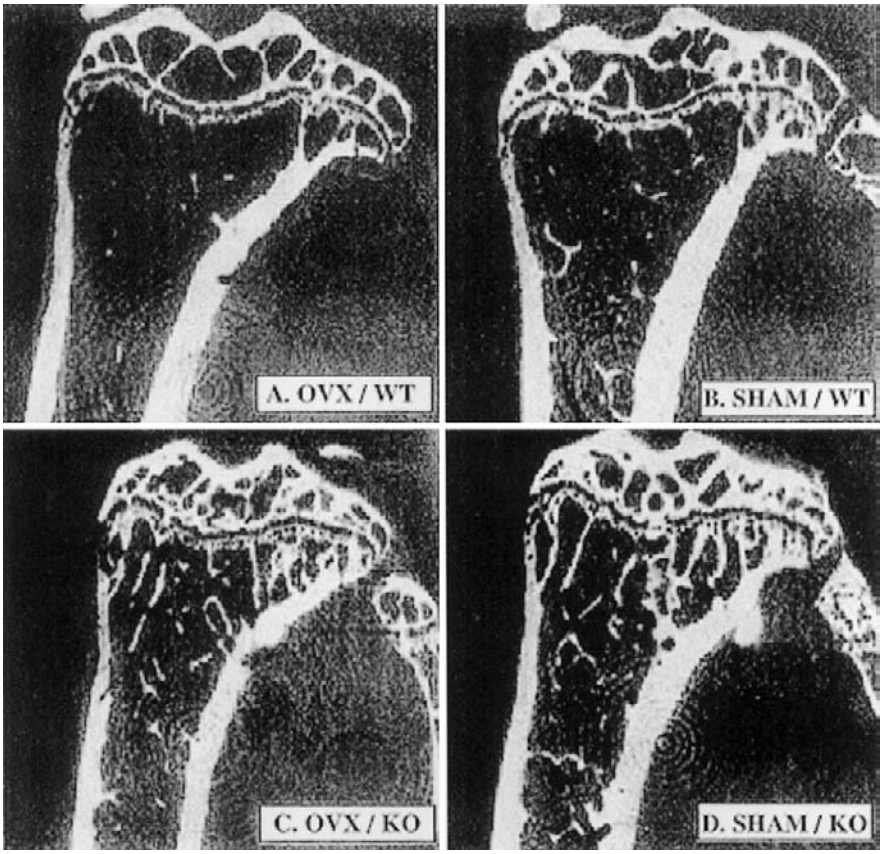

Figure 1 OPN deficiency suppresses ovariectomy-induced bone loss. Micro-CT images of the dissected tibia from ovariectomized and sham-operated mice, both wild type and OPN deficient, are shown. See Yoshitake *et al.* (1998) for further details.

Morphological examination also supported the suppression of ovariectomy-induced bone resorption in OPN-deficient mice. The number of osteoclasts was increased about threefold 4 weeks after ovariectomy in wild-type mice. In contrast, basal levels of osteoclast number were relatively high in OPN-deficient mice and were not increased even after depletion of estrogen. The large number of osteoclasts, together with the increased bone volume in OPN-deficient mice, is superficially paradoxical. However, it could be due to a feedback system in the body that maintains serum calcium levels tightly by increasing osteoclast number to compensate for the reduced efficiency of the osteoclasts to resorb calcium from bone. Even with such compensation, a defect in the ability of osteoclasts to resorb bone is suggested by the relatively large trabecular bone volume in sham-operated OPN-deficient mice (Fig. 1; Yoshitake *et al.,* 1999).

Osteopontin Facilitates Resorption and Angiogenesis of Ectopically Implanted Bone Discs

Angiogenesis is important for bone resorption because osteoclast progenitors are derived from hematopoietic precursor cells. However, it is not known whether OPN promotes bone resorption by stimulating angiogenesisis or by stimulating bone resorption via signaling through the bone matrix. Studies of ectopic bone (disc-shaped pieces punched out of the calvaria) implantation revealed a relationship between OPN and bone resorption associated with vascularization (Asou *et al.,* 2001). Wild-type bone implanted intramuscularly in the back of the wild-type mice was resorbed by about 25%. In contrast, bone from OPN-deficient mice implanted into OPN-deficient mice exhibited significantly less resorption (5%). Thus, about five-fold more bone was resorbed in the presence of OPN; this is illustrated in Fig. 2. The promotion of the resorption of ectopically implanted bone by OPN was associated with a larger number of osteoclasts attached to the surface of the wild-type bone than that in OPN-deficient bone. Furthermore, the number of CD34-positive vessels in the vicinity of bones implanted in OPN-deficient mice was reduced compared to the number of vessels in wild-type bones, suggesting that OPN deficiency may lead to a reduction in neovascularization of the ectopically implanted bones, and consequently a reduction in the number of osteoclasts and subsequent bone resorption efficiency. It is also possible that OPN may promote the survival of endothelial cells on the bone matrix.

Implantation of bone into muscle is suitable for the evaluation of bone resorption because of the higher vascularity of the tissues. However, detailed examination of the vascularization is difficult in intramuscular implantation experiments. Therefore, subcutaneous implantation was used

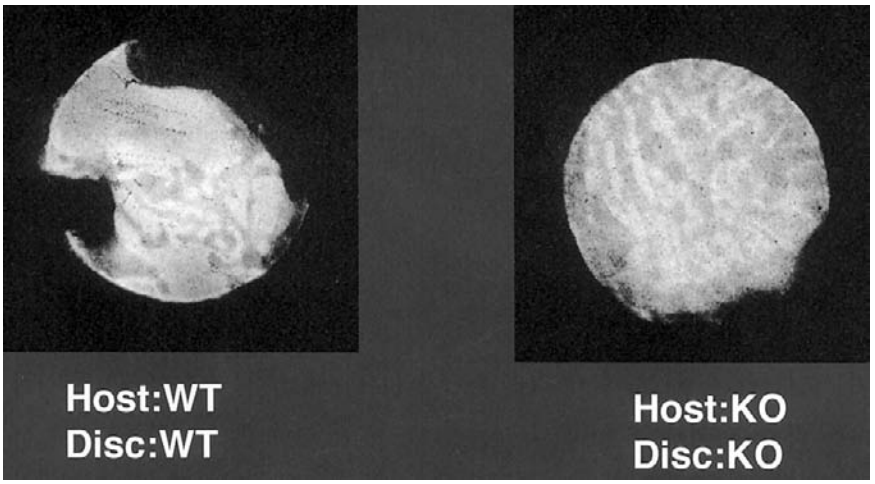

Figure 2 OPN is required for ectopic bone resorption. Bone discs derived from the calvaria were implanted intramuscularly as described by Asou *et al.* (2001). Four weeks after implantation, the discs were removed and examined using soft X-rays.

to examine vascularization without making histological sections (Asou *et al.,* 2001). Significant vascularization was observed in both wild-type and OPN-deficient mice. Interestingly, the length of the blood vessels and the number of branch points in the vasculature on the surface of the implanted bone were both decreased in the OPN-deficient bone relative to wild-type bone. This observation further supports the notion that OPN facilitates vascularization of bone tissue. In these subcutaneous implantation experiments the absence of OPN reduced the TRAP-positive area on the bone disc. These observations indicate that OPN *in vivo* facilitates vascularization in association with osteoclast recruitment, thereby stimulating bone resorption.

Cross mixing of the genotypes of implanted bone disc and host mouse indicated that when either the implanted bone disc or the host mouse was deficient in OPN, then bone resorption, as well as vascularization efficiency, was reduced to a value intermediate between wild-type and OPN-deficient mice. Overall, data indicated the importance of OPN for vascularization during bone resorption. Because the growth plate in OPN-deficient mice is mostly normal it is intriguing to know how the mechanisms involved in vascularization and/or chondroclast accumulation in growth plate metabolism are different from those involved in bone resorption and the related vascularization.

Parathyroid Hormone-Induced Bone Resorption in Organ Culture Requires the Presence of Osteopontin

As ovariectomy experiments suggest that OPN-deficient mice are resistant to bone loss, and ectopic bone implantation experiments indicate that these mice exhibit a reduced efficiency in bone resorption, it was suspected that OPN deficiency may cause a direct suppression of osteoclastic activity to resorb bone matrix. However, direct action of OPN in the process of bone resorption cannot be verified conclusively by ovariectomy experiments or ectopic bone resorption experiments per se. In this regard, bones in organ culture stimulated by parathyroid hormone have shown that OPN is directly responsible for bone resorption in the microenvironment of bone without influences from other humoral factors or vascularization (Ihara *et al.,* 2001). In these experiments, the release of $^{45}Ca^{2+}$ into the medium from $^{45}Ca^{2+}$-labeled forelimb bones excised from newborn OPN-deficient mice and cultured in the presence or absence of parathyroid hormone was measured.

The basal level of calcium release from organ cultures of forelimb bones of OPN-deficient mice was similar to that of wild-type bones. As reported previously, the presence of parathyroid hormone increased Ca^{2+} release from the cultured wild-type bones. However, as shown in Fig. 3, in the organ cultures of OPN-deficient forelimb bones, the increase in calcium release was not observed even in the presence of parathyroid hormone. Because parathyroid hormone increases osteoclast activities via stimulation of the expression of receptor activator of NFκB ligand (RANKL), soluble RANKL in combination with M-CSF was used to stimulate bone resorption. However, OPN-deficient bones failed to respond to RANKL and M-CSF, indicating that the deficiency is downstream of RANKL. Analysis of TRAP-positive cells in the cultured bones indicated that PTH treatment increased the number of these cells in wild-type bones. However, such an increase was not observed in the case of OPN-deficient bones, suggesting that the deficit resided in the inability to increase osteoclast number in the local environment of the bone rudiments. Because bone marrow cells or spleen cells taken from OPN-deficient mice were able to generate similar numbers of osteoclast-like TRAP-positive multinucleated cells with normal morphology compared to wild-type cells in culture in the presence of RANKL and M-CSF, the intrinsic ability of the progenitors to develop into osteoclasts per se is apparently not impaired in OPN-deficient mice. Actin ring formation and the distribution of Src appeared similar in osteoclasts developed in the presence of soluble RANKL and M-CSF in cultures of

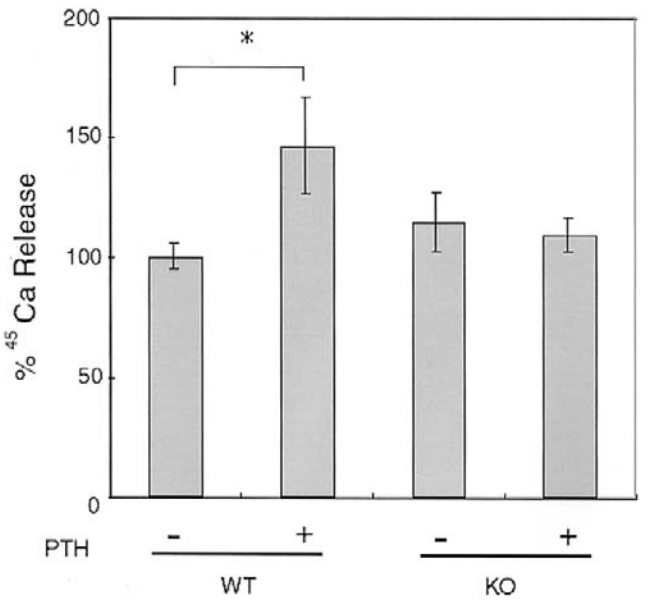

Figure 3 PTH fails to stimulate bone resorption in organ culture in the absence of OPN. Fetal forearm bones labeled with $^{45}Ca^{2+}$ were incubated in the presence of 10^{-7} *M* parathyroid hormone. After 6 days the amount of $^{45}Ca^{2+}$ in the medium was quantified. The percentage calcium released is determined relative to control cultures incubated in the absence of hormone (Ihara *et al.,* 2001).

spleen cells regardless of the presence or absence of OPN. These observations suggest that in the absence of OPN in the microenvironment, PTH is unable to stimulate the formation of TRAP-positive cells; enabling PTH action could therefore be one mechanism by which OPN promotes bone resorption.

Osteopontin and Metastatic Disease

Many types of tumor cells express OPN. OPN levels in the serum of patients bearing highly metastatic tumors have been known to be high (Koeneman *et al.,* 1999; Liaw and Crawford, 1999; Carey *et al.,* 1999; Goodison *et al.,* 1999). It has been suggested that tumor cells produce OPN that could protect tumor cells themselves against the attack by macrophages by suppressing their production of nitric oxide (NO), which can kill tumor cells (Hwang *et al.,* 1994; Denhardt and Chambers, 1994; Feng *et al.,* 1995; Rollo *et al.,* 1996). Another possibility is that OPN may be a positive factor for the attachment of tumor cells and/or may promote proliferation of the tumor cells. Melanoma cells are known to be highly metastatic, and the prognosis of patients bearing those tumors is poor. Bone is one of the sites of melanoma tumor metastases. Once tumor cells metastasize to bones, the mass due to the growth of the tumor causes severe pain and eventually destroys bone tissue, resulting in debilitating fractures. Thus, elucidation of the role of OPN in the process of tumor metastasis is important.

B16 murine melanoma cells attached more effectively to culture dish coated with recombinant OPN than with glutathione *S*-transferase (GST) control, while their proliferation was not affected by the presence of OPN (Nemoto *et al.,* 2001). The β_4 integrin and CD44 were detected in B16 melanoma cells, consistent with a previous report that these molecules may be involved in B16 cell attachment. An experimental metastasis assay based on the injection of B16 melanoma cells via an intracardiac route revealed a reduction in the number of melanoma tumors in the bones (5.4 $\pm$ 1.7) of OPN-deficient mice compared to the number in wild-type mice (11.5 $\pm$ 2.5). Figure 4 (see also color plate) shows an example. Injection of B16 cells into the left ventricle, which also gives rise to metastasis in nonskeletal tissues, yielded 6.5 $\pm$ 2.8 tumors in the adrenal glands of OPN-deficient mice and 17.8 $\pm$ 5.5 in wild-type animals. In the liver the number of melanoma metastasis was 102.6 $\pm$ 53.0 for wild-type mice and 62.2 $\pm$ 32.2 for OPN-deficient mice though the difference was not statistically significant (Nemoto *et al.,* 2001).

As reported previously, a different injection route for experimental metastasis ends up with the different efficiency of metastasis to the different tissues in OPN-deficient mice. When B16 melanoma cells were injected via the femoral vein, most of the metastases were found in the lung. The number of lung metastases in OPN-deficient mice was 37.8 $\pm$ 11.4, whereas the number in wild-type mice reached up

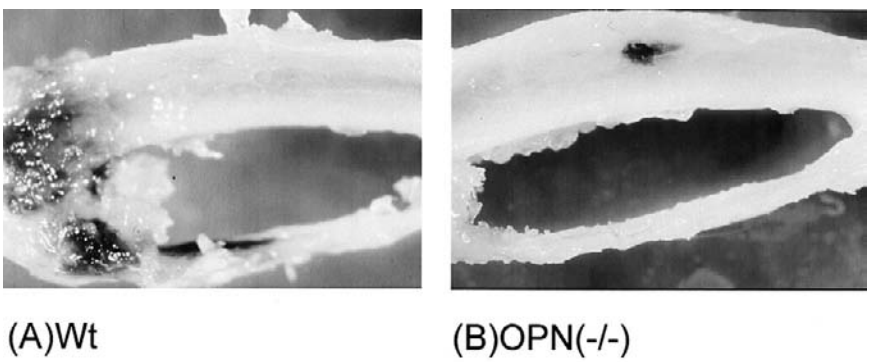

Figure 4 Experimental tumor metastasis to bone is facilitated in the presence of OPN. The B16 tumor cell metastases on the bones of wild-type and OPN-deficient mice observed 2 weeks after intracardiac injection of melanoma cells (Nemoto *et al.,* 2001). (See also color plate.)

to 126.7 ± 42, again indicating suppression by the absence of OPN ($p < 0.05$). Overall, these experimental metastasis data clearly indicated that the presence of OPN promotes the metastasis of B16 melanoma cells to bone as well as to soft tissues such as lung regardless of the route of injection. In B16 cells, expression of OPN *per se* was very low and hardly detectable by Northern blot analyses compared to MC3T3-E1, an osteoblastic cell line that expresses high levels of OPN. Although the possibility that B16 melanoma cells still produce sufficient OPN to contribute to the metastatic process cannot be excluded, the clear difference in the number of experimental metastases seen in wild-type host animals compared to OPN-deficient host animals indicates that at least the presence of OPN in the host makes a difference in the metastatic process (Nemoto *et al.,* 2001).

Metastasis of a tumor in an animal is a complex event initiated by the detachment of the cells from the primary tumor, followed by invasion into the vasculature (or lymphatics) and movement to other locations in the body where the tumor cells extravasate and establish themselves at a new site. Proliferation of the cells at that site and vascularization of the resulting tumor by the host animal produce an expanding tumor mass. Experimental metastasis by injection into the vascular system does not test the first steps in this process, steps that may also involve OPN. Even in the later part of the process, how OPN functions to promote metastasis remains to be elucidated. Among vascular tissues, endothelial cells express $\alpha_v\beta_3$ integrin. Thus, vascularization may be one of the steps affected by the absence of OPN. Unfortunately, our injection model did not allow us to examine the vascularization process because the animals started to die before the tumor foci were large enough to see an effect of vascularization. Within the limit of this model, it appears that OPN can promote the metastasis of tumor cells to various skeletal and nonskeletal sites. It is known that the efficiency of metastasis can vary depending on the tumor cells and tissues. The reduction of melanoma metastases in OPN-deficient mice observed in bone and lung suggests the involvement of a common mechanism operating in both tissues, for instance, possibly based on host macrophages. When tumor cells invade, host stromal cells have been suggested to produce OPN to attract macrophages, which in turn may suppress tumor formation (Crawford *et al.,* 1998). Our data suggest that OPN may be required in the initial attachment phase when the tumor cell is colonizing a new site.

Role of Osteopontin in Mediating Mechanical Stress

OPN is expressed in cells of the osteoblastic lineage, and possibly those including osteocytes (Noble and Reeve, 2000), which are exposed to mechanical stress (Terai *et al.,* 1999). Because chondrocytes express receptors for cell attachment molecules, i.e. $\alpha_v\beta_3$ integrins, they are also candidates for the perception of mechanical stress *in vivo* (Loeser, 2000). In addition, proximal kidney tubules express OPN in response to renin–angiotensin following mechanical stimulation, such as cell stretch. When an antisense oligonucleotide was introduced to block angiotensinogen or angiotensin 2 type I receptor expression, there was a significant decreased in OPN mRNA expression compared to unstretched cells (Ricardo *et al.,* 2000).

Smooth muscle cells also express osteopontin and respond to mechanical stress. Pulsatile pressure increases the proliferation of differentiated smooth muscle cells; in contrast, cells expressing low levels of smooth muscle cell differentiation markers exhibit decreased cell growth and decreased MAPK signaling in response to the mechanical stress (Cappadona *et al.,* 1999).

Integrin-binding forces in intact cells have been measured by using atomic-force microscopy. In cells attached to hexapeptides, 32–97 pN were measured. In contrast, for larger molecules such as OPN and BSP, the experiments showed different binding affinities. Therefore, the context of the RGD sequence has considerable influence on the final binding strength of the receptor interaction (Lehenkari and Horton, 1999).

In tooth movement, only 3.3% of the osteocytes in the inter radicular septum of rats expressed OPN in the absence of the pressure. However, upon the application of pressure, the number was increased to 87.5% within 48 hrs after initiation of the tooth movement. This movement was followed by a 17-fold increase in the number of osteoclasts on the pressure side. These responses were inhibited upon injection of an RGD peptide (Terai *et al.,* 1999). In another model of bone stress, distraction osteogenesis, chondrocyte-like cells in the osteotomized area expressed OPN, osteocalcin, and alkaline phosphatase; this region also includes many osteoblastic cells and preosteoblastic cells that are also expressing OPN at the boundary between fibrous tissue and new bone. The levels of OPN, osteonectin, bone matrix Gla protein, and osteocalcin mRNA expression were enhanced remarkably by the distraction force (0.25 mm/12 hrs) (Sato *et al.,* 1998).

In chondrogenic cells, induction of OPN expression by mechanical stress was found to be dependent on integrin receptors because OPN expression and the response to mechanical stimuli were blocked by the absence of fibronectin, and by the presence of an RGD competitor. (Carvalho *et al.,* 1998). These data indicate that ostopontin expression is enhanced in response to mechanical stimuli and that it is mediated by certain integrins recognizing fibronectin.

As mentioned, OPN expression is regulated by mechanical stress, whereas OPN itself is also involved in bone resorption, and possibly in bone formation. These observations suggest that OPN plays one or more roles during metabolic changes in response to mechanical loading. The question of whether OPN is involved in the mechanical stress-mediated regulation of bone metabolism was tested in a tail suspension model, which is one of the representative models to study unloading effects on bone metabolism (Vico, 1998; Vico *et al.,* 1998, 2000; Bikle and Halloran, 1999; Marie *et al.,* 2000). Unloading causes suppression of

bone formation in growing rodents; however, it has not been clear whether the bone resorption side is affected by tail suspension-based unloading in rodents.

To examine the role of OPN in regulation of bone loss induced by unloading, OPN-deficient mice were subjected to tail-suspension. Micro-CT analysis of the metaphyseal region of the long bones indicated an increase in sparsity in wild-type mice after unloading as expected. However, no such increase in sparsity was observed in OPN-deficient mice. Quantification of the fractional trabecular bone volume indicated about 50% reduction in the wild-type animals. In contrast, no such reduction in bone volume was observed in OPN-deficient mice (Ishijima *et al.,* 2001). Biochemically, a reduction in bone volume was reflected by the increase in the bone resorption marker deoxypyridinolin, which is secreted in the urine of the mice. In wild-type mice, deoxypyridinolin secretion was increased. However, no such increase was detected in the tail-suspended OPN-deficient mice, indicating that systemic bone resorption due to tail suspension was suppressed in OPN-deficient mice.

The cellular basis for the alteration in unloading-induced bone loss in OPN-deficient mice was revealed by histomorphometric analysis. As expected, the number of osteoclasts was increased by about 150% in wild-type mice; this increase was not observed in OPN-deficient mice. In parallel to the number of osteoclasts, the osteoclast surface was also increased in wild-type but not in OPN-deficient mice. This inability of osteoclasts to respond to unloading may be due to a defect in the signaling system to support osteoclastogenesis. However, *in vitro* osteoclastogenesis experiments using RANKL, and M-CSF indicated that TRAP-positive multinucleated cell formation was similar regardless of unloading or loading and/or difference in genotypes. Thus, suppression of the response to unloading in the case of osteoclastogenesis in OPN-deficient mice would be due to the signaling prior to osteoclastogenesis, as the intrinsic ability for osteoclastogenesis in the precursor cells per se does not seem to be impaired (Ihara *et al.,* 2001). Therefore, certain extracellular signaling could be lost in OPN-deficient mice. This possibility was also suggested by the analysis of the osteoblastic cells.

Osteoblasts are regarded as central players in regulating bone metabolism because they express receptors for parathyroid hormone, prostaglandins, and vitamin D—all major humoral factors that regulate bone metabolism. In addition, they are thought to be the cells that respond to mechanical stress. Analysis of osteoblastic activity in tail-suspended mice indicated suppression of bone formation rate as well as mineral apposition rate in wild-type mice, as has been reported previously. However, as illustrated in Fig. 5 (see also color plate), a reduction in the values of these two parameters of osteoblastic bone formation did not occur when OPN-deficient mice were subjected to unloading by tail suspension (Ishijima *et al.,* 2001). Because reduction of bone formation was not affected in experiments where bisphosphonate was administered to tail-suspended animals to

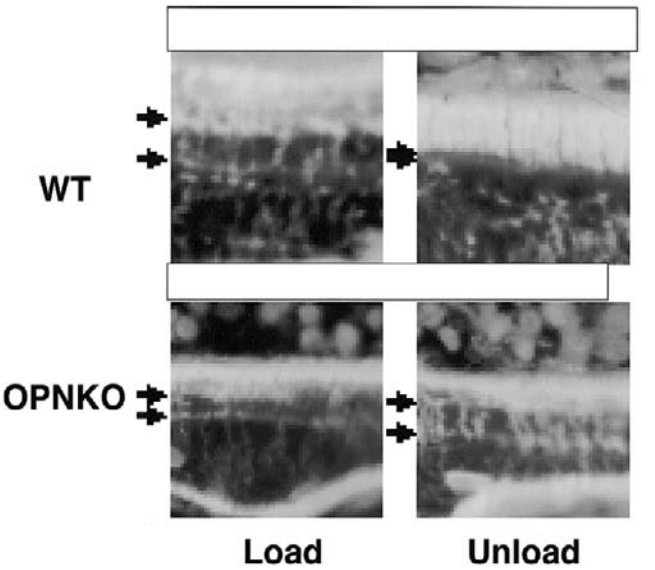

Figure 5 The reduction in bone formation induced in wild-type mice by mechanical stress does not occur in OPN-deficient mice. The hindlimbs of the mice were unloaded for 4 weeks prior to performing a calcein double-label analysis of the bone at the end of the tibia. Arrows indicate the lines of calcein labeling (light green). See Ishijima *et al.* (2001) for further details. (See also color plate.)

block bone resorption due to unloading, the two phenomena appear to be either regulated independently; alternatively, the bone resorption aspect could be downstream of the bone formation aspect. However, this may not be the case as increase in bone could be observed earlier than resorption after tail suspension relative to alterations in bone formation.

The inability of osteoblasts to respond to unloading in OPN-deficient mice suggests that OPN is involved in mediating the signaling induced by tail suspension to suppress the function of osteoblastic cells. This suppression, in turn, could give another signal to increase osteoclastic activities during the loss of bone mass in tail-suspended mice. Bone resorption due to unloading occurs immediately after the exposure of animals and human to agravity or to unloading conditions. However, it is not known whether such an early response of the osteoclasts to unloading is a direct or indirect phenomenon. Because osteoblastic activity, i.e., bone formation, can be detectable morphologically only after a relatively long stimulation period (a week or two) compared to bone resorption, and because the bone formation rate, though slow to change, is the most reliable marker for bone formation *in vivo,* it is possible that initial signals elicited by osteoblasts in the early period of time after exposure to loading and/or unloading have not yet been recognized by the current techniques.

To obtain further insight into the mechanism of the effects of mechanical stress, events occurring immediately after loading or unlaoding must be investigated by using methodology suitable to detect small changes in the metabolism or to detect signals elicited by the cells in the local environment. By such analyses it is still to be elucidated whether the absence of OPN by itself could impair sens-

ing of the mechanical stress in the case of OPN-deficient mice directly in the osteoblasts or indirectly by prohibiting osteoblastic cells via producing other possible loading-induced signals or by delivering the molecular messenger molecules to activate osteoclasts in response to unloading. Analyses of the events that are taking place at the interface between cells and extracellular components, as well as in the intracellular signaling process resulting in modified gene expression or protein function in the early period of unloading in the case of OPN-deficient mice, may yield clues to long-standing questions regarding how bone mass is lost in response to unloading. Studies have suggested that adherent bone cells unable to synthesize OPN tended to have a defect in their ability to respond to a fluid flow stimulus (Ishijima *et al.,* 2000; Denhardt, Krishna, Semeins, and Klein-Nulend, unpublished results).

Summary

As reviewed in the chapter, OPN plays a critical role in the maintenance of bone, especially as a molecule involved in the response of bones to external stress. It is also involved in other homeostatic defense mechanism in the mammalian organism. Further investigations are required to elucidate the molecular mechanisms of OPN action in mediating responses to inflammation, mechanical stress, angiogenesis, and accelerated bone resorption. Understanding the pathways of OPN signaling will contribute to the development of novel measures to cure patients suffering from many bone diseases, and other afflictions, in our aging modern society.

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CHAPTER 16

Bone Proteinases

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Introduction

This chapter surveys our knowledge of the proteinases expressed in bone. Although previously the osteoclast had been considered to be the main producer of proteinases in bone, it has become increasingly clear that osteoblasts play a significant role in the production of many of these proteinases. For example, it is true that the osteoclast secretes abundant lysosomal proteinases, especially cathepsin K (Vaes, 1988; Xia *et al.,* 1999; Yamaza *et al.,* 1998) and produces some of the neutral proteinases, e.g., matrix metalloproteinase-9 (MMP-9; Wucherpfennig *et al.,* 1994). However, osteoblasts, like their related cells fibroblasts, are able to secrete a host of proteinases, including neutral proteinases such as serine proteinases, plasminogen activators, and metalloproteinases such as collagenase-3, as well as lysosomal proteinases, e.g., cathepsins. Thus, osteoblasts, like fibroblasts, have the capacity to not only synthesize a range of matrix proteins, including type I collagen, but also have the ability to remodel their own extracellular matrix by the secretion of a range of proteinases. Proteinases can be classified into four groups: metalloproteinases e.g., collagenase-3; serine proteinases, e.g., plasminogen activator; cysteine proteinases, e.g., cathepsin K; and aspartic proteinases, e.g., cathepsin D.

This subdivision is based on the structure and the catalytic mechanism of the active site involving particular amino acid residues and/or zinc. In the following review of the proteinases synthesized in bone, we deal with each group according to this subdivision in the order just given. For some, much more is known than for others and they have warranted their own section.

Metalloproteinases

Matrix metalloproteinases (MMPs) are an important group of neutral proteinases thought to be involved in bone growth and bone remodeling. According to structural and functional characteristics, human MMPs can be classified into at least six different subfamilies of closely related members: collagenases, type IV collagenases (gelatinases), stromelysins, matrilysins, membrane-type MMPs (MT-MMPs), and other MMPs (Matrisian, 1992; Woessner, 1991; Vu and Werb, 2000). All matrix metalloproteinases are active at neutral pH, require Ca^{2+} for activity and contain Zn^{2+} in their active site. The catalytic domain of MMPs contains the conserved sequence HEXGH, which is believed to be the zinc-binding site. Metalloproteinases are secreted or inserted into the cell membrane in a latent form caused by the presence of a conserved cysteine residue in the prosegment, which completes the tetrad of zinc bound to three other residues in the active site. Cleavage of this pro-piece by other proteolytic enzymes (e.g., trypsin, plasmin, cathepsins, or other unknown activators) causes a loss of ~10 kDa of the pro-piece; this disrupts the cysteine association with the zinc and results in a conformational change in the enzyme yielding activation. Metalloproteinases all have homology to human fibroblast collagenase (collagenase-1, MMP-1) and all are inactivated by tissue inhibitors of metalloproteinases (TIMPs).

Activation of MMPs can occur via the plasminogen activator/plasmin pathway. Plasminogen activators convert plasminogen to plasmin, which subsequently can activate prostromelysin to stromelysin and procollagenase to col-

lagenase. The activated MMPs can then degrade collagens and other extracellular matrix proteins.

Apart from the regulation of secretion, activation, and/or inhibition, MMPs are substantially regulated at the transcriptional level (Matrisian, 1992; Crawford and Matrisian, 1996). Several MMPs contain specific regulatory elements in their promoter sequences. Human and rat stromelysin-1 and -2 contain activator protein-1 (AP-1) and polyoma enhancer activator-3 (PEA-3)-binding sites that may be important for basal levels and inducibility. AP-1 and PEA-3 consensus sequences have also been found in human, rabbit, and rat collagenase genes (Brinckerhoff, 1992; Selvamurugan *et al.,* 1998). The transcription factors Fos and Jun form heterodimers and act through the AP-1 sequence (Lee *et al.,* 1987; Chiu *et al.,* 1988; Angel and Karin, 1991), whereas c-*ets* family members bind at the PEA-3 sequence (Wasylyk *et al.,* 1993). The urokinase plasminogen activator gene also contains AP-1 and PEA-3-binding sites and, as a result, agents acting through these sites could lead to coordinate expression of many of these genes (Matrisian, 1992). Glucocorticoids and retinoids can suppress metalloproteinase synthesis at the transcriptional level (Brinckerhoff, 1992) by forming a complex with AP-1 transcription factors and inhibiting their action (Jonat *et al.,* 1990; Yang-Yen *et al.,* 1990). More recently, a new transcription factor-binding site has been identified in the collagenase-3 promoter as well as other bone-specific genes such as osteocalcin and osteopontin. This site is referred to as the runt domain (RD)-binding site or polyomavirus enhancer-binding protein-2A/osteoblast-specific element-2/nuclear matrix protein-2 binding site (Geoffroy *et al.,* 1995; Merriman *et al.,* 1995). Members of the core-binding factor (CBF) protein family (recently renamed RUNX by the Human Genome Organization), such as the osteoblastic transcription factor, CBFA1/RUNX2, bind to these RD sites (Kagoshima *et al.,* 1993). CBF/RUNX proteins are capable of binding to DNA as monomers, but can also heterodimerize with CBFB, a ubiquitously expressed nuclear factor (Kanno *et al.,* 1998; Ogawa *et al.,* 1993). CBFA1/RUNX2 is essential for the maturation of osteoblasts, and targeted disruption of the CBFA1/RUNX2 gene in mice produces skeletal defects that are essentially identical to those found in human cleidocranial dysplasia (Banerjee *et al.,* 1997; Ducy *et al.,* 1997; Mundlos *et al.,* 1997; Otto *et al.,* 1997).

Stromelysin

Stromelysin or MMP-3 degrades fibronectin, gelatin, proteoglycans, denatured type I collagen, laminin, and other extracellular matrix components (Chin *et al.,* 1985; Galloway *et al.,* 1983). Mesenchymal cells, such as chondrocytes and fibroblasts, are commonly found to secrete stromelysin (Matrisian, 1992). Transin, the rat homologue of human stromelysin, was originally discovered in fibroblasts transformed with the polyoma virus (Matrisian *et al.,* 1985). One importance of stromelysin comes from its implication in the activation of procollagenase (Murphy *et al.,* 1987), and the enzyme is thought to play a role, together with collagenase, in the destruction of connective tissues during disease states (Brinckerhoff, 1992).

Stromelysin is regulated by growth factors, oncogenes, cytokines, and tumor promoters. Epidermal growth factor (EGF) has been shown to increase stromelysin transcription through the induction of Fos and Jun, which interact at the AP-1 site in the promoter (McDonnell *et al.,* 1990). Platelet-derived growth factor is also important in the induction of stromelysin (Kerr *et al.,* 1988a). The protein kinase C activator, phorbol myristate acetate (PMA), is a notable stimulator of stromelysin transcription (Fini *et al.,* 1987; Brinckerhoff, 1992). Transforming growth factor-β (TGF-β), however, causes an inhibition of transin (rat stromelysin) expression (Matrisian *et al.,* 1986; Kerr *et al.,* 1988b) through a TGF-β inhibitory element (Kerr *et al.,* 1990).

In bone, stromelysin has been shown to be produced by normal human osteoblasts (Meikle *et al.,* 1992) after stimulation with PTH or monocyte-conditioned media (cytokine-rich). Similarly, Rifas *et al.* (1994) have shown that two human osteosarcoma cell lines (MG-63 and U2OS) secrete stromelysin and this may be increased by treatment with PMA, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α), but these authors were not able to find the enzyme in medium conditioned by cultured normal human osteoblasts. Mouse osteoblasts and osteoblastic cell lines also produce stromelysin-1 and demonstrate enhanced expression with $1,25(OH)_2D_3$, interleukin-1, or interleukin-6 treatment (Thomson *et al.,* 1989; Breckon *et al.,* 1999; Kusano *et al.,* 1998). There have also been reports that this stromelysin is expressed by osteoclasts (Witty *et al.,* 1992).

Type IV Collagenases (Gelatinases)

Type IV collagenases or gelatinases are neutral metalloproteinases requiring Ca^{2+} for activity and are involved in the proteolysis and disruption of basement membranes by degradation of type IV, V, and denatured collagens. There are two types of gelatinases, 72-kDa gelatinase (gelatinase A) or MMP-2 (Collier *et al.,* 1988) and 92-kDa gelatinase (gelatinase B) or MMP-9 (Wilhelm *et al.,* 1989). There are very distinct differences between the two gelatinases. The 72-kDa gelatinase has been found complexed to TIMP-2 (Stetler-Stevenson *et al.,* 1989), whereas the 92-kDa gelatinase has been found complexed to TIMP-1 (Wilhelm *et al.,* 1989). Regulation of the two gelatinases is also very distinct. Analysis of the genomic structure and promoter of the 72-kDa gelatinase has revealed that this gene does not have an AP-1 site or TATA box in the 5′ promoter region as all the other MMPs have been shown to have (Huhtala *et al.,* 1990). This enzyme is also not regulated by PMA and, in many cases, seems to be expressed constitutively rather than in a regulated fashion. In contrast, the 92-kDa gelatinase has a promoter very similar to the other MMPs and is regulated similarly (Huhtala *et al.,* 1991). Nevertheless, expression and activity of both types

of gelatinase are markedly stimulated by interleukin-1 (Kusano *et al.,* 1998).

In bone, as is to be expected, 72-kDa gelatinase is expressed constitutively by many osteoblastic preparations (Murphy *et al.,* 1989; Overall *et al.,* 1989; Rifas *et al.,* 1989, 1994; Meikle *et al.,* 1992) and is unchanged by treatment with any of the agents tested. The zymogen form of 72-kDa gelatinase is also resistant to activation by serine proteases, but MT1-MMP can initiate the activation of the 72 kDa progelatinase by cleavage of the Asn66-Leu peptide bond (Sato *et al.,* 1994).

The 92-kDa gelatinase is secreted by three osteosarcoma cell lines (TE-85, U2OS, and MG-63) (Rifas *et al.,* 1994) and, in some of the cell lines, can be stimulated by PMA, IL-1β, and TNF-α, analogous to these authors' observations regarding stromelysin. Similarly, they were unable to identify secreted 92-kDa gelatinase in the media of normal human osteoblasts or the human osteosarcoma cell line SaOS-2, which has been shown to have retained many characteristics of highly differentiated osteoblasts. Likewise, Meikle *et al.* (1992) found very little immunohistochemical staining for 92-kDa gelatinase in normal human osteoblasts. In fact, this enzyme has been found to be highly expressed by rabbit and human osteoclasts (Tezuka *et al.,* 1994a; Wucherpfennig *et al.,* 1994; Vu *et al.,* 1998). Indeed, a lack of expression of the 92 kDa gelatinase in mature osteoclasts of c-*fos*-null mice may be one of the reasons the animals exhibit an osteopetrotic phenotype (Grigoriadis *et al.,* 1994). Furthermore, studies of mice with a targeted inactivation of the gene indicate that the 92-kDa gelatinase plays a role in regulating endochondral bone formation, particularly of the primary spongiosa, possibly by mediating capillary invasion. Mice containing a null mutation in the 92-kDa gelatinase gene exhibit delays in vascularization, ossification, and apoptosis of the hypertrophic chondrocytes at the skeletal growth plates (Vu *et al.,* 1998). These defects result in an accumulation of hypertrophic cartilage in the growth plate and lengthening of the growth plate. These defects are reversible, and by several months of age the affected mice have an axial skeleton of normal appearance. It was postulated that the 92-kDa gelatinase is somehow involved in releasing angiogenic factors such as vascular endothelial growth factor (VEGF) that is normally sequestered in the extracellular matrix (Gerber *et al.,* 1999)

Membrane-Type Matrix Metalloproteinases

While most matrix metalloproteinases are secreted, a newly identified subtype called membrane-type matrix metalloproteinases (MT-MMPs) are inserted into the cell membrane (Sato *et al.,* 1997; Pei, 1999). These proteases contain a single transmembrane domain and an extracellular catalytic domain. Characteristically, MT-MMPs have the potential to be activated intracellularly by furin or furin-like proteases through recognition of a unique amino acid sequence: Arg-Arg-Lys-Arg111 (Sato *et al.,* 1996). To date, six MT-MMPs have been described, and MT1-MMP, MT2-MMP, and MT3-MMP have been shown to have a wide range of activities against extracellular matrix proteins (Pei and Weiss, 1996; Will *et al.,* 1996; Velasco *et al.,* 2000). A mouse cDNA homologue to MT4-MMP (mMT4-MMP) has been cloned (English *et al.,* 2000). MT4-MMP has the least degree of sequence identity to the other family members and has TNF-α convertase activity but does not activate pro-MMP2 (Puente *et al.,* 1996). Conversely, MT5-MMP and MT6-MMP may facilitate tumor progression through their ability to activate pro-MMP2 at the membrane of cells from tumor tissue (Llano *et al.,* 1999; Velasco *et al.,* 2000). As mentioned earlier, MT1-MMP (MMP-14) serves as a membrane receptor or activator of MMP-2 and possibly other secreted MMPs (Sato *et al.,* 1994). Further, studies indicate that MT1-MMP may also function as a fibrinolytic enzyme in the absence of plasmin and facilitate the angiogenesis of endothelial cells (Hiraoka *et al.,* 1998). MT1-MMP is highly expressed in embryonic skeletal and periskeletal tissues and has been identified in osteoblasts by *in situ* hybridization and immunohistochemistry (Apte *et al.,* 1997; Kinoh *et al.,* 1996). Targeted inactivation of the MT1-MMP gene in mice produces several skeletal defects that result in osteopenia, craniofacial dysmorphisms, arthritis, and dwarfism (Holmbeck *et al.,* 1999; Zhao *et al.,* 2000). Several of the notable defects in bone formation include delayed ossification of the membranous calvarial bones, persistence of the parietal cartilage vestige, incomplete closure of the sutures, and marked delay in the postnatal development of the epiphyseal ossification centers characterized by impaired vascular invasion. Histological observation suggested that the progressive osteopenia noted in these animals may be attributed to excessive osteoclastic resorption and diminished bone formation. This finding was supported by evidence that osteoprogenitor cells isolated from the bone marrow of these mutant mice demonstrate defective osteogenic activity.

Collagenases

Collagenases generally cleave fibrillar native collagens I–III at a single helical site and do so at neutral pH (Matrisian, 1992). The resultant cleavage products denature spontaneously at 37° C and become substrates for many enzymes, particularly gelatinases. The collagenase subfamily of human MMPs consists of three distinct members: fibroblast collagenase-1 (MMP-1), neutrophil collagenase-2 (MMP-8), and collagenase-3 (MMP-13) (Goldberg *et al.,* 1986; Freije *et al.,* 1994). An additional collagenase, called collagenase-4, has been identified in *Xenopus laevis* (Stolow *et al.,* 1996). At the present time, only one rat/mouse interstitial collagenase has been studied thoroughly and shown to be expressed by a range of cells, including osteoblasts. This collagenase has a high degree of homology (86%) to human collagenase-3 and is aptly named collagenase-3 (Quinn *et al.,* 1990). Rat collagenase-3 is secreted by osteoblasts, smooth muscle cells, and fibroblasts, in proenzyme form at

58 kDa, and is subsequently cleaved to its active form of 48 kDa (Roswit *et al.,* 1983). Efforts to isolate murine homologues of human collagenase-1 had been unsuccessful until recently, when two MMP-1-like genes, called Mcol-A and Mcol-B, which had 58 and 74% nucleotide sequence identity with human MMP-1, were identified within the MMP gene cluster on mouse chromosome 9 (Balbin *et al.,* 2001). When the cDNAs were expressed, however, only Mcol-A, and not Mcol-B, could degrade native type I and II collagens into the typical fragments and also degrade casein and gelatins (Balbin *et al.,* 2001). A murine ortholog of collagenase-2 has been identified by two groups (Lawson *et al.,* 1998; Balbin *et al.,* 1998). A role for Mcol-A, Mcol-B, or murine collagenase-2 in bone cell function has not been demonstrated, although human collagenase-2 is expressed in chondrocytes and other skeletal cells. In the report of Balbin *et al.* (2001), the expression of Mcol-A was limited to early embryos. It has also been shown that other MMPs [MMP-2 or gelatinase A (GelA or 72-kDa gelatinase) and MMP-14 or MT1-MMP, respectively] can function as collagenases *in vitro*. The collagenolytic activity of MMP-2 was demonstrated by using recombinant protein or after purifying the enzyme free of the TIMPs (Aimes and Quigley, 1995). An expressed soluble form of MMP-14 also has collagenase activity (Ohuchi *et al.,* 1997). These MMPs (-1, -2, -8, -13, and -14) all cleave each of the triple helical interstitial collagens at the same locus and therefore must also be considered to be collagenases.

In developing rat calvariae, we have found ample amounts of collagenase-3 by immunohistochemistry 14 days after birth (Davis *et al.,* 1998). These are always in select areas, mostly associated with sites of active modeling. At the cellular level, staining is associated with osteocytes and bone-lining cells that have the appearance of osteoblasts. Supporting these observations, Delaissé *et al.* (1988) have extracted abundant amounts of collagenase from developing mouse tibiae and calvariae. Originally, there was controversy regarding the cellular origin of bone collagenase. The osteoclast was reported to show immunohistochemical staining for collagenase (Delaissé *et al.,* 1993), but it was not determined whether this was a gene product of the osteoclast or was, perhaps, produced by osteoblasts/osteocytes and bound by the osteoclast through a receptor (see later). *In situ* hybridization of 17- to 19-gestational-day rat fetal long bones showed collagenase-3 expression only by chondrocytes, bone surface mononuclear cells, and osteocytes adjacent to osteoclasts; there was no evidence of expression in osteoclasts (Fuller and Chambers, 1995). Similarly, Mattot *et al.* (1995) showed expression of mouse collagenase-3 in hypertrophic chondrocytes and in cells of forming bone from humeri of mice at the 18th gestational day. Interestingly, the latter group found very little expression in any tissue other than mature cartilage and bone in mouse embryos. Related to this issue of whether osteoblasts or osteoclasts are a source of collagenase-3, it has been known for some time that bone explants from osteopetrotic mice (lack active osteoclasts) continue to produce abundant collagenolytic activity, either unstimulated or stimulated by bone-resorbing hormones (Jilka and Cohn, 1983; Heath *et al.,* 1990). Our interpretation from the evidence presented earlier is that the osteoblast/osteocyte and hypertrophic chondrocytes are the source of collagenases in skeletal tissue, whereas the osteoclast does not appear to express these genes. It should also be noted that the expression of MMP-13 assayed by *in situ* hybridization was strikingly reduced (Lanske *et al.,* 1996), although not absent, in the distal growth plate and midshafts of bones from PTH/PTHrP receptor−/−mouse embryos (Lanske *et al.,* 1998).

The remodeling of the fracture callus mimics the developmental process of endochondral bone formation. Excess tissue accumulates as callus prior to endochondral ossification followed by osteoclast repopulation. In collaboration with Dr. Mark Bolander, we demonstrated profuse concentrations of metalloproteinases in the fracture callus of adult rat long bones (Partridge *et al.,* 1993). The predominant cells observed to stain for collagenase-3 are hypertrophic chondrocytes during the phase of endochondral ossification; marrow stromal cells (putative osteoblasts) when the primary spongiosa is remodeled; and osteoblasts/osteocytes at a time when newly formed woven bone is being remodeled to lamellar bone. The consistent observation here is a role for this enzyme when a collagenous matrix must undergo substantial, rapid remodeling. This would indicate that the adult long bone has the ability to produce profuse levels of collagenase-3, but only when challenged, e.g., by a wound-healing situation.

Liu *et al.* (1995) have demonstrated that a targeted mutation encoding amino acids around the collagenase cleavage site in both alleles of the endogenous mouse type I collagen gene Col1a1 that results in resistance to collagenase cleavage leads to dermal fibrosis and uterine collagenous nodules. Nevertheless, these animals are able to develop normally to adulthood. Some of the major abnormalities only become apparent with increasing age. Studies of these mice revealed that homozygous mutant (r/r) mice have diminished PTH-induced bone resorption, diminished PTH-induced calcemic responses, and thicker bones (Zhao *et al.,* 1999). These observations imply that collagenase activity is necessary not only in older animals for rapid collagen turnover, but also for PTH-stimulated bone resorption. There were further observations regarding the abnormal skeletal phenotype and the effects of PTH in r/r mice (Zhao *et al.,* 2000). As early as 2 weeks of age, empty osteocyte lacunae were evident in the calvariae and long bones from r/r mice, and the number of empty lacunae increased with increasing age. Many persisting osteocytes, as well as periosteal cells in r/r calvariae, were *TUNEL* positive, whereas few *TUNEL*-positive cells were seen in +/+ calvariae. Evidence also indicates that collagenase cleavage takes place in periosteocytic ECM in wild type but not in r/r calvariae. Thus, normal osteocytes (and osteoblasts) and osteoclasts might bind to cryptic epitopes that are revealed by the collagenase cleavage of type I collagen by liganding the $\alpha_v\beta_3$ integrin to maintain their viability and, if such

signals are not induced (as postulated for the osteoclastic defect in r/r mice), they would undergo apoptosis and their lacunae would empty. Young r/r mice are also noted to develop thickening of the calvariae through the deposition of new bone predominantly at the inner periosteal surface; an increased deposition of endosteal trabecular bone was found in long bones in older r/r mice. Judging from the pattern of calcein labeling, the increased bone deposition in untreated r/r mice was accounted for by a marked activation of bone-forming surfaces. This pattern in untreated r/r mice resembled that in wild-type mice treated with PTH and might therefore be ascribable to secondary hyperparathyroidism, although significant differences in circulating levels of PTH in r/r compared to wild-type mice were not observed. Thus, the failure of collagenase to cleave type I collagen in r/r mice was associated with increased osteoblast and osteocyte apoptosis, yet, perhaps paradoxically, increased bone deposition.

Related to work in the whole animal, we have shown, together with Drs. Jane Lian and Gary Stein, that collagenase-3 is expressed late in differentiation in an *in vitro* mineralizing rat osteoblast culture system (Shalhoub *et al.,* 1992; Winchester *et al.,* 1999, 2000). When osteoblasts derived from fetal rat calvariae are grown in this culture system, they undergo development from an immature preosteoblast to a mature, differentiated osteoblast, which exists within a mineralized extracellular matrix [reviewed in Stein and Lian (1993) and Stein *et al.* (1990)]. The appearance of the enzyme in late differentiated osteoblasts may correlate with a period of remodeling of the collagenous extracellular matrix.

These observations regarding the differentiation of rat osteoblasts may explain the very low levels of human collagenase- 1 observed in cultures of normal human osteoblasts (Rifas *et al.,* 1989) where mRNAs and proteins were isolated from cells at confluence, but apparently not from mineralized cultures. Alternatively, the cultures may predominantly express the human homologue of rat collagenase-3, human collagenase-3 (rather than collagenase-1), which has been shown to be expressed by human osteoblasts, chondrocytes, and in synovial tissue, particularly in pathological conditions such as osteoarthritis (Johansson *et al.,* 1997; Mitchel *et al.,* 1996; Reboul *et al.,* 1996, Wernicke *et al.,* 1996). At the time that Rifas and colleagues conducted the work on human osteoblasts, human collagenase-3 had not been identified.

Canalis' group has conducted considerable research on the hormonal regulation of collagenase-3 in rat calvarial osteoblasts, including demonstrating stimulation by retinoic acid (Varghese *et al.,* 1994). More recently, they have demonstrated that triiodothyronine (T_3), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) all stimulate collagenase-3 transcription (Pereira, *et al.,* 1999, Rydziel, *et al.,* 2000; Varghese, *et al.,* 2000). Interestingly, they have also shown that insulin-like growth factors (IGFs) inhibit both basal and retinoic-stimulated collagenase expression (Canalis *et al.,* 1995) by these cells.

We have conducted many studies with the clonal rat osteosarcoma line UMR 106-01, which has been described as osteoblastic in phenotype (Partridge *et al.,* 1980, 1983). This cell line responds to all of the bone-resorbing hormones by synthesizing collagenase-3 (Partridge *et al.,* 1987; Civitelli *et al.,* 1989). In contrast to the physiological regulation of collagenase in fibroblasts (Woessner, 1991), synoviocytes (Brinckerhoff and Harris, 1981), and uterine smooth muscle cells (Wilcox *et al.,* 1994), the control of expression of this enzyme in bone and osteoblastic cells appears to have some distinct differences. First, it is stimulated by all the bone-resorbing hormones (Partridge *et al.,* 1987; Delaissé *et al.,* 1988), which act through different pathways, including protein kinase A (PKA; PTH and PGs), protein kinase C (PKC; PTH and PGs), tyrosine phosphorylation (EGF), and direct nuclear action [$1,25(OH)_2D_3$; retinoic acid]. Second, glucocorticoids do not inhibit stimulation by PTH (Delaissé *et al.,* 1988; T. J. Connolly, N. C. Partridge, and C. O. Quinn, unpublished observations) whereas retinoic acid stimulates collagenase-3 expression rather than inhibiting it (Delaissé *et al.,* 1988; Connolly *et al.,* 1994; Varghese *et al.,* 1994). Last, in rat osteosarcoma cells, PMA is unable to elicit a pronounced stimulatory effect on collagenase-3 gene expression.

Among the bone-resorbing agents tested, PTH is the most effective in stimulating collagenase-3 production by UMR cells. A single 10^{-7} *M* PTH dose significantly stimulates transient collagenase-3 secretion with maximal enzyme concentrations achieved between 12–24 hr (Partridge *et al.,* 1987; Civitelli *et al.,* 1989). This level is maintained at 48 hr, decreases to 20% of the maximum by 72 hr, and is ultimately undetectable by 96 hr. Because the enzyme is stable in conditioned medium and because experiments showed that this disappearance was not due to extracellular enzymatic degradation, we hypothesized that collagenase-3 was removed from the media through a cell-mediated binding process. Binding studies were conducted with ^{125}I-collagenase-3 at 4° C, which revealed a specific receptor for rat collagenase-3. This novel receptor is saturable, has high affinity ($K_d = 5$ nM), and has 12,000 receptors per UMR cell (Omura *et al.,* 1994). Further, we showed that binding of collagenase-3 in this fashion is responsible for the rapid internalization and degradation of collagenase-3. The processing of collagenase-3 in this system requires receptor-mediated endocytosis and involves sequential processing by endosomes and lysosomes (Walling *et al.,* 1998). In addition to UMR cells, we identified a very similar collagenase-3 receptor on normal, differentiated rat osteoblasts, rat and mouse embryonic fibroblasts, and human chondrocytes (Walling *et al.,* 1998; Barmina *et al.,* 1999). These results indicate that the function of the collagenase-3 receptor is to limit the extracellular abundance of collagenase-3 and, consequently, breakdown of the extracellular matrix.

Further investigation of the collagenase-3 receptor system has led us to conclude that collagenase-3 binding and internalization require a two-step mechanism both involving a specific collagenase-3 receptor and a member of the

low-density lipoprotein (LDL) receptor-related superfamily. For example, our ligand blot analyses demonstrate that ^{125}I-labeled collagenase-3 binds specifically to two proteins (approximately 170 and 600 kDa) present in UMR 106-01 cells (Barmina *et al.,* 1999). Of these two binding proteins, 170 kDa appears to be a high-affinity primary-binding site and the 600-kDa protein appears to be the low-density lipoprotein receptor-related protein responsible for mediating internalization. The LDL receptor superfamily represents a diverse group of receptors, including the LDL receptor, the low-density lipoprotein-related receptor protein (LRP), the VLDL receptor, and the gp330 receptor (Krieger and Herz, 1994). These plasma membrane receptors have a number of common features. All have a single membrane-spanning domain and several stereotyped repeats, both complement-like (for ligand binding) and EGF-like (for ligand dissociation). Each receptor in this family participates in receptor-mediated endocytosis, whereby the receptor–ligand complex is directed (via an NPXY signal in the receptor) to clathrin-coated pits and then internalized. Ligands bound by these receptors include LDL, VLDL, uPA-or tPA-PAI-1 complexes, tPA, lactoferrin, activated α_2-macroglobulin/proteinase complexes, apolipoprotein E-enriched β-VLDL, lipoprotein lipase, *Pseudomonas* exotoxin A, and vitellogenin (Krieger and Herz, 1994).

The striking stimulation of collagenase-3 secretion by bone-resorbing agents in UMR cells was shown to be paralleled by an even more striking induction of collagenase-3 mRNA. To undertake these studies, we isolated a cDNA clone to rat collagenase-3 (Quinn *et al.,* 1990). Examination of poly(A^+)RNA from PTH-treated UMR cells using this clone as a probe showed a ~l80-fold induction of collagenase-3 mRNA 4 hr after PTH treatment (Scott *et al.,* 1992) with a lag period of between 0.5 and 2 hr before collagenase-3 steady state mRNA levels rose above basal.

Nuclear run-on studies showed a comparable increase in transcription of the gene 2 hr after treatment with PTH. The PTH-induced increase in collagenase-3 transcription was completely inhibited by cycloheximide, whereas the transcriptional rate of β-actin was unaffected by inclusion of the protein synthesis inhibitor (Scott *et al.,* 1992). These results demonstrate that the PTH-mediated stimulation of collagenase-3 transcription requires *de novo* synthesis of a protein factor(s).

We used second messenger analogs to test which signal transduction pathway is of primary importance in the PTH-mediated transcriptional induction of the collagenase-3 gene. The cAMP analogue, 8BrcAMP, was capable of inducing collagenase-3 transcription to levels close to that of PTH. In contrast, neither the PKC activator, PMA, nor the calcium ionophore, ionomycin, when used alone, resulted in any increase in collagenase-3 gene transcription similar to that elicited by PTH after 2 hr of treatment (Scott *et al.,* 1992).

Thus, we demonstrated that PTH increases the transcription of collagenase-3 in rat osteoblastic osteosarcoma cells primarily by stimulation of the cAMP signal transduction pathway. Furthermore, the effect requires protein synthesis and a 1- to 1.5-hr lag period, suggesting that the transcriptional activation of the collagenase-3 gene may be the result of interactions with immediate early gene products. We next discovered that PTH transiently increases the mRNA expression of the AP-1 protein subunits c-fos and c-jun (Clohisy *et al.,* 1992). Both mRNA species were maximally induced within 30 min, well before the maximal transcription rate of 90 min for collagenase-3. Later we determined that PTH is responsible for phosphorylation of the cAMP response element-binding (CREB) protein at serine 133 (Tyson *et al.,* 1999). Once phosphorylated, the CREB protein binds a cAMP response element (CRE) in the c-fos promoter and activates transcription (Pearman *et al.,* 1996).

To further identify and delineate the signal transduction pathways involved in the PTH regulation of the collagenase-3 gene in osteoblastic cells, genomic clones of the rat collagenase-3 gene were isolated. The collagenase-3 gene has 10 exons (Rajakumar *et al.,* 1993), encoding a mRNA of ~2.9 kb, which in turn encodes the proenzyme with a predicted molecular weight of the core protein of 52 kDa (Quinn *et al.,* 1990). A large stretch of promoter region was isolated in one of these clones, and from this a series of deletion and point mutants were generated to identify the PTH-responsive region and subsequently the primary response genes, which convey the hormonal signal and bind to this region(s) of the collagenase-3 gene. The minimum PTH regulatory region was found to be within 148 bp upstream of the transcriptional start site (Selvamurugan *et al.,* 1998). This region contains several consensus transcription factor recognition sequences including SBE (Smad binding element), C/EBP (CCAAT enhancer-binding protein site), RD (runt domain-binding sequence), p53, PEA-3 (polyoma enhancer activator-3), and AP (activator protein)-1 and -2. The AP-1 site is a major target for the Fos and Jun families of oncogenic transcription factors (Chiu *et al.,* 1988; Lee *et al.,* 1987; Angel and Karin, 1991). The RD site is a target for core-binding factor proteins, specifically CBFA1/RUNX2. Mice containing a targeted disruption of the CBFA1/RUNX2 gene die at birth and lack both skeletal ossification and mature osteoblasts (Ducy *et al.,* 1997; Komori *et al.,* 1997; Otto *et al.,* 1997). These mutant mice also do not express collagenase-3 during fetal development, indicating that collagenase-3 is one of the target genes regulated by CBFA1/RUNX2 (Jimenez *et al.,* 1999). Additional experiments on the collagenase-3 promoter determined that both native AP-1 and RD sites and their corresponding binding proteins, AP-1 and CBFA1/RUNX2-related proteins, were involved in PTH regulation of the collagenase-3 promoter. Using gel-shift analysis, we further showed enhanced binding of c-Fos and c-Jun proteins at the AP-1 site upon treatment with PTH (Selvamurugan *et al.,* 1998), although there was no significant change in the level of CBFA1/RUNX2 binding to the RD site. We determined that PTH induces PKA-mediated posttranslational modification of CBFA1/RUNX2 and leads to enhanced collagenase-3 promoter activity in UMR cells (Selvamurugan *et al.,* 2000b). The binding of members of the AP-1 and CBF/RUNX families to their corresponding binding sites in the collagenase-3

promoter also appears to regulate collagenase-3 gene expression during osteoblast differentiation (Winchester *et al.,* 2000). As discussed earlier, collagenase-3 expression is regulated by a variety of growth factors, hormones, and cytokines, but the effects of these compounds appear to be cell type specific. Data obtained in breast cancer and other cell lines suggest that the differential expression of and regulation of collagenase-3 in osteoblastic compared to nonosteoblastic cells may depend on the expression of AP-1 factors and post-translational modifications of CBFA1/RUNX2 (Selvamurugan and Partridge, 2000; Selvamurugan *et al.,* 2000a). The close proximity of the AP-1 and RD sites and their cooperative involvement in the activation of the collagenase-3 promoter suggests that the proteins binding to these sites may also physically interact. Recent work indicates this to be the case, as CBFA1 directly binds c-Fos and c-Jun in both *in vitro* and *in vivo* experiments (D'Alonzo *et al.,* 2000).

Plasminogen Activators

The plasminogen activator (PA)/plasmin pathway is involved in several processes, including tissue inflammation, fibrinolysis, ovulation, tumor invasion, malignant transformation, tissue remodeling, and cell migration. The PA/plasmin pathway is also thought to be involved in bone remodeling by osteoblasts and osteoclasts. The pathway results in the formation of plasmin, another neutral serine proteinase, which degrades fibrin and the extracellular matrix proteins fibronectin, laminin, and proteoglycans. In addition, plasmin can convert matrix metalloproteinases, procollagenase, and prostromelysin to their active forms (Eeckhout and Vaes, 1977). Plasminogen has been localized to the cell surface of the human osteosarcoma line MG63, where its activity was enhanced by endogenous cell bound uPA (Campbell *et al.,* 1994).

The PA/plasmin pathway is regulated by members of the serpin family in addition to various hormones and cytokines. The primary function of this family of inhibitors is to neutralize serine proteinases by specific binding to the target enzyme. Serpins are involved in the regulation of several processes, including fibrinolysis, cell migration, tumor suppression, blood coagulation, and extracellular matrix remodeling (Potempa *et al.,* 1994). Members of this pathway involved in the regulation of the PA/plasmin pathway are plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2), which regulate uPA and tPA; protease nexin-1, which regulates thrombin, plasmin, and uPA; and α_2-antiplasmin, which regulates plasmin. Active PAI-1 combines with uPA and tPA, forming an equimolar complex (Levin, 1986), exerting its inhibition through interactions with the active site serine. PAI-1 has been detected in media of cultured human fibrosarcoma cells (Andreasen *et al.,* 1986) and primary cultures of rat hepatocytes and hepatoma cells. PAI-1 was also detected from conditioned medium of rat osteoblast-like cells and rat osteosarcoma cells (Allan *et al.,* 1990).

Urokinase-Type Plasminogen Activator

The urokinase-type plasminogen activator is secreted as a precursor form of ~55 kDa (Nielsen *et al.,* 1988; Wun *et al.,* 1982). It is activated by cleavage into a 30-kDa heavy chain and a 24-kDa light chain, joined by a disulfide bond, with the active site residing in the 30-kDa fragment. Urokinase has a Kringle domain, serine proteinase-like active site, and a growth factor domain (GFD). The noncatalytic NH_2-terminal fragment contains the GFD and Kringle domain and is referred to as the amino-terminal fragment (ATF). Rabbani *et al.* (1990) demonstrated that ATF stimulated proliferation and was involved in mitogenic activity in primary rat osteoblasts and the human osteosarcoma cell line, SaOS-2. The GFD of the ATF is necessary for the binding of uPA to its specific receptor.

Tissue-Type Plasminogen Activator

The tissue-type plasminogen activator is secreted as a single-chain glycosylated 72-kDa polypeptide. This enzyme has been found in human plasma and various tissue extracts, as well as in normal and malignant cells. The cleavage of tPA forms a 39-kDa heavy chain and a 33-kDa light chain linked by a disulfide bond. The heavy chain has no proteinase activity, but contains two Kringle domains that assist in binding fibrin to plasminogen (Banyai *et al.,* 1983; Pennica *et al.,* 1983). Furthermore, the heavy chain contains a finger domain involved in fibrin binding (van Zonneveld *et al.,* 1986) and a GFD with homology to human and murine epidermal growth factor.

Plasminogen Activators in Bone

Plasminogen activator activity is increased in normal and malignant osteoblasts as well as calvariae by many agents, including PTH, $1,25(OH)_2D_3$, PGE_2, IL-1α, fibroblast growth factor, and EGF (Hamilton *et al.,* 1984, 1985; Thomson *et al.,* 1989; Pfeilschifter *et al.,* 1990; Cheng *et al.,* 1991; Leloup *et al.,* 1991; De Bart *et al.,* 1995). It should be noted that work suggests that PAs are not necessary for PTH- and $1,25(OH)_2D_3$-induced bone resorption (Leloup *et al.,* 1994). Expression of tissue-type plasminogen activator, urokinase-type plasminogen activator, plasminogen activator inhibitor-1, plasminogen activator inhibitor-2, protease nexin, and urokinase receptor isoform 1 (uPAR1) were detected in mouse osteoclasts using the reverse transcriptase–polymerase chain reaction (RT-PCR) (Yang *et al.,* 1997). Deletion of tPA, uPA, PAI-1, and plasminogen genes in mice can lead to fibrin deposition, some growth retardation, and inhibition of osteoclast ability to remove noncollagenous proteins *in vitro*, but no other significant effects on bone were reported (Carmeliet *et al.,* 1993, 1994; Bugge *et al.,* 1995; Daci *et al.,* 1999). There are conflicting data as to whether the increase in osteoblastic PA activity is due to an increase in the total amount of one or both of the PAs or is due to a decline in the amount

of PAI-1. All possible results have been observed, depending on which osteoblastic cell culture system is used or the method of identification of the enzymes. The latter have been difficult to assay categorically because there have not been abundant amounts of specific antibodies available for each of the rat PAs. Similarly, different groups have found the predominant osteoblastic PA to be uPA whereas others have obtained results indicating it to be tPA.

A range of agents have also been found to inhibit the amount of osteoblastic PA activity. These include glucocorticoids, TGF-β, leukemia inhibitory factor, and IGF-I (Allan *et al.,* 1990, 1991; Cheng *et al.,* 1991; Hamilton *et al.,* 1985; Lalou *et al.,* 1994; Pfeilschifter *et al.,* 1990). Where it has been examined, in many of these cases the decline is due to a substantial increase in PAI-1 mRNA and protein. Nevertheless, some of these agents also markedly enhance mRNA abundance for the PAs (Allan *et al.,* 1991), although the net effect is a decline in PA activity.

Cysteine Proteinases

The major organic constituent of the ECM of bone is fibrillar type I collagen, which is deposited in intimate association with an inorganic calcium/phosphate mineral phase. The presence of the mineral phase not only protects the collagen from thermal denaturation but also from attack by proteolytic enzymes (Glimcher, 1998). The mature osteoclast, the bone-resorbing cell, has the capacity to degrade bone collagen through the production of a unique acid environment adjacent to the ruffled border through the concerted action of a vacuolar proton pump ([V]-type H^+-ATPase) (Chakraborty *et al.,* 1994; Bartkowicz *et al.,* 1995; Teitelbaum, 2000) and a chloride channel of the Cl-7 type (Kornak *et al.,* 2001). Loss-of-function mutations in the genes that encode either this proton pump (Li *et al.,* 1999; Frattini *et al.,* 2000; Kornak *et al.,* 2000) or the chloride channel (Kornak *et al.,* 2001) lead to osteopetrosis. At the low pH in this extracellular space adjacent to the ruffled border, it is possible to leach the mineral phase from the collagen and permit proteinases that act at acid pH to cleave the collagen (Blair *et al.,* 1993). Candidate acid-acting proteinases are cysteine proteinases such as cathepsin K. Cathepsin K is highly expressed in osteoclasts (Drake *et al.,* 1996; Bossard *et al.,* 1996). Cysteine proteinases contain an essential cysteine residue at their active site that is involved in forming a covalent intermediate complex with its substrates (Bond and Butler, 1987). The enzymes are either cytosolic or lysosomal. The latter have an acidic pH optimum and make up the majority of the cathepsins. These enzymes are regulated by a variety of protein inhibitors, including the cystatin superfamily (Turk and Bode, 1991) and α_2-macroglobulin (Barrett, 1986). Their extracellular abundance must consequently be regulated by cell surface receptors for α_2-macroglobulin as well as the lysosomal enzyme targeting mannose-6-phosphate/IGF-II receptors.

Involvement of lysosomal cysteine proteinases in bone resorption has been indicated by many studies showing that inhibition of these enzymes prevents bone resorption *in vitro* as well as lowering serum calcium *in vivo* (Delaissé *et al.,* 1984; Montenez *et al.,* 1994). A more recently identified cathepsin, cathepsin K (Tezuka *et al.,* 1994b), was found to have substantial effects on bone. Mice containing a targeted disruption of cathepsin K were developed and found to exhibit an osteopetrotic phenotype characterized by excessive trabeculation of the bone marrow space (Saftig *et al.,* 1998, 2000). Additionally, cathepsin K mutations have been linked to pycnodysostosis, a hereditary bone disorder characterized by osteosclerosis, short stature, and defective osteoclast function (Gelb *et al.,* 1996). Immunohistochemistry revealed that the majority of the cysteine proteinases (cathepsins B, K, and L) and the aminopeptidases (cathepsins C and H) are products of osteoclasts (Ohsawa *et al.,* 1993; Yamaza *et al.,* 1998; Littlewood-Evans *et al.,* 1997), although immunoreactive staining for cathepsins B, C, and H was also seen in osteoblasts and osteocytes. It is notable that the most potent collagenolytic cathepsin at acid pH, cathepsin L, was strongly expressed in osteoclasts and very weakly in osteoblasts. Mathieu *et al.* (1994), however, have detected both cathepsins B and L as proteins secreted by their immortalized osteogenic stromal cell line, MN7. Oursler *et al.* (1993) have also demonstrated that normal human osteoblast-like cells produce cathepsin B and that dexamethasone can increase expression and secretion of this lysosomal enzyme by these cells. Interestingly, they also showed that dexamethasone treatment causes activation of TGF-β and, by the use of lysosomal proteinase inhibitors, ascribed a role for cathepsins B and D to activation of this growth factor.

Aspartic Proteinases

These lysosomal proteinases contain an aspartic acid residue at their active site and act at acid pH. Very little investigation has been conducted on these enzymes in bone cells except for the observations that cathepsin D, a member of this family, can be found by immunohistochemical staining in osteoblasts and osteocytes (Ohsawa *et al.,* 1993), and expression of this enzyme is increased markedly by dexamethasone treatment of human osteoblasts in culture (Oursler *et al.,* 1993).

Conclusions

The osteoblast has the ability to produce proteinases of all four classes, but far more is known about their production of collagenase and plasminogen activators, at least *in vitro*. We still do not know the absolute role of any of these osteoblastic enzymes *in vivo*. Further work with knockouts of the respective enzymes are likely the only way we will deter-

mine their required functions. These roles may not be restricted to assisting in the resorption process but may include functions to regulate bone development. Additionally, the osteoclast produces MMP-9 and cathepsin K, which appear to have similar roles in the two diverse processes.

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CHAPTER 17

Integrins and Other Cell Surface Attachment Molecules of Bone Cells

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Introductory Remarks: Adhesion and Bone Cell Function

Osteoclasts are the main cells responsible for the breakdown of the extracellular matrix of bone during normal and pathological bone turnover (see Chapters 7 and 8; Chambers, 2000). Osteoclastic bone resorption involves a series of developmental and regulatory steps that include the proliferation and homing to bone of hemopoietic progenitor cells; their differentiation into postmitotic osteoclast precursors, which express features of mature osteoclasts; fusion to form multinucleated cells; and migration of osteoclasts to the area of bone to be remodeled. Osteoclasts attach to the bone surface and polarize to create three discrete areas of plasma membrane: (1) the basolateral membrane, which faces the marrow space and is not in contact with the bone; (2) the "tight sealing", or clear, zone that is closely apposed to the bone matrix; and (3) the ruffled border, a highly convoluted area of plasma membrane, which faces the bone matrix and is surrounded by the sealing zone. The sealing zone (see later) forms a diffusion barrier and permits the localized accumulation of high concentrations of protons and proteases secreted via the ruffled border into an extracellular resorption compartment underneath the cell. Many of these steps involve adhesion between mature osteoclasts and osteoclast precursors and other cell types in the bone/bone marrow compartment and with components of the extracellular matrix of bone (some of these possible functional events are summarized in Table I).

Bone (re)modeling (see Chapters 3, 4, and 19) involves the coordinated response of osteoblasts, osteocytes, and osteoclasts. Osteoblasts (see Chapters 4 and 5) and bone-lining cells form a near-continuous layer covering the periosteal, endosteal, and trabecular bone; interactions between these cells and the organic matrix of bone are important determinants of osteoblast proliferation and differentiation. Osteocytes (see Chapter 6) are found in lacunae, set within the bone matrix, and are joined both to their neighbors and cells lining the bone surfaces by cytoplasmic processes, which pass through fine channels or canaliculi. Together, this interconnecting network of osteoblasts, bone-lining cells, and osteocytes provides a possible mechanism for the detection of physical or mechanical changes and the coordination of osteosynthetic and resorptive activity leading to remodeling. Cell–cell and cell–matrix communication is central to this process, and by inference, cell adhesion molecules will be key players in these events, both in normal skeletal homeostasis, growth, and development and in pathological situations where the balance between resorption and remodeling becomes disturbed (see Table I).

Connective tissue cells in general, and bone and cartilage cells in particular, are surrounded by an abundance of extracellular matrix. Chondroblasts, osteoblasts, and, to a lesser extent, osteocytes are responsible for the synthesis of the majority of the organic components of this matrix, whereas osteoclasts mainly degrade the matrix. The function of bone and cartilage cells reflects the matrix components that surround them; conversely, the composition of the matrix, i.e., the structure of cartilage and bone, is highly dependent

Table I Summary of Possible Functions of Cell Adhesion Receptors in Bone

Osteoclast development and function
Migration of committed osteoclast precursors from the bone marrow to sites of future resorption, exiting via specialized endothelial barriers
Homing to "bone" (using chemo-, haptotactic signals) and ingress across vascular endothelium
Recognition of, adhesion to, and migration upon "bone" matrix proteins
Fusion of postmitotic osteoclast precursors
Regulatory intercellular interactions with osteoblasts, leucocytes, and other cell types in marrow space; presentation of growth factors from extracellular matrix stores
Signal transduction (and control of osteoclast function) by interaction with matrix (via RGD and other sequences)
Cellular polarization, cytoskeletal (re)organization, tight sealing zone formation, and bone resorption
Cessation of resorption by detachment from matrix, cell migration, and regulation of osteoclast survival versus apoptosis
Osteoblasts (and osteocytes)
Transduction of mechanical signals within skeleton to regulate cell function
Adhesion to, and migration on, bone matrix, including unmineralized osteoid
Regulation of osteoblast maturation from mesenchymal stem cells
Regulation of mature cell function (gene expression, matrix synthesis, protease secretion, etc.)
Interaction with other bone cells (e.g., osteoclasts) and cells in the bone marrow compartment (e.g., marrow stroma, leukocytes)
Chondrocytes
Response to mechanical forces (e.g., in articular cartilage)
Maintenance of tissue integrity by matrix synthesis and assembly
Regulation of chondrocyte proliferation, maturation, gene expression, and cell survival
Mediation of response in cartilage to injury and disease

on the cellular function of chondroblasts, osteoblasts, and osteoclasts.

Cell–matrix interactions associated with osteoclastic bone resorption have been researched extensively (reviewed in Väänänen and Horton, 1995; Horton and Rodan, 1996; Helfrich and Horton, 1999; Duong *et al.,* 2000). Much less is known about cell–cell and cell–matrix interactions in osteoblasts and related populations, although there has been considerable progress since the first published analysis (Horton and Davies, 1989; Helfrich and Horton, 1999; Bennett *et al.,* 2001b). The best defined of these adhesive interactions are mediated by a particular class of cell adhesion molecule, the integrin receptors. Integrins are now known to be major functional proteins of osteoclasts and have become targets for potential therapeutic intervention in bone diseases such as osteoporosis. The balance of this chapter reflects this bias, but some discussion of the nature and function of other adhesion proteins, and cell adhesion receptors in other bone cell types, is included for completeness.

Overview of Cell Adhesion Molecule Structure

Adhesion Receptors and Their Ligands

Molecular and immunological approaches have led to considerable advances in our understanding of the range of cell membrane molecules that are capable of mediating cell adhesion. Detailed sequence and structural analysis (reviewed in Barclay *et al.,* 1997; Isacke and Horton, 2000) has enabled many of them to be grouped into "families," with related structure based on their content of highly homologous domains. Thus, for example, the immunoglobulin (Ig) superfamily, which formed the first class of homologous adhesion proteins to be identified, is characterized by an Ig domain of about 70–100 amino acids arranged between two sheets of antiparallel β strands, which is found in more than 100 molecules (Barclay *et al.,* 1997). The major groupings of adhesion receptor families are summarized in Table II; this identifies some specific examples, their regions of homology by which they are defined, their ligands, and the nature and specificity of their interactions with receptors. Individual members of the families have a diverse range of structures, tissue distribution, and functions, and it is outside the scope of this chapter to provide information other than in outline. The reader is referred to Barclay *et al.* (1997) and Isacke and Horton (2000) for further details; as integrin receptors form a major focus of this chapter, some basic structural information is provided in greater depth later.

Similar methods have been applied to elucidate the structure of the molecules recognized by cell adhesion proteins, i.e., their ligands; these include components of the extracellular matrix and plasma proteins and cell-associated matrix proteins (Table II; Ayad *et al.,* 1998) and cell membrane- associated "counterreceptors" (e.g., the "ICAMs," Table II). As with adhesion receptors, a range of structural domains are recognizable within their ligands, some of which have clearly defined functions; e.g., the well-characterized Arg-Gly-Asp (RGD) peptide motif, originally described in the protein fibronectin and now known to be present widely in many matrix proteins (Pierschbacher and Ruoslahti, 1984; Ruoslahti, 1996). The function of others, despite their frequency, remains unclear; thus, the function of EGF repeats in, for example, laminin is unknown. Interestingly, some of the domains that have been found in extracellular matrix proteins can also be identified in adhesion receptors (Table II), suggesting a shared function; e.g., hyaluronidate-binding sites have been found in both the matrix proteoglycan, versican, and the "homing" receptor, CD44.

The diversity of the types and combinations of cellular receptors and the complexity of the molecular structure of the extracellular matrix are reflected in the large number of functions that have been ascribed to "cell adhesion molecules." These include both true adhesive interactions, which are clearly seen in, for instance, cell-to-cell interactions regulating the immune response and the integrity of epithelial barriers, or via the increasingly identified signaling pathways

Table II Classes of Cell Adhesion Receptors and Their Ligands

Family	Homology region in receptor	Examples	CD No.	Ligands	Recognition motif in ligand/ counterreceptor	Extracellular matrix components with shared homology domain
Integrin	PEGG (all β chains) I domain (CD11, $\alpha 1 \alpha 2$)	gpIIbIIIa LFA-1 $\alpha_v\beta_3$ $\alpha_2\beta_1$ $\alpha_4\beta_1$	CD41/61 CD11/18 CD51/61 CD49b/29 CD49d/29	Blood proteins ICAM counterreceptor Matrix, blood proteins Collagen Fibronectin	RGD, KQAGDV ICAMs, etc. RGD DGEA, GER EILDV	Collagen VI, von Willebrand factor, cartilage matrix protein (integrin I domain)
Ig superfamily	Ig fold	ICAMs, VCAM N-CAM CD2	CD54, etc.	Heterophylic interaction Homophylic LFA-3 counterreceptor	Multiple KYSFNYDGSE	Perlecan (Ig fold) Fibronectin, tenascin, thrombospondin (N-CAM type III repeat)
Selectins	C-type lectin, EGF repeat Complement regulatory protein domain	L-, P-selectin	CD62	Glycam-1, PSGL-1, CD34, etc.	Sialyl Le^x (CD15), etc.	Aggrecan, versican (lectin) Laminin, tenascin, thrombospondin, aggrecan, veriscan (EGF repeat) Aggrecan, versican (complement regulatory domain)
Cadherins	LDRE repeat (110 amino acid module)	E-, N-cadherin		Homophylic	HAV	
Leucine-rich glycoproteins (LRG)	Leucine repeat (24 amino acid repeat)	Platelet gpIb	CD42b	Blood proteins	von Willebrand factor, thrombin	Biglycan, decorin
Mucins	Mucin side chain	Leukosialin	CD43 CD34	Selectins		Muc-1
CD36 family		Platelet gpIV	CD36	Thrombospondin, collagen	SVTCG (for thrombospondin)	Aggrecan, versican, link protein
CD44	Hyaluronidate-binding site		CD44	Hyaluronic acid, etc.		Aggrecan, versican, link protein

mediated by adhesion receptors, including integrins and cadherins. This includes events mediated through linkages to the F-actin cytoskeleton, leading to changes in cell shape and motility, and activation of Src family and other tyrosine kinases or mobilization of intracellular calcium, resulting in other functional changes downstream, such as activation of early response genes or protease secretion (termed "outside-in" signaling). Similarly, intracellular events can lead to the modification of receptor affinity and activity ("inside-out" signaling; e.g., the platelet integrin gpIIbIIIa will only bind fibrinogen after alterations in integrin conformation following activation on ligand binding to other nonintegrin receptors such as via the thrombin receptor).

Integrin Structure

Integrins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Hynes, 1992; see also Isacke and Horton, 2000) are heterodimeric proteins whose constituent polypeptide chains, α and β, are linked noncovalently. Although originally identified by antibodies or direct purification, the primary structure of most integrin subunits has been deduced by cDNA cloning. To date, 17 different mammalian α subunits and 9 β subunits have been identified, forming 23 distinct heterodimers.

Both integrin subunits are transmembrane, N-glycosylated glycoproteins with a large extracellular domain, a single hydrophobic transmembrane region, and a short cytoplasmic domain (apart from β_4, which has a large intracellular domain not found in other integrins). Electron microscopy of several purified integrin dimers shows an extended structure with dimensions of approximately 10 by 20 nm, formed by an N-terminal globular "head" composed by the association of the two subunits, connected to the membrane by two "stalks."

α subunits vary in size from 120 to 180 kDa, and analysis of their cDNA sequences reveals several features in common. All contain seven homologous, tandem repeat sequences of approximately 60 amino acids length, with the last three or four containing putative divalent cation-binding sites showing similarity to the EF-hand loop structure seen in calmodulin. These sites are of critical importance to both ligand binding and subunit association. Some integrins contain an inserted, or "I," domain of approximately 200 amino acids between the second and third repeats (see Table II) that is involved in ligand binding. Other integrin α subunits are cleaved posttranslationally near the transmembrane domain.

β subunits are 90 to 110 kDa in size, apart from the 210-kDa β_4 chain. Their cDNA sequences show a high cysteine content (e.g., 56 Cys residues in β_3), largely concentrated in four 40 amino acid long segments that are internally disulfide bonded. Several conserved motifs in β chains are involved in ligand binding and interaction with cytoskeletal elements.

Integrins recognize highly specific peptide recognition sequences, such as the Arg-Gly-Asp (RGD) sequence present in fibronectin (Pierschbacher and Ruoslahti, 1984), in adhesion to extracellular matrix proteins, or Ig family members such as VCAM and ICAM in intercellular interactions. Cross-linking studies using radioactively labeled RGD peptide probes for the integrins $\alpha_v\beta_3$ and gpIIbIIIa, respectively (see Isacke and Horton, 2000), and mutational analysis have shown the ligand-binding site to be composed of distinct, relatively short elements in the N termini of both α and β subunits. When taken with the requirement for an "I" domain for ligand binding in some integrins, these data suggest that the interaction site depends on the composite structure formed by interplay of the two chains of the receptor, with ligand specificity reflecting subunit usage.

Integrins are linked to the F-actin cytoskeleton via interaction of the β subunit with actin-binding proteins, including α-actinin, vinculin, and talin. The cytoplasmic domain of the β subunit also associates with a signaling complex comprising kinases and phosphatases and various adaptor proteins. Ligand binding leads to the activation of one or more intracellular signal transduction pathways, which, in turn, contribute to the regulation of differentiation, cytoskeletal organization, and other aspects of cell behavior. Most information regarding signaling via integrins (Clark and Brugge, 1995; Dedhar *et al.,* 1999; Giancotti and Ruoslahti, 1999; Coppolino and Dedhar, 2000; Schlaepfer and Hunter, 1998) has come from studies in cells that produce focal contacts *in vitro;* here, the focal adhesion kinase FAK is targeted to focal adhesions, where it associates with the cytoskeleton and is activated by autophosphorylation. Downstream signaling pathways include association of Src family kinases with phosphorylated FAK and engagement of the Ras-MAP kinase pathway.

Cadherins

Cadherins (see Isacke and Horton, 2000; Chapter 18) are a large family of calcium-dependent transmembrane proteins that are associated with the catenin-linked actin cytoskeleton; they play prominent roles in morphogenesis and intercellular adhesion and signaling. Cadherins share several regions of high homology, with the greatest found in the short cytoplasmic domain; they have molecular masses of around 100–130 kDa. The extracellular domain contains repeats ("cadherin repeats") of around 110 amino acids, which contain negatively charged, calcium-binding motifs in the first three repeats and conserved cysteine residues in the fifth repeat. The ligand-binding site of cadherins is the conserved HAV motif located at the N-terminal region of the molecule in the first conserved extracellular repeat. Cadherins are divided into two main subsets of receptors: classical cadherins and protocadherins. The latter differ from classical cadherins in that they do not have a propeptide sequence and contain a variable number of cadherin repeats; this group includes a number of *Drosophila* gene products with cadherin-like sequences and a growing group of cadherin homologues with atypical functions and complexity increased by alternate splicing.

Cadherins are mediators of cell–cell adhesion and bind ligand mainly in a homophilic manner, although heterophilic binding between different cadherin molecules and with integrins occurs. On the cell surface, cadherins tend to be concentrated at cell–cell junctions, where they can associate with members of the Src kinase family, leading to the activation of signaling pathways.

CD44

CD44, also known as the hyaluronan or "homing" receptor, forms a family of transmembrane glycoproteins (see Isacke and Horton, 2000) with molecular masses of 80–200 kDa. They share an N-terminal region that is related to the cartilage proteoglycan core and link proteins. Alternative splicing of 20 exons and extensive posttranslational modification such as glycosylation and addition of chondroitin sulfate produces the wide variety of CD44 proteins. Chondroitin sulfate-containing variants can bind fibronectin, laminin, and collagen in addition to the extracellular matrix glycosaminoglycan hyaluronan, and CD44 binding to osteopontin has also been reported. CD44 functions, therefore, in a variety of ways, including cell–cell interaction, such as homing and endothelial transmigration of lymphocytes, and also cell–matrix adhesion. Malignant transformation of cells leads to the upregulation of CD44 expression, and metastatic tumors often express an altered repertoire of CD44 variants.

Immunoglobulin Superfamily

The immunoglobulin (Ig) family of receptors (see Isacke and Horton, 2000) all share a basic motif consisting of an Ig fold of between 70 and 110 amino acids organized into two antiparallel β sheets, which seem to serve as a scaffold upon which unique determinants can be displayed. There is considerable variation in the primary structure of the members of this family, and hence in their molecular weights, but their tertiary structure is well conserved. There are well over 100 members of this family currently known, all with different numbers of the basic Ig repeats. Their functions are wide ranging, with some members functioning as true signal-transducing receptors, whereas others have predominantly adhesive functions. Ligands for Ig family members include other Ig family members (identical, as well as nonidentical members), but also members of the integrin family and components of the extracellular matrix.

Selectins

Selectins (see Isacke and Horton, 2000) are a family of three closely related glycoproteins (P- and E- selectin expressed in endothelial cells and L-selectin expressed in leukocytes). Their common structure consists of an N-terminal Ca^{2+}-dependent lectin type domain, an EGF domain, and variable numbers of short repeats homologous to complement-binding sequences, a single transmembrane region, and a short cytoplasmic domain. Their molecular masses range from 75 to 140 kDa, with variably glycosylated forms expressed in different cell types. In general, the function of selectins is in leukocyte trafficking where they are involved in the earliest stages of leukocyte extravasation. Here, binding of the selectin ligand on the leukocyte to selectins expressed on the endothelial surface results in "rolling" of leukocytes over the endothelial surface, functions that have now been confirmed in knockout mice. Selectin ligands are specific oligosaccharide sequences in sialated and, often, sulfated glycans, such as sialyl-Lewis^x, although there is still considerable uncertainty about the natural ligands for selectins. The signal transduction pathways linked to selectins are only partially elucidated and include activation of the MAPK pathway.

Syndecans

Syndecans are a family of four cell surface proteoglycans (see Isacke and Horton, 2000), modified by heparan sulfate glycosaminoglycan chains on their extracellular domain. Their single transmembrane domain and cytoplasmic domains are highly conserved and are involved in signal transduction events and link to cytoskeletal elements. Syndecans function predominantly as coreceptors for other receptors, including integrins; this is thought to occur via the cytoplasmic domains of associated receptors rather than the syndecan molecule itself. They also bind members of the fibroblast growth factor family, which need heparan sulfate for signaling, and hence syndecans are involved in the regulation of cell growth and proliferation. Syndecans can also function as cell–matrix receptors, binding various matrix proteins (e.g., syndecan-1 binds to type I collagen, fibronectin, tenascin-C). In different cell types, syndecans have different patterns of glycosaminoglycans attached to their core protein and these influence ligand-binding capabilities. Thus, in one cell type, syndecan-I may contain heparan sulfate as well as chondroitin sulfate side chains and bind collagen, whereas in others a different binding pattern is observed where it only has heparan sulfate side chains.

Distribution and Function of Adhesion Receptors in Bone

There is a recent and fairly extensive literature on the expression of cell adhesion molecules by the stromal and matrix-forming components of the skeleton—osteoblasts, osteocytes, and chondrocytes (see later). For each cell type, a number of receptors, including integrins, have been detected. Although there is as yet no clear concensus as to their molecular phenotype, increasing data support a functional role for adhesion molecules in bone formation and cartilage homeostasis. There is a clearer picture for osteoclasts. Here, only three integrins have been described, and there is little evidence for expression of other adhesion proteins by mature osteoclasts other than CD44 (Athanasou

and Quinn, 1990; Hughes *et al.,* 1994; Nakamura *et al.,* 1995) and possibly some cadherin family members (Mbalaviele *et al.,* 1995, 1998; Ilvesaro *et al.,* 1998; see Chapter 18). Moreover, there is a strong functional correlate by which the antagonism of osteoclast integrins leads to a downregulation of osteoclastic bone resorption, an effect with clinical implications (see later).

Osteoclasts

Role of Integrins in Osteoclastic Bone Resorption

The first suggestion that adhesion receptors played a functional role in osteoclastic bone resorption was obtained when the monoclonal antibody 13C2 (Horton *et al.,* 1985) was found to inhibit bone resorption *in vitro* by human osteoclasts from the giant cell tumor of bone (osteoclastoma) (Chambers *et al.,* 1986). It was later established that the inhibitory effect was mediated via the $\alpha_v\beta_3$ vitronectin receptor, a member of the integrin family of cell adhesion molecules (Davies *et al.,* 1989).

Subsequent detailed phenotypic (reviewed in Horton and Davies, 1989; Horton and Rodan, 1996; Helfrich and Horton, 1999) and biochemical analyses (Nesbitt *et al.,* 1993) demonstrated that mammalian osteoclasts express three integrin dimers: $\alpha_v\beta_3$, the "classical" vitronectin receptor; $\alpha_2\beta_1$, a collagen/laminin receptor; and $\alpha_v\beta_1$, a further "vitronectin receptor" (data summarized in Horton and Rodan, 1996; Helfrich and Horton, 1999; but first demonstrated for β_3 by Beckstead *et al.,* 1986 and Horton, 1986) (see Fig. 1, see also color plate, and Table III). Mostly, the findings have been consistent among studies, and, where analysis has been possible, across species. Low to undetectable levels of $\alpha_v\beta_5$ are found in mature mammalian osteoclasts (Shinar *et al.,* 1993; Nesbitt *et al.,* 1993). There have, however, been reports that osteoclasts may express α_3 (Grano *et al.,* 1994) and α_5 (Steffensen *et al.,* 1992; Hughes *et al.,* 1993; Grano *et al.,* 1994), although this has not been a general finding. Some differences have been noted with avian osteoclasts, which additionally express β_2 integrins (Athanasou *et al.,* 1992), $\alpha_5\beta_1$, and, unlike in mammals, $\alpha_v\beta_5$ (Ross *et al.,* 1993); these latter integrins act as fibronectin receptors.

Adhesion of osteoclasts to the bone surface involves the interaction of osteoclast integrins with extracellular matrix proteins within the bone matrix. This has been studied in *in vitro* phenotypic analysis, cell adhesion assays, and organ cultures from several species (Horton and Davies, 1989; Flores *et al.,* 1992, 1996; Ross *et al.,* 1993; Ek-Rylander *et al.,* 1994; Sato *et al.,* 1990, 1994; Horton *et al.,* 1991, 1993, 1995; Helfrich *et al.,* 1992; Van der Pluijm *et al.,* 1994; Gronowicz and Derome, 1994; Hultenby *et al.,* 1993). The $\alpha_v\beta_3$ vitronectin receptor mediates RGD peptide-dependent adhesion to a wide variety of proteins containing the RGD sequence, including bone sialoproteins and several extracel-

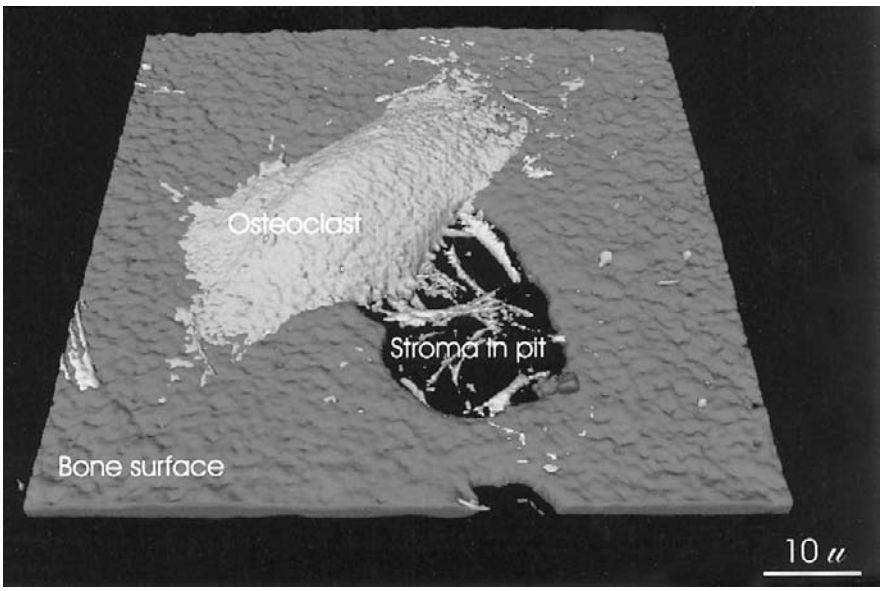

Figure 1 A three-dimensional image of a site of osteoclastic bone resorption. The isosurface image of an *in vitro* site of osteoclastic resorption was constructed from a series of optical sections gathered by immunofluorescence confocal microscopy (Leica TCS NT) (Nesbitt *et al.,* 2000) using Bitplane software. Immunostaining shows the $\alpha_v\beta_3$ integrin in green, the matrix proteins at the bone surface in blue, and the cortical F-actin in the surrounding stromal cells in red. The osteoclast (predominantly stained green) is resorbing through bone (in blue), and a trail of resorption (in black) appears behind the osteoclast in which the stromal cells (which do not express $\alpha_v\beta_3$ and thus show as red) are seen to follow. Original magnification: ×630; scale bar 10 μm. [A further example of a 3D image of osteoclasts can be viewed in an interactive format in the on-line publication by Lehenkari *et al.* (2000) at http://www-ermm.cbcu.cam.ac.uk/00001575h.htm.] (See also color plate.)

Table III Integrin and Other Receptors Expressed by Mature Human Osteoclasts[a]

	Receptor/integrin chain
Present[b]	
	$\alpha_v\beta_3$ ("vitronectin receptor")
	$\alpha_2\beta_1$
	$\alpha_v\beta_1$
	CD44
"Not detected"	
	α_1, α_{3-9} (VLAs), α_E
	β_2 and CD11, a, b, c (LFAs), α_d
	β_{4-8}
	gpIIb (αIIb)

[a]Data summarized from immunological analysis of human and rodent species (reviewed in Horton and Rodan, 1996) and biochemistry of human giant cell tumor osteoclasts (Nesbitt *et al.,* 1993). Some reports have suggested the presence of $\alpha_5\beta_1$ (Grano *et al.,* 1994; Hughes *et al.,* 1993; Steffensen *et al.,* 1992) and $\alpha_3\beta_1$ (Grano *et al.,* 1994) in osteoclasts. Some differences have been noted in avian osteoclasts (Athanasou *et al.,* 1992). α_{10} has not been examined for expression by osteoclasts. Aside from a publication describing expression of a truncated form of β_3 (Kumar *et al.,* 1997) in osteoclasts, no detailed analysis of "splice variants" has been reported.

[b]There are limited data on expression of "cadherins" in osteoclasts (Mbalaviele *et al.,* 1995, 1998; Ilvesaro *et al.,* 1998).

lular matrix and plasma proteins. In addition, mammalian (Helfrich *et al.,* 1992), but not avian (Ross *et al.,* 1993), osteoclasts adhere to type I collagen. Osteoclasts also express $\alpha_2\beta_1$ and $\alpha_v\beta_1$ integrins, and we have shown that β_1, but not β_3, mediates osteoclast adhesion to native collagens, mainly via $\alpha_2\beta_1$ (Helfrich *et al.,* 1996). Interestingly, osteoclast integrin-mediated adhesion to collagen is sensitive to RGD peptides, unlike collagen binding by integrins of other cells (Helfrich *et al.,* 1996).

The demonstration that antibodies recognizing the vitronectin receptor block osteoclast adhesion, combined with the limited integrin repertoire of these cells, suggested that it may be possible to influence bone resorption *in vitro,* either by RGD-containing peptides or by function-blocking antibodies to osteoclast integrins (reviewed in Horton and Rodan, 1996; Helfrich and Horton, 1999). The observation that the RGD sequence containing snake venom protein, echistatin, blocked bone resorption confirmed this hypothesis (Sato *et al.,* 1990). Subsequently, these findings were confirmed using linear and cyclic RGD peptides, peptidomimetic agents (Engelman *et al.,* 1997), snake venom proteins, and antibodies to α_v and β_3 components of the vitronectin receptor and, more recently, by the use of antisense oligodeoxynucleotides (Villanova *et al.,* 1999) in a variety of *in vitro* systems: resorption of bone slices, bone rudiment coculture, and calvarial or fetal long bone organ culture from chick, mouse, rat, rabbit, and human species (reviewed in Horton and Rodan, 1996; Helfrich and Horton, 1999). Blockade of the $\alpha_2\beta_1$ integrin with antibodies also inhibits bone resorption *in vitro* in isolated osteoclast assays (Helfrich *et al.,* 1996).

The snake venom peptides, echistatin and kistrin, have both been shown to induce hypocalcaemia in rats *in vivo* (Fisher *et al.,* 1993; King *et al.,* 1994): the former in the PTH-infused thyroparathyroidectomy model and the latter in parathyroid hormone-related protein (PTHrP)-induced hypercalcemia. Small cyclic RGD-containing peptides and peptidomimetics (Engelman *et al.,* 1997) also induce hypocalcemia in the former model. The inhibition seen *in vivo,* taken with the RGD sequence specificity observed with mutant (non-RGD sequence containing) echistatin (Fisher *et al.,* 1993; Sato *et al.,* 1994), suggests that integrins are mediating their hypocalcemic effect by inhibiting osteoclastic bone resorption. Direct action on an osteoclast integrin was first demonstrated in two *in vivo* experiments. First, a function-blocking antibody, F11, to the rat β_3 chain of the osteoclast $\alpha_v\beta_3$ integrin is hypocalcemic in the rat thyroparathyroidectomy model (Crippes *et al.,* 1996). Second, infusion of echistatin or peptidomimetics totally blocks the acute loss of trabecular bone seen in secondary hyperparathyroidism (Masarachia *et al.,* 1998) and following ovariectomy in the mouse (Yamamoto *et al.,* 1998; Engelman *et al.,* 1997). This latter observation strongly suggests that the inhibitory effect of RGD occurs via a direct action on bone, most likely via the $\alpha_v\beta_3$ integrin on osteoclasts, although other mechanisms cannot be totally excluded.

Evidence concerning the role of α_v and β_3 integrins in bone biology has been obtained from examining the phenotype of knockout mice (Bader *et al.,* 1998; McHugh *et al.,* 2000). From the foregoing, it would have been predicted that deletion of either component of the vitronectin receptor would produce a severe bone phenotype. Somewhat surprisingly, skeletal development was essentially normal in both sets of mice at birth. Perinatal mortality due to vascular abnormalities made further analysis of the role of α_v integrin(s) impossible in the α_v knockout mouse (Bader *et al.,* 1998). The β_3 knockout mouse (McHugh *et al.,* 2000) had the expected platelet defect of human Glanzmann thrombasthenia. However, only relatively mild skeletal changes—osteosclerosis and growth plate abnormalities—were seen on aging; the predicted osteopetrosis was not observed. Some data have been presented showing abnormal osteoclast function, but further analysis of this interesting phenotype, and of bone metabolism in patients with Glanzmann thrombasthenia, is awaited.

Nonintegrin Receptors in Osteoclasts

Studies have been carried out to assess the expression of nonintegrin adhesion receptors in osteoclasts. Earlier data (Horton and Davies, 1989) suggested the absence of a range of adhesion receptor families aside from integrins. More recently, data have been published indicating that osteoclasts express cadherin family members, including E-cadherin (Mbalaviele *et al.,* 1995, 1998; Ilversaro *et al.,* 1998) and the 67-kDa laminin receptor Mac-2 (Takahashi *et al.,* 1994). Additionally, CD44 is highly expressed in osteoclasts, although at the basolateral membrane and not at points of contact with the bone matrix (Athanasou and Quinn, 1990;

Hughes *et al.,* 1994; Nakamura *et al.,* 1995; Nakamura and Ozawa, 1996). There is no published information on the expression of selectins or syndecans by osteoclasts. A selectin-mediated mechanism for extravasation through endothelia of hemopoietic cells is well established; thus, selectins may be of interest to bone biologists, as they would be prime candidates for a role in osteoclast precursor migration to sites in bone to undergo their final differentiation.

While some of these proteins are not major components of mature osteoclasts, or only present on a subpopulation of "immature" osteoclasts, it is possible that they could be involved in osteoclast development, fusion, or functional maturation from hemopoietic stem cells. Whether novel, "osteoclast-restricted" nonintegrin adhesion receptors exist remains to be established.

Adhesion Molecules and Osteoclast Development

The question of which adhesion receptors are expressed, if any, during the development of osteoclasts from stem cells to committed, mononuclear, postmitotic precursors (functional mononuclear osteoclasts) has been difficult to address. This, in part, reflects the difficulty in isolating these cells prior to fusion and association with bone, although they are identifiable within the periosteum of developing bone anlagen as TRAP-positive, calcitonin-binding mononuclear cells that express the vitronectin receptor. Otherwise, evidence has been indirect and gained by using antibody or peptide inhibition in short-term murine and human peripheral blood or bone marrow cultures. Interpretation of such studies can prove problematic, as inhibitory effects can easily be indirect via other cell types critical for osteoclast differentiation, such as osteoblasts or marrow stromal cells. Thus, rodent osteoclast development *in vitro* is inhibited by the RGD-containing snake venom protein echistatin (Nakamura *et al.,* 1998b), implying a role for the vitronectin receptor or other RGD-sensitive integrin receptors. In contrast, osteoclast size or numbers are not altered greatly in rodents treated chronically with $\alpha_v\beta_3$ antagonists, suggesting no major influence on osteoclast differentiation or fusion *in vivo*. Studies with antibodies to $\alpha_2\beta_1$ (Helfrich *et al.,* 1996), presently limited to resorption and adhesion assays, suggest that a role in osteoclast fusion for this class of integrin is a distinct possibility. E- (but not P- or N-) cadherin has been reported to be expressed by human and rodent osteoclasts (Mbalaviele *et al.,* 1995, 1998). Function-blocking antibodies to E-cadherin and adhesion blocking "HAV peptide" inhibit osteoclast formation and fusion *in vitro,* as well as resorption by mature osteoclasts, supporting the view that this class of receptor may be active *in vivo* (Mbalaviele *et al.,* 1995; Ilvesaro *et al.,* 1998); however, because there is strong evidence for many cadherin types in osteoblasts (see later; Chapter 18), indirect effects may be more likely. There is also evidence for the involvement of β_2 integrins (Mac-1, Duong *et al.,* 1995; and LFA-1, Kurachi *et al.,* 1993) and α_4 (Duong *et al.,* 1994) and their respective counterreceptors ICAM-1 (Kurachi *et al.,* 1993; Duong *et al.,* 1995) and VCAM-1 (Duong *et al.,* 1994). Antibodies to CD44 have been shown to inhibit osteoclast formation in mouse marrow cultures, but bone resorption by differentiated osteoclasts was unaffected (Kania *et al.,* 1997). Because of the widespread distribution of CD44 in the bone/bone marrow compartment, it is difficult to assess whether this was a direct effect or mediated via other accessory cells in this *in vitro* system.

Knowledge of the range of receptors involved in osteoclast maturation prior to terminal function is important, as imbalances could lead to bone diseases such as osteoporosis. Moreover, the identification of novel (and possibly "osteoclast-specific") adhesion proteins on osteoclast precursors could well lead to the development of new therapeutic strategies.

Adhesion Molecules and Function of the Osteoclast Clear Zone

Osteoclasts resorb bone after a series of cellular polarization events. These compartmentalize the cell and are essential for bone resorption to proceed. After osteoclast attachment to the bone surface, the cell initiates a cytoskeletal rearrangement and creates a zone that separates dorsal (basolateral) and ventral plasma membranes. This clear, or "tight sealing," zone is "organelle free" (hence the term clear zone), rich in actin filaments, and is closely apposed to the bone surface (Holtrop and King, 1977). This membrane domain of the resorbing osteoclast maintains close apposition to the bone surface and encloses a further specialized secretory membrane, the ruffled border, and "isolates" the acidic microenvironment of the resorption lacuna (see Chapter 8). Protons and proteases cross the ruffled border and solubilize the adjacent bone matrix through demineralization and proteolytic activity. Subsequently, the bone matrix, including calcium and type I collagen fragments, is liberated and a resorption compartment forms beneath the cell.

The finding that osteoclast attachment to matrix-coated glass or bone is interrupted by integrin inhibitors led to the suggestion that the osteoclast tight seal may be mediated by integrins. Some data have supported the view that the vitronectin receptor is enriched in clear zones of resorbing osteoclasts (Reinholt *et al.,* 1990; Hultenby *et al.,* 1993; Nakamura *et al.,* 1996), as well as podosomes of osteoclasts cultured on glass (Zambonin-Zallone *et al.,* 1989; reviewed in Aubin, 1992). Others, however, have been unable to confirm this finding, reporting that the vitronectin receptor is undetectable in the sealing zone (Lakkakorpi *et al.,* 1991, 1993; Nakamura *et al.,* 1999; Duong *et al.,* 2000). Väänänen and Horton (1995) have argued previously that the dimensions of the integrin molecule, when compared to a membrane to bone gap of 2–10 nm, preclude a direct involvement of integrins in the maintenance of a "tight seal" during resorption, as opposed to a role in initial osteoclast attachment and cell movement, which is not in dispute. The molecular mech-

anism of the *attachment* process in the established clear zone of a resorbing, nonmigratory osteoclast thus remains to be established (Väänänen and Horton, 1995) and is likely to involve both cell autonomous characteristics of the osteoclast in combination with chemical and physical features of the bone matrix (Nakamura *et al.,* 1996), as described later.

Early work by Lucht suggested, using an *in vivo* model, that there is no tight sealing zone, as the endocytic marker, horseradish peroxidase, could be detected in the ruffled border area as early as 5 min after injection into the animal (Lucht, 1972). However, generation of a low pH zone underneath the actively resorbing osteoclast, against a substantially different surrounding media, requires the presence of a diffusion barrier. An alternate hypothesis is that the apposition of the osteoclast to the bone surface is not as tight as predicted, less than with high resistance, ion-impermeable epithelial tight junctions, and that the restriction of ionic movement from under the resorbing osteoclast is a combined feature of substrate (i.e., bone matrix) and osteoclast activity. Proton pumping by the osteoclast alters the properties of the bone surface (Delaissé *et al.,* 1987; Everts *et al.,* 1988), which one could envisage would result in swelling and physicochemical modification of the extracellular matrix. In fact, incubation of type I collagen under mild acidic conditions *in vitro* leads to the formation of a gel (Chandrakasan *et al.,* 1976), a process that one could also envisage taking place *in vivo* within the resorption pit. Generation of a collagenous gel at the resorption site would have a triple effect: (i) acting as a diffusion barrier by increasing the viscosity of the medium in immediate apposition to the resorption site, which in turn would decrease the diffusion coefficient of all substances in this area; (ii) acting as an ion-exchange matrix, as, due to the basic pI of collagen, the resorption pit would acquire an overall positive charge, which could serve as a trap for negatively charged molecules; and (iii) providing a matrix for hydrophobic interactions through the high content of nonpolar amino acid residues (~74%) in type I collagen. Released protons are likely to be tethered readily to the inorganic components of the bone matrix, which would reduce their free concentration and their mobility. As a consequence, a localized low pH zone would be established only at the site of proton release that could be sufficient to activate secreted lysosomal enzymes. The presence of such a pH gradient has also been suggested to accommodate the different pH requirements of collagenase and lysosomal enzymes in the resorption area (Delaissé *et al.,* 1993). During the resorption process, the low pH zone, followed by the collagenous gel zone, would advance further into the bone matrix, thus providing a localized microenvironment for bone resorption without the need for a static lateral tight seal (Fig. 2, see also color plate). An efficient osteoclast endocytotic mechanism would be responsible for the removal of the reaction products before they leave the resorption area by diffusion. In this model, the two processes of cell movement and resorption could occur simultaneously, events that would be difficult to integrate if stable, epithelium-like tight junctions were the basis of the osteoclast tight adhesion. This dynamic model also accounts for the apparently contrary role of integrins in osteoclast resorption. Without the need for a sterically close membrane–matrix contact zone, integrins could well be involved in the establishment and functioning of the sealing zone, in addition to their role in initial cell attachment and migration (Väänänen and Horton, 1995).

To test this hypothesis, Stenbeck and Horton (2000) examined the permeability of the osteoclastic sealing zone

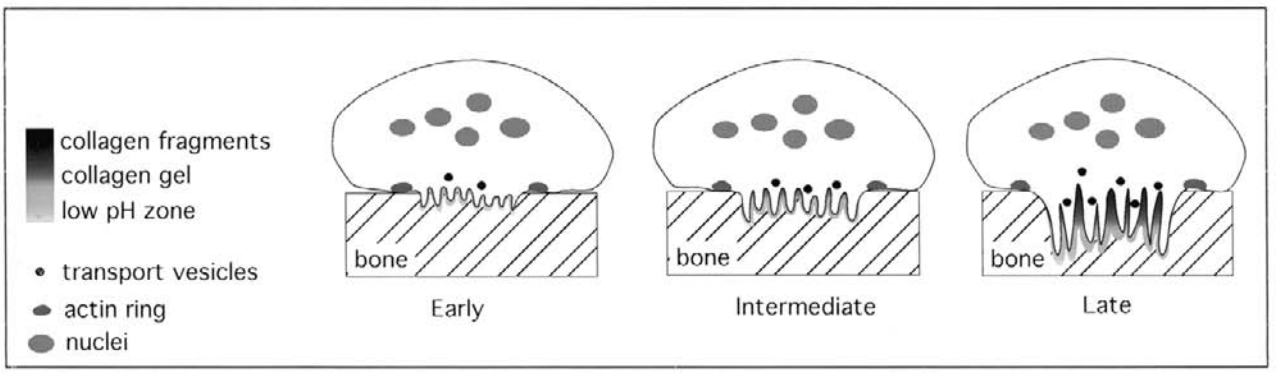

Figure 2 Model of the osteoclast sealing zone. During the early stages of resorption, the proton pump (VATPase) is inserted into the osteoclast plasma membrane that is enclosed by the actin ring (green). Proton extrusion by the VATPase leads to the formation of a localized low pH zone that dissolves the mineral content of the bone (yellow area). Proton movement is restricted by binding to hydroxyapatite. Lysosomal enzymes, secreted during the intermediate stages of resorption, digest the organic content of the bone in the low pH zone (red area). In the later stages of resorption, the ruffled border expands deep into the bone matrix by fusion of transport vesicles with the plasma membrane. The low pH zone moves with expansion of the ruffled border further into the bone matrix. The area behind the low pH zone consists of a collagenous "gel" that is endocytosed by the osteoclast (dark brown). This further restricts solute diffusion from below the osteoclast. However, because the net concentration of secreted and resorbed components is a balance between generation rate and limited diffusion rather than the presence of an impermeable barrier, externally added small molecules have access to the resorption area. Modified from Stenbeck and Horton (2000), with permission. (See also color plate.)

during bone resorption and migration in rabbit and human osteoclasts plated on dentine and bone. Using a series of fluorescent dyes of known molecular weight and different surface charge, it was established that negatively charged molecules with M_r up to 10,000 accumulate rapidly underneath actively resorbing osteoclasts. Live cell imaging shows that access underneath the osteoclasts occurred as early as 30 sec after the addition of the low molecular weight markers, as predicted by the dynamic sealing model proposed earlier (summarized in Fig. 2).

$\alpha_v\beta_3$-Mediated Signal Transduction

It has become increasingly clear, in a wide variety of cellular systems, that integrins can act as receptors capable of transducing signals. This results in biochemical changes within cells and induction of cell-specific early response genes and activation of nuclear transcription ("out-side-in" signaling), and in the modification of integrin activity ("inside-out" signaling) in response to signals generated via other receptor systems. Osteoclasts from a variety of species also respond to integrin ligands, either RGD-containing peptides or proteins or via antibody-mediated receptor cross-linking, in a number of ways indicative of direct signal transduction or changes mediated via cytoskeletal rearrangement (reviewed by Duong *et al.,* 2000; see Chapter 9). These include release of intracellular calcium stores, induction of protein tyrosine phosphorylation, and reorganiztion of the structural and signaling components of the cytoskeleton. Mammalian osteoclasts respond to integrin ligation with a prompt increase in intracellular calcium (Paniccia *et al.,* 1993; Shankar *et al.,* 1993; Zimolo *et al.,* 1994), whereas this is accompanied by a slower decrement in avian cells (Miyauchi *et al.,* 1991); the reason for this species difference is unclear. Reorganization and activation of cytoskeleton-associated tyrosine kinases are key downstream steps following osteoclast adhesion. c-Src has been found to be essential for osteoclast polarization and resorption in knockout mice (Soriano *et al.,* 1991), and c-Cbl has been suggested to act as a key mediator of c-Src action (Tanaka *et al.,* 1996). However, studies by Duong *et al.* (1998) have identified the focal adhesion kinase family member PYK2 as the major adhesion-dependent tyrosine kinase in osteoclasts. In contrast, FAK (focal adhesion kinase) is a minor kinase in osteoclasts (Tanaka *et al.,* 1995a; Duong *et al.,* 1998; Lakkakorpi *et al.,* 1999) when compared to its dominant role in adherent mesenchymal cells. Ligand binding or receptor clustering induces PYK2 phosphorylation by Src kinase in osteoclasts, and c-Src, PYK2, and actin form a stable complex on osteoclast adhesion. PYK2 colocalizes with F-actin in the ring-like structures characteristic of resorbing osteoclasts. Here, it associates directly with phosphorylated $p130^{cas}$ (Nakamura *et al.,* 1998a; Lakkakorpi *et al.,* 1999), which acts as an adaptor protein in the integrin–PYK2 signaling pathway. There is also evidence for recruitment of phosphatidyl inositol 3-kinase (PI3-K) to the cytoskeleton (Hruska *et al.,* 1995; Lakkakorpi *et al.,* 1997) where it associates with gelsolin (Chellaiah and Hruska, 1996).

The downstream effects of integrin-mediated signaling in osteoclasts have not been analyzed adequately (but see Chapter 9). Possibilities include roles in the onset of cellular polarization, transcytosis, and bone resorption; regulation of cell motility; and cessation of resorption and induction of adhesion-related apoptosis (Ruoslahti and Reed, 1994).

Integrins, Collagen-Binding Proteins and Transcytosis in Osteoclasts

Osteoclasts use transcytosis to remove degraded matrix from the active sites of bone resorption (Nesbitt and Horton, 1997; Salo *et al.,* 1997), enabling the osteoclast to maintain the integrity of the enclosed resorption site and facilitate cell migration and penetration into bone. Degraded bone matrix is endocytosed along the ruffled border and transported through the osteoclast in a vesicular pathway toward the basolateral surface of the cell; finally, it enters the extracellular space via a specialized exocytotic site located at the cell apex (Salo *et al.,* 1996). Thus, the transcytotic process utilized by osteoclasts is similar to that in epithelium and endothelium (Mostov *et al.,* 2000).

Reports have suggested that integrins may participate in transcytosis in an epithelial cell model (Ivanenkov and Menon, 2000) where the transport of adenovirus across cells was increased by RGD peptides. If osteoclasts use a similar RGD-dependent mechanism in transcytosis, then candidate receptors involved in the uptake of the bone matrix at the ruffled border would include $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins, which, respectively, bind native and denatured collagens (Nesbitt *et al.,* 1993; Helfrich *et al.,* 1996). The proteolysis of collagenous matrix exposes cryptic RGD sites (Holliday *et al.,* 1997) during bone resorption, and engagement with the $\alpha_v\beta_3$ integrin could initiate endocytosis and subsequent transcytosis of the denatured collagenous matrix. Conversely, higher concentrations of RGD peptides, produced after extensive matrix proteolysis, could inactivate matrix transcytosis and, thus, lead to the cessation of resorption.

Another group of collagen-binding proteins in osteoclasts (Nesbitt *et al.,* 1994) are the annexins, a family of calcium-dependent phospholipid-binding proteins that exhibit a wide tissue distribution (reviewed by Raynal and Pollard, 1994). Annexins have been shown to participate in endocytosis, transcytosis, and exocytosis in several polarized cells (Burgoyne, 1994; Creutz, 1992; Wilton *et al.,* 1994), in addition to their role in a number of other cellular processes (Moss, 1997; Siever and Erickson, 1997). Evidence shows that annexin II participates in matrix transcytosis during bone resorption (Nesbitt and Horton, 1999). It is found at the cell surface of resorbing osteoclasts and colocalizes with degraded bone matrix in the resorption pit; it is also highly expressed within the basolateral cell body and at apical exocytotic sites. Furthermore, the addition of exogenous

annexin II to resorption cultures increases transcytosis of bone matrix and bone resorption by osteoclasts (Nesbitt and Horton, 1999). The ability of annexins to associate with membrane phospholipids, together with the collagen-binding capacity of osteoclast annexin II, may enable annexins to complex solubilized, degraded collagen with membrane structures associated with the transcytotic pathway, such as the ruffled border, intracellular transport vesicles, or the apical exocytotic site.

$\alpha_v\beta_3$ Integrin as a Therapeutic Target for Bone Disease

Early Studies and Rationale

Osteoporosis places a large and growing medical and financial burden on health services in developed countries; however, it remains a clinical area where, despite recent advances in therapy and diagnosis, there are still unmet needs. While potent drugs have been developed, e.g., bisphosphonates (see Chapter 78), the pharmaceutical industry is still developing novel antiresorptive agents. The $\alpha_v\beta_3$ vitronectin receptor presents a key step in the process on bone resorption *(vide supra),* which is being exploited by the pharmaceutical industry.

The development (for reviews, see Horton and Rodan, 1996; Hartman and Duggan, 2000; Miller *et al.,* 2000) of a number of orally active, nonpeptidic integrin antagonists, particularly based on modification of the RGD peptide motif identified in fibronectin by Pierschbacher and Ruoslahti in 1984, suggests that treatment of a range of bone diseases may be susceptible to strategies that involve the blockade of integrin function or modulation of their expression. The development of $\alpha_v\beta_3$ antagonist drugs has been aided considerably by the prior existence of an analogous set of agents that have been developed for use in thrombosis (see references in Hartman and Duggan, 2000; Miller *et al.,* 2000); the platelet integrin fibrinogen receptor, gpIIbIIIa/$\alpha_{IIb}\beta_3$, which is structurally related to the $\alpha_v\beta_3$ integrin on osteoclasts and shares the same β chain, is targeted. These are the first of the integrin antagonist "drugs" that have been approved for clinical use (Coller, 1997; Phillips and Scarborough, 1997; Theroux, 1998) and they form the paradigm for potential application to bone disease.

The functional role of $\alpha_v\beta_3$ in osteoclast biology, first examined by Horton and colleagues in antibody studies over a decade ago, has been confirmed in a large battery of *in vitro* systems and *in vivo* proof of concept studies *(vide supra).* Target specificity is aided by the *in vivo* distribution of $\alpha_v\beta_3$, which is expressed at high levels in osteoclasts (Horton, 1997). Much lower levels are found in platelets and megakaryocytes, kidney, vascular smooth muscle, some endothelia, and placenta (Horton, 1997). Thus, the therapeutic drug levels that would influence osteoclastic bone resorption are less likely to modify $\alpha_v\beta_3$ function at other sites. In certain pathological situations, though, tissue levels of $\alpha_v\beta_3$ are increased; for example, tumor microvessels show increased levels of $\alpha_v\beta_3$, as do melanoma cells when they metastasize (Horton, 1997), and these features are being exploited.

Strategies for Therapeutic Modification of Integrin Function

From basic principles, there are two main strategies for inhibiting cell adhesion molecule function therapeutically (Table IV). First, a direct approach: competitive antagonists of the receptor–ligand interaction can be developed, and this has been the usual pharmaceutical approach with the aim of producing orally active, synthetic nonpeptide mimetic agents. They have been identified by a variety of standard industry techniques, as summarized in Table IV (e.g., see Ferguson and Zaqqa, 1999; Wang *et al.,* 2000). Other approaches, such as using receptor-specific antibodies, peptides, and naturally occurring protein antagonists, together with molecular engineering, have generally been used in proof of principle experiments rather than as clinical drug candidates, although there are some notable examples of protein therapeutics in the field (for examples, see Table IV). Directly acting antagonists have entered clinical trial to modify activation-dependent platelet aggregation in thrombotic conditions via the integrin platelet fibrinogen receptor, gpIIbIIIa/$\alpha_{IIb}\beta_3$. Thus, ground-breaking trials [EPIC, EPILOG etc. (Tcheng, 1996)] have demonstrated efficacy of the humanized anti-gpIIIa monoclonal antibody 7E3 (ReoPro) in various ischemic heart conditions (Coller 1997). Results from trials with RGD mimetics [e.g., lamifiban, tirofiban (Ferguson and Zaqqa, 1999; Wang *et al.,* 2000)] and the cyclic KGD peptide integrilin have, though, been less impressive (Theroux, 1998). As with gpIIbIIIa-specific agents, the possibility of developing osteoclast $\alpha_v\beta_3$ (vitronectin receptor) antagonists as resorption inhibitors in bone disease was initially demonstrated *in vitro* using a variety of techniques to disrupt receptor function, and small molecule inhibitors of $\alpha_v\beta_3$ are now at the late stage of preclinical development or entering the early stages of clinical trial evaluation (Hartman and Duggan, 2000; Miller *et al.,* 2000). Thus, general principles for the use of adhesion receptor antagonists in disease have been established, and useful drugs are thus likely to be available for a wide variety of indications in the future.

The second approach is indirect, with the aim of modifying expression or intracellular function (such as signal transduction) of cell adhesion molecules, especially integrins. Some examples of such strategies are given in Table IV. The furthest advanced are the use of antisense oligonucleotide inhibitors of receptor protein synthesis. Because inhibitors of ICAM-1 expression are finding promise in the treatment of various inflammatory diseases, such as of the bowel or eye, then modulation of α_v expression by an antisense approach (Villanova *et al.,* 1999) could be a promising strategy. Likewise, a number of agents to block the function of c-Src, a cellular kinase that acts downstream in the signaling pathway of integrin receptors in bone cells, are being developed for the treatment of osteoporosis, based on the earlier

Table IV Strategies for Therapeutic Modification of Integrin Adhesion Receptor Function *in Vivo*

Direct approaches
Naturally occurring protein inhibitors and their engineered derivatives (e.g., RGD-containing snake venoms and proteins from ticks, leeches, etc.)[a]
Blocking antibodies, and their engineered derivatives, to adhesion molecules[b]
Arg-Gly-Asp (RGD) peptides and their chemical derivatives (e.g., designed to improve specificity and stability)[c]
Nonpeptidic mimetics,[d] produced via different compound selection strategies[e]
Indirect approaches
Altered receptor synthesis via use of antisense oligonucleotides[f]
Inhibition of adhesion receptor expression via regulatory cytokines and their receptors
Modification of integrin receptor function via regulatory integrin-associated proteins
Modulation of integrin receptor affinity (i.e., activation) for ligands
Modification of downstream receptor-associated signaling (e.g., c-Src and other kinases, adhesion-associated apoptosis genes)

[a] Echistatin has been used as a proof of concept inhibitor of $\alpha_v\beta_3$ in bone disease studies (Fisher *et al.,* 1993; Yamamoto *et al.,* 1998). Barbourin snake venom protein contains KGD instead of RGD and is the basis of selective inhibitors of platelet gpIIbIIIa (Phillips and Scarborough, 1997).

[b] Antibodies to gpIIbIIIa (i.e., 7E3, ReoPro, Centocor Inc) formed the first cell adhesion receptor inhibitor licensed for clinical use (in the various vascular/thrombotic condition, see Tcheng, 1996; Coller, 1997). A humanized $\alpha_v\beta_3$ antibody (clone LM609) is currently in clinical trial for cancer acting via induction of apoptosis in tumor vessels.

[c] Integrilin (Cor Therapeutics Inc), a cyclic KGD-containing peptide gpIIbIIIa inhibitor, is in clinical trial (Phillips and Scarborough, 1997; Coller, 1997), as are RGD-derived cyclic peptides with selectivity for $\alpha_v\beta_3$ [cyclic RGDfVA, E. Merck (Haubner *et al.,* 1996)].

[d] A number of companies have intravenous and orally active nonpeptidic gpIIbIIIa antagonists in clinical trial for platelet-related disorders (Phillips and Scarborough, 1997; Coller, 1997; Theroux, 1998). Analogous mimetics are in late preclinical development for inhibition of $\alpha_v\beta_3$ (Horton and Rodan, 1996; Hartman and Duggan 2000; Miller *et al.,* 2000) in bone disease and cancer.

[e] Structure–function, combinatorial chemistry, phage display, compound/natural product library screening, etc. (Lazarus *et al.,* 1993; Pasqualini *et al.,* 1995; Corbett *et al.,* 1997; Hoekstra and Poulter, 1998).

[f] Antisense therapeutics directed against adhesion receptors are in clinical trials; antisense oligonucleotides to α_v block bone resorption *in vitro* (Villanova *et al.,* 1999).

finding in knockout mice that c-Src plays a central role in osteoclastic bone resorption (Soriano *et al.,* 1991).

Current Drug Development Status of $\alpha_v\beta_3$ Antagonists for Use in Bone and Other Diseases

The action in bone models of several candidate mimetic $\alpha_v\beta_3$ antagonists has been reported by a number of companies, and their evolution has been reviewed by Hartman and Duggan (2000) and Miller *et al.* (2000); as yet, these are not drugs but still agents used for proof of concept and pharmaceutical experiments. Compounds based on a variety of proprietary scaffolds, which have all shown varying efficacy and specificity for $\alpha_v\beta_3$ in the number of *in vitro* screening assays, have inhibitory effects on the calcemic response in thyroparathyroidectomized rodents and bone-sparing responses in ovariectomy and other rodent models of increased bone turnover. Drug candidates with optimized pharmacokinetics/dynamics are about to enter clinical trials for bone disease and for other indications where $\alpha_v\beta_3$ is involved in disease pathogenesis.

Positive findings in proof of concept studies using small molecule mimetics in models of bone metabolism underline $\alpha_v\beta_3$ antagonists as promising candidates for a new class of bone disease therapeutics, although they still require optimization. Further, the expression, albeit at lower levels, of $\alpha_v\beta_3$ in other tissues suggests that their inhibition could produce unwanted side effects: for example, will they, on chronic administration, interfere with wound healing, the function of the related α_v integrins in respiratory tract or intestinal epithelium, or platelet ($\alpha_{IIb}\beta_3$-mediated) aggregation?

Finally, although $\alpha_v\beta_3$ antagonists have been developed for use in bone diseases, other clinical targets also show promise (such as rheumatoid arthritis, angiogenesis in eye diseases and cancer, vascular restenosis following coronary angioplasty, and direct targeting of tumours expressing $\alpha_v\beta_3$). These are all being investigated for possible new applications of $\alpha_v\beta_3$ antagonist drugs.

Osteoblasts

Osteoblasts are uniquely involved with the synthesis and maintenance of the bone matrix and lie in direct contact with the specialized extracellular matrix of bone; they also respond to the mechanical forces exerted on the skeleton and to the growth factors that regulate their function and that are entrapped in the surrounding extracellular matrix. Thus, it is likely that cell adhesion receptors play several important roles in bone cell function and that the maintenance of osteoblast adhesion would be a major function for the integrin family of matrix receptors. Some of the possible roles of adhesion receptors in osteoblasts are listed in Table I.

It is well established that osteoblast differentiation, maturation, and their function are regulated by their interaction with matrix components such as type I collagen (e.g., Lynch *et al.,* 1995; Masi *et al.,* 1992). The role of integrins and other adhesion molecules in the regulation of osteoblast precursor differentiation and functional maturation has yet, though, to be adequately investigated; however, preliminary data suggest that RGD-sensitive, β_1 integrins are involved (*vide infra;* Globus *et al.,* 1998; Gronowicz and Derome, 1994). Osteoblasts also partake in a wide variety of cell–cell interactions in the bone/bone marrow compartment (see Table I). Intercellular adhesion is likely to be conducted by adhesion proteins such as cadherins (see Chapter 18), but knowledge of their role in osteoblast function is limited (Babich and Foti, 1994). A role for integrins

in such interactions cannot be excluded, and the integrin counterreceptors, ICAM-1 and VCAM-I, as well as the CD2 counterreceptor, LFA-3, are all expressed by osteoblasts. Evidence shows that these receptors are involved in interactions of osteoblasts with T lymphocytes and subsequent secretion of cytokines (Tanaka *et al.,* 1995b); such events may be important in the regulation of skeletal turnover in inflammation.

Mechanical strain sensing within the skeleton is probably performed by the "bone-lining cells" that are not synthesizing matrix and lie on the bone surface and/or by the osteocyte network lying within the bone (reviewed elsewhere in this volume; see Chapters 2 and 6). Cell membrane integrins interacting with matrix could act as force transducers to modify cellular behavior (both locally in osteocytes and distantly via osteocyte processes interacting with osteoblasts on bone surfaces); this phenomenon has been demonstrated in other cell systems and is elegantly discussed by Ingber and colleagues (Wang and Ingber, 1994; Ruoslahti, 1997). There is some data on the nature of the structure of the matrix in the osteocyte lacunae, including adhesion proteins synthesized by osteocytes themselves (see references in Aarden *et al.,* 1996), and evidence has demonstrated a role for β_1 (but not β_3) integrins in osteocyte adhesion to a number of bone matrix proteins (Aarden *et al.,* 1996). A limited phenotypic analysis has been performed on human tissue and shows expression of β_1 integrins (Hughes *et al.,* 1993). CD44 has been found at a high level in osteocytes but in lower amounts in osteoblasts (Hughes *et al.,* 1994; Nakamura *et al.,* 1995), although its function has not been investigated. We thus can conclude that mechanical sensing by osteocytes could be mediated via integrin–matrix or other cell adhesion receptor coupling, but currently there is no experimental evidence to support this conclusion.

β_1 Integrins and Osteoblasts

A diverse range of integrins, particularly of the β_1 class, have been shown to be expressed by osteoblasts, including α_1 through α_5 (Brighton and Albelda, 1992; Clover and Gowen, 1992; Clover *et al.,* 1992; Grzesik and Gehron Robey, 1994; Horton and Davies, 1989a,b; Hughes *et al.,* 1993; Majeska *et al.,* 1993; Pistone *et al.,* 1996; Ganta *et al.,* 1997; Gronthos *et al.,* 1997; reviewed by Bennett *et al.,* 2001a), although there is some contradiction between different studies as to the specific heterodimers expressed. This may reflect the heterogeneity of osteoblast-like populations (see Chapter 4) and includes the possibility that cells at successive stages of osteoblast differentiation, from fetal or adult bone or from different anatomical sites, show different patterns of integrin expression. Nevertheless, trends are emerging. While they may express α_v integrins, osteoblastic cells differ from osteoclasts in that β_1 integrins appear to have the major functional role, which has been underscored by *in vivo* data (Zimmerman *et al.,* 2000). Several of these β_1 integrins have a high affinity for extracellular matrix components found in bone and adjacent matrix, such as collagen types I and III and fibronectin.

Collagen Receptors

The $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ integrins bind collagen, and immunological studies confirm that they are expressed by osteoblastic cells *in vivo* and *in vitro*. Collagen type 1 is the dominant bone matrix protein, and interactions involving these receptors are strong candidates for a role in regulating osteoblast behavior. Furthermore, functional studies have demonstrated that $\alpha_2\beta_1$–ligand binding leads to expression or upregulation of markers of osteoblastic differentiation (Xiao *et al.,* 1998). Cultures of osteoblast-like cells in the presence of inhibitors of α_2 function, such as blocking antibodies, modulate $\alpha_2\beta_1$-dependent expression of osteoblast markers (Takeuchi *et al.,* 1997). Others have shown that ligand binding by the α_2 integrin modulates cell motility and contraction of collagen gels *in vitro* (Riikonen *et al.,* 1995).

$\alpha_3\beta_1$ heterodimers bind collagen, fibronectin, and several other extracellular matrix proteins and are expressed by early osteoblastic cells from human bone (Bennett *et al.,* 2001a). Function-perturbing antibodies against the α_3 integrin inhibit the formation of mineralized nodules in rat calvarial osteoblast cultures (Moursi *et al.,* 1997).

Integrins and Fibronectin

Of the integrins that are fibronectin receptors, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and α_v heterodimers are expressed in bone. Most are capable of binding a broad range of extracellular matrix proteins in bone, using both RGD-dependent (Puleo and Bizios, 1991) and independent mechanisms.

In vitro studies using an osteoblast culture model have shown that the selective fibronectin receptor, $\alpha_5\beta_1$, is expressed by cells of the osteoblast lineage, and evidence shows that it is important in both the development and maintenance of bone. Interruption of binding with blocking antibodies leads to inhibition of bone nodule formation by osteoprogenitor cells in rat calvarial cultures (Moursi *et al.,* 1996, 1997). In mature cells, $\alpha_5\beta_1$–ligand binding appears to be necessary for cell survival and receptor blockade leads to osteoblast apoptosis (Globus *et al.,* 1998).

Fibronectin is a normal constituent of human bone, but data on its distribution within the bone matrix are sparse, and it has been reported absent from mature lamellar bone (Carter *et al.,* 1991). Supporting evidence from rodent tissues suggests that fibronectin synthesis and expression are restricted to developing or immature bone (Weiss and Reddi, 1980; Cowles *et al.,* 1998). It is, therefore, possible that $\alpha_5\beta_1$–ligand interaction is a feature of bone formation during development or repair and may not play a prominent role in the turnover and maintenance of mature lamellar bone. There is also some evidence for the involvement of the $\alpha_5\beta_1$ integrin in mechanical sensing by osteoblasts, at least *in vitro* (Salter *et al.,* 1997).

Integrins and Mesenchymal Precursor Cells

$\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins are both collagen receptors, with $\alpha_1\beta_1$ showing higher affinity for type IV over type I collagen (Kern *et al.,* 1993). Type IV collagen is a feature of the endothelial basal lamina, and the α_1 integrin has been reported in mesenchymal stem cells (Owen, 1998) with osteogenic potential (Bruder *et al.,* 1998a). In addition, both type IV collagen and laminin are synthesized by pericytes (Doherty *et al.,* 1998), which have been suggested as putative osteogenic precursor cells, and expression of these proteins is lost as cells mature. It is possible, therefore, that the $\alpha_1\beta_1$ heterodimer has a role in cell–matrix interactions involving mesenchymal precursor cells associated with small blood vessels in the bone/bone marrow microenvironment. Support for this also comes from the finding that osteoprogenitors, in contrast to more mature calvarial-derived cells, showed preferential binding to laminin, a component of the endothelial basement membrane (Roche *et al.,* 1999). Furthermore, mesenchymal precursor cells express the α_6 integrin (Bruder *et al.,* 1998a), which binds laminin, a major component of the basement membrane.

α_v Integrins

Several studies report expression of α_v in cells of the osteoblast lineage. However, the published literature varies with regard to which β subunit is utilized (the balance of data favoring $\alpha_v\beta_5$ expression), and staining appears more prominent in osteoblasts than osteocytes (Hughes *et al.,* 1993; Grzesik and Robey, 1994).

Nonintegrin Cell Adhesion Molecules in Osteoblasts

Cadherins

Osteoblasts and bone-lining cells form gap and adherens type cell junctions with each other and with the osteocytes (Palumbo *et al.,* 1990, Doty, 1981). Cadherins are among the best characterized cell–cell adhesion molecules (Isacke and Horton, 2000), localizing to sites of intercellular attachment. The expression pattern and function in osteoblasts of cadherins are covered in depth in Chapter 18 and is only reviewed briefly herein. Cells of the osteoblast lineage express a limited repertoire of cadherins, including N-cadherin, cadherin-4, cadherin-6, and cadherin-11 (Okazaki *et al.,* 1994; Babich and Foti 1994; Cheng *et al.,* 1998; Mbalaviele *et al.,* 1998; Ferrari *et al.,* 2000). N-cadherin has been histochemically localized to well-differentiated osteoblasts lining the bone surface, but not osteocytes, in fetal rat calvaria and blocking N-cadherin binding decreased bone nodule formation *in vitro* (Ferrari *et al.,* 2000).

The biological significance of cadherin-mediated cell–cell interactions in bone remains largely unexplored. However, evidence shows that cadherins function synergistically with other cell adhesion molecules to influence cell behavior. They may, for example, modulate the function of connexins, proteins associated with gap junction function, and osteoblasts are known to communicate via such mechanisms (Yellowley *et al.,* 2000) (see Chapter 18). In common with other tissues, variations in cadherin expression have been observed in association with malignancy. Reduced N-cadherin and anomalous cadherin-11 expression have been associated with high-grade metastatic osteosarcomas (Kashima *et al.,* 1999), suggesting an important role for these molecules in intercellular adhesion.

CD44

Immunohistochemical studies of human tissues showed CD44 expression in osteocytes, but not osteoblasts or bone-lining cells (Hughes *et al.,* 1994). This is consistent with other mammalian models in which strong expression has been observed in osteocytes, with weaker staining in cells earlier in the osteoblast lineage (Jamal and Aubin, 1996; Nakamura *et al.,* 1995; Nakamura and Ozawa, 1996; Noonan *et al.,* 1996). The functional significance of CD44 expression is unknown. *In vitro,* osteoblastic cells have, indeed, been shown to be able to bind to, and degrade, hyaluronate in the transition zone from cartilage to bone in the growth plate and utilize a CD44-dependent mechanism (Pavasant *et al.,* 1994). There are a variety of other known ligands for CD44, e.g., type I collagen, fibronectin, laminin, and osteopontin, and these are also produced by both osteoblasts and osteocytes (Aarden *et al.,* 1996) and colocalize with CD44, indicating that a much wider range of functions for this molecule may exist.

Immunoglobulin Superfamily

Although sparse, there are data relating to the expression of members of this family by osteoblasts and related cells. ICAM-1 and -2, VCAM-1, and LFA-3 expression has been reported in human bone cells (Tanaka *et al.,* 1995b; Bruder *et al.,* 1998a) and they may play a role in the interaction of osteoclast precursors with stromal cells in the bone/bone marrow microenvironment (Tanaka *et al.,* 2000). Expression of the activated leukocyte cell adhesion molecule (ALCAM) has been reported in undifferentiated human mesenchymal cells and may have a functional role in osteoblast differentiation (Bruder *et al.,* 1998a,b); likewise, NCAM is expressed transiently during ossification (Lee and Chuong, 1992).

Selectins

There is, as yet, little information on the expression of members of the selectin family in osteoblasts. However, there is evidence for L-selectin expression by human mesenchymal stem cells (Bruder *et al.,* 1998a), but not E- or P-selectin. These molecules are normally associated with leukocyte trafficking across endothelia, but the function of L-selectin in osteoblasts is unknown.

SYNDECANS

Syndecans -1, -2, and -4 have been identified in human marrow stromal and osteoblast-like cells *in vivo* and *in vitro* (Schofield *et al.,* 1999; Birch and Skerry, 1999), and syndecan-3 is expressed during periosteal development (Koyama *et al.,* 1996). Studies using a rat organ culture model demonstrated coincident expression of syndecan-2 and -4 with fibroblast growth factor receptors *in vitro* and a similar spatiotemporal expression *in vivo* (Molteni *et al.,* 1999). This suggests that members of the syndecan family have a role in presentating growth factors during skeletal development, but this remains to be investigated.

Adhesion Receptors in Cartilage

The role of cell adhesion molecules in cartilage is relatively unclear, although some of their putative functions are summarized in Table I. These may include roles in chondrocyte proliferation and cartilage differentiation during fetal development (see Chapter 3); responses to mechanical forces (e.g., in articular cartilage or menisci); maintenance of tissue architecture and integrity, including matrix synthesis and assembly; or cell adhesion, regulation of chondrocyte gene expression, and cell survival. Additionally, there is likely to be a role for cell adhesion molecules in the response in cartilage to injury and disease (Forster *et al.,* 1996; Lapadula *et al.,* 1997; Millward-Sadler *et al.,* 2000; Ostergaard *et al.,* 1998). The differing distribution of both integrin and matrix proteins (Salter *et al.,* 1995) in the zones of cartilage suggests a role in chondrocyte differentiation from mesenchymal precursors (Hirsch and Svoboda, 1996; Tavella *et al.,* 1997; Shakibaei *et al.,* 1995) and/or interaction with matrix, or a specialized function such as response to mechanical stresses. There have been few studies to investigate these possibilities.

Integrins in Chondrocytes

As with the osteoblast lineage, the reported integrin phenotype of chondrocytes is complex, with additional inconsistency between publications (Durr *et al.,* 1993; Enomoto *et al.,* 1993; Loeser *et al.,* 1995; Salter *et al.,* 1992, 1995; Woods *et al.,* 1994; Ostergaard *et al.,* 1998; reviewed in Helfrich and Horton, 1999). A synthesis of the literature suggests that human chondrocytes express the β_1 integrins α_1, α_2, α_3, α_5, and α_6, but not α_4; β_2, β_4, and β_6 are absent, and analysis of β_{7-9} and CD11 has not been reported. Some studies have shown high expression of α_v integrin; as in osteoblasts, this is mainly as $\alpha_v\beta_5$ and not the $\alpha_v\beta_3$ dimer seen in osteoclasts, although a subpopulation of superficial articular chondrocytes has been found to be $\alpha_v\beta_3$ positive (Woods *et al.,* 1994). A new collagen type II-binding integrin, first identified in chondrocytes, $\alpha_{10}\beta_1$, has been reported (Camper *et al.,* 1998). Differences in reported integrin expression patterns could well relate, in part, to a variation in sampling site, use of fetal versus adult material, species differences, or influences of disease on phenotypes; indeed the first possibility is born out by the study of Salter *et al.* (1995) where the distribution of integrin clearly differs by site (human articular, epiphyseal, and growth plate chondrocytes were studied). Likewise, changes have been reported in *in vitro*-cultured chondrocytes (Loeser *et al.,* 1995; Shakibaei *et al.,* 1993).

Extensive studies have, though, been performed to address the role of $\alpha_5\beta_1$ in chondrocyte interaction with fibronectin (Durr *et al.,* 1993; Enomoto *et al.,* 1993; Shimizu *et al.,* 1997; Enomoto-Iwamoto *et al.,* 1997; Homandberg and Hui, 1994; Xie and Homandberg, 1993). Function-blocking antibodies and RGD peptides have been shown to inhibit cell adhesion to fibronectin and its fragments, thus modifying chondrocyte behavior and cartilage function. Likewise, chondrocyte recognition of collagen, including types I, II, and VI collagen, has been studied *in vitro* (Durr *et al.,* 1993; Enomoto *et al.,* 1993; Shimizu *et al.,* 1997; Enomoto-Iwamoto *et al.,* 1997; Holmvall *et al.,* 1995) and shown to be mediated via several β_1 integrins: $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ (Shakibaei, *et al.,* 1993; Loeser, 1997; Holmvall *et al.,* 1995).

Data have highlighted the functional interaction between integrins and mechanical strain in cartilage, although the mechanisms are likely to differ considerably from those obtained in osteoblasts due to the major differences in strain magnitude and exposure frequency to which joint cartilage is exposed. Thus, Salter and colleagues (Wright *et al.,* 1997; Millward-Sadler *et al.,* 1999; Lee *et al.,* 2000) have shown that integrin $\alpha_5\beta_1$ acts as a mechanoreceptor in chondrocytes, with strain inducing a variety of downstream signaling events and cytokine secretion. Differences were, additionally, observed in osteoarthritic versus normal cartilage (Millward-Sadler *et al.,* 2000).

There is also increasing evidence for a connection between chondrocyte adhesion to extracellular matrix proteins, especially fibronectin, and chondrocyte–synovial cell interaction (Ramachandrula *et al.,* 1992). Here, integrin expression and function are regulated by inflammatory cytokines and growth factors, resulting in the release of matrix metalloproteinases (Arner *et al.,* 1995) and hence cartilage breakdown (Xie and Homandberg, 1993; Yonezawa *et al.,* 1996). Such events are likely to be involved in the pathogenesis of the cartilage destruction seen in osteoarthritis and rheumatoid arthritis.

Nonintegrin Cell Adhesion Molecules of Cartilage

CADHERINS

There is some evidence for differential expression of cadherins (N-cadherin and cadherin-11) in prechondrocytic cells of developing limb primordia, but cadherins appear not to be found in mature cartilage (Simonneau *et al.,* 1995; Oberlender and Tuann, 1994; Tavella *et al.,* 1994). There is no published literature on cadherin in human cartilage development.

CD44

CD44 is expressed by cartilage and has been studied for a variety of sites and species (Hughes *et al.,* 1994; Noonan *et al.,* 1996; Stevens *et al.,* 1996). The predominant isoform detected is the standard CD44H variant (Salter *et al.,* 1996). There is some evidence from the use of function-blocking antibodies showing that CD44 is involved in chondrocyte pericellular matrix assembly (Knudson, 1993). The range of extracellular matrix molecules recognized by CD44 in cartilage is unclear, although interaction with hyaluronan is likely. CD44 is upregulated during cartilage catabolism (Chow *et al.,* 1995) induced by inflammatory cytokines, and chondrocytes have been shown to actively take up hyaluronan via CD44-mediated endocytosis (Hua *et al.,* 1993). Thus, it is reasonable to speculate that this molecule plays a regulatory role in cartilage matrix turnover in health and disease (Neidhart *et al.,* 2000).

IG FAMILY MEMBERS

N-CAM is distributed similarly to N-cadherin in early cartilage development (Tavella *et al.,* 1994; Hitselberger Kanitz *et al.,* 1993); again, there are no data for mature human cartilage. A further Ig superfamily molecule, ICAM-1, is expressed by chondrocytes, particularly after activation by inflammatory cytokines (Bujia *et al.,* 1996; Davies *et al.,* 1991), which may play a role in mediating

Table V Key Roles for Cell Adhesion Molecule Interactions in Bone

Receptor	Cell type	Ligand(s) bound[a]	Known/potential functions
Integrins			
$\alpha_v\beta_3$	Osteoclast	Vitronectin, osteopontin, bone sialoprotein, fibronectin, fibrinogen, denatured collagen, etc.	Matrix adhesion Signal transduction Osteoclast polarization ? cessation of resorption
$\alpha_2\beta_1$	Osteoclast	Native collagens	Matrix adhesion
	Osteoblast	Native collagens	Matrix adhesion Osteoblast differentiation
	Chondrocytes	Type II collagen	Matrix adhesion
$\alpha_5\beta_1$	Osteoblast	Fibronectin (RGD)	Osteoblast differentiation
	Chondrocyte	Fibronectin (RGD)	Cartilage breakdown
Cadherins			
N- and other cadherins	Osteoblasts	N-cadherin, etc.	Osteoblast development
	Chondrocyte	N-cadherin, etc.	Cartilage development
E-cadherin	Osteoclast	E-cadherin	? Osteoclast differentiation
Ig superfamily			
ICAM-1	Osteoblast	LFA-1 on leukocytes	Osteoblast differentiation Production of cytokines
	Chondrocyte	LFA-1 on leukocytes	Cartilage breakdown
VCAM-1	Osteoblast	α_4 integrins on leukocytes	Osteoblast differentiation Production of cytokines
Cell surface proteoglycans			
Syndecan-1	Osteoblast, osteocyte	Type I collagen, tenascin-C	? Matrix adhesion ? Osteoblast differentiation ? Role in mechanosensing
Syndecan-3	Chondrocyte	Tenascin-C	Cartilage development
CD44	Osteoclast	? Hyaluronate, ? osteopontin, ? type I collagen, ? fibronectin	Osteoclast formation ? Osteoclast migration ? Osteoclast–osteoblast interaction
	Osteoblast	Hyaluronate	Hyaluronate degradation
	Osteocyte	? Hyaluronate, ? osteopontin, ? type I collagen, ? fibronectin	? Matrix adhesion ? Role in mechanosensing
	Chondrocyte	Hyaluronate	Pericellular matrix assembly

[a] There is no definitive information on the natural ligands in bone or cartilage for these molecules. The range of ligands demonstrated to be bound in *in vitro* adhesion assays is shown.

T-cell–chondrocyte interactions at sites of inflammatory joint destruction (Horner *et al.,* 1995; Seidel *et al.,* 1997).

SYNDECANS

Syndecan-3 is highly expressed in proliferating chondrocytes, below the tenascin-C-rich layer of articular chondrocytes; decreased levels are found in hypertrophic cartilage (Shimazu *et al.,* 1996). High levels are also found in forming perichondrium (and later in periosteum) in the developing avian limb (Seghatoleslami *et al.,* 1996) and it has been suggested that syndecan-3 is involved with tenascin-C in establishing, or maintaining, boundaries during skeletogenesis (Koyama *et al.,* 1995).

Concluding Remarks: Modulation of Integrin Function in Bone—New Therapeutic Possibilities for Bone Disease

Bone and cartilage cells express a wide variety of adhesion molecules (summarized with their known and potential functions in Table V). Integrin expression has been studied extensively, but, generally, there is less information on expression of other adhesion molecule family members. There is also little information on the expression and function of adhesion molecules of all classes during skeletal cell development, largely because we currently lack adequate markers to identify immature bone cells. Adhesion receptors fulfill many functions in the skeleton, and these are frequently linked to a variety of intracellular signaling pathways, leading to a central regulatory role for this class of molecules in bone metabolism. Knowledge of their role in bone resorption and cartilage integrity is extensive, although a function for cell adhesion receptors in bone formation has only been defined recently. Although no unique osteoblast, osteoclast, or chondrocyte adhesion molecule has been identified to date, therapeutic strategies based on selectively inhibiting highly expressed receptors, such as the $\alpha_v\beta_3$ integrin in osteoclasts, have proved to be successful in regulating excessive bone resorption. Better knowledge of the expression of adhesion molecules in bone and cartilage pathology is required, and elucidation of the role of cell–matrix interactions in the aetiology of skeletal disease will, therefore, remain a research challenge for the foreseeable future.

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CHAPTER 18

Intercellular Junctions and Cell–Cell Communication in Bone

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Introduction

The organization of cells in tissues and organs is controlled by molecular programs that afford cells the ability to recognize other cells and the extracellular matrix and to communicate with their neighbors. Adhesive interactions are essential not only in embryonic development, but also in a variety of other biologic processes, including the differentiation and maintenance of tissue architecture and cell polarity, the immune response and the inflammatory process, cell division and death, tumor progression and metastases (Takeichi, 1993; Goodenough *et al.,* 1996). Cell–cell and cell–matrix adhesion are mediated by four major groups of molecules: cadherins, immunoglobulin-like molecules, integrins, and selectins (Hynes *et al.,* 1992; Gumbiner, 1996). Cadherins are an integral part of *adherens junctions,* which along with tight junctions and desmosomes, constitute the so-called anchoring junctions, which join cells by anchorage through their cytoskeletons (Alberts *et al.,* 1994). A special type of intercellular junction are *gap junctions,* which do not provide cell anchorage but allow direct communication via specialized intercellular channels, and thus they are defined as communicating junctions (Lowenstein, 1981). Recent findings of naturally occurring mutations of gap junction proteins in several pathologic conditions (Paul, 1995) and the development of mouse models with disrupted gap junctional communication indicate that gap junctions are critical for cell fate specification, migration, differentiation, and tissue morphogenesis. In addition to cell–cell adhesion molecules and gap junctional communication, mechanically induced "calcium waves" represent a short-range signaling system that allows cell-to-cell propagation of locally generated signals that diffuse through cell networks.

Bone development occurs by aggregation and condensation of immature osteoprogenitor cells in specific areas to form cartilaginous scaffolds and in the adult skeleton bone remodels via repeated sequences of bone resorptive and formative cycles, which in turn requires a coordinated cellular activity among osteoblasts, osteoclasts, and osteocytes. The cooperative nature of bone modeling and remodeling requires efficient means of intercellular recognition and communication that allow cells to sort and migrate, synchronize their activity, equalize hormonal responses, and diffuse locally generated signals. Thus, as differentiated osteoclasts represent a real syncytium, the result of mononuclear precursor fusion, osteoblasts and osteocytes are interconnected in a "functional syncytium" via intercellular adhesive and communicating junctions. This chapter reviews current knowledge about the role of direct cell–cell interactions in the development and remodeling of the skeletal tissue, focusing on cell–cell adhesion via cadherins and other cell adhesion molecules, cell–cell communication via gap junctions, and short-range calcium signals, or calcium waves.

Cell–Cell Contact via Cell Adhesion Molecules

Adherens Junctions and the Cadherin Superfamily

Cadherins are single chain integral membrane glycoproteins that mediate calcium-dependent cell–cell adhesion (Kemler, 1992; Takeichi, 1995). About 30 members of this large superfamily of cell adhesion molecules have been cloned (Tanihara *et al.,* 1994; Takeichi, 1995; Gumbiner, 1996). Although cadherins were originally named after their tissue of origin—N-cadherin (Ncad) from the nervous system, E-cadherin (Ecad) from epithelial cells, etc.—new family members are identified sequentially as cadherin-4 (cad4) through cadherin-14 (Suzuki *et al.,* 1991). Cadherins have a molecular mass of about 120 kDa and are composed of a long extracellular domain (EC), a single transmembrane-spanning domain, and a relatively small intracellular (IC) C-terminus tail. Calcium-binding sites are located in the EC, which is composed of five repeats (EC1 through EC5), and confer the ability to bind to the same cadherin on neighboring cells. A further classification into two cadherin types has been proposed based on relatively minor structural differences (Tanihara *et al.,* 1994): type I, which includes, among others, Ncad, Ecad, Mcad, and cad4 (the human homologue of mouse Rcad), and type II, comprising cad5 through cad12. Another group of cadherins includes those lacking the intracellular tail, i.e., Tcad and cad13, whose function is still obscure. This now large family of molecules is sometimes referred to as "classical" cadherins to distinguish them from protocadherins, also members of this superfamily, perhaps representing ancestor molecules (Sano *et al.,* 1993; Suzuki, 1996), and from desmocollins and desmogleins, which also differ from the typical cadherins in their cytoplasmic domain (Buxton *et al.,* 1992).

Crystallographic analysis has provided mechanistic insights into the steric arrangement and Ca^{2+} dependency of the adhesion structure formed by cadherins (Shapiro *et al.,* 1995; Pertz *et al.,* 1999). The most recent model derived from the crystallization of Ecad ectodomains envisages the formation of *cis* homodimers as Ca^{2+} concentration increases to > 1 m*M*. Upon Ca^{2+} binding and dimerization, the two cadherins participating in the dimer become rigid and form an X-shaped assembly, interfacing through their EC1 and EC2 domains. Further steric rearrangement at higher Ca^{2+} concentration allows Trp2 to dock into the hydrophobic pocket formed in part by the His-Ala-Val (HAV) domain—thought to be critical for cell adhesion (Blaschuk *et al.,* 1990)—of an opposing cadherin, thus forming a *trans* homodimer and generating a "zipper" structure of multiple *cis* dimers on opposing membranes (Fig. 1). It is unclear whether the same domain mediates adhesion among type II cadherins, where the motif is changed to QAV (cad11) or QAI (cad6) (Suzuki *et al.,* 1991). The cytoplasmic tail, highly conserved among cadherins, is associated with specific proteins, β-catenin and plakoglobin, which connect the cadherin molecule to the actin cytoskeleton either directly or via α-catenin. The latter in turn interacts with a number of proteins, such as α-actinin, ZO-1, vinculin, and other molecules (Yamada *et al.,* 1997) (Fig. 1). The assembly of cadherins and their associated cytoskeletal elements form the junctional structures known as *adherens junctions.* Both β-catenin and plakoglobin are targets of tyrosine kinases that regulate their phosphorylation state, leading to inhibition or strengthening of the adhesion complex, respectively (Grunwald, 1993). Regulation of adhesion is also controlled by cadherin binding to other proteins, most importantly, $p120^{ctn}$ and IQGAP1. The former is a member of the armadillo family of proteins, which also includes β-catenin and plakoglobin (Hatzfeld, 1999). The other interacting protein, IQGAP1, mediates regulatory signals from the Rho family of small GTPases, particularly Rac1 and Cdc42 (Kaibuchi *et al.,* 1999). Cadherins are not only part of adhesion structures, they can also function as signaling molecules. One mechanism of cadherin-mediated signaling is via β-catenin, which can translocate to the nucleus where it functions as a transcriptional activator through interaction with lymphoid enhancer factor-1 and T-cell factor-1,3,4, all part of the *Wnt* signaling cascade (Ben Ze'ev *et al.,* 1998).

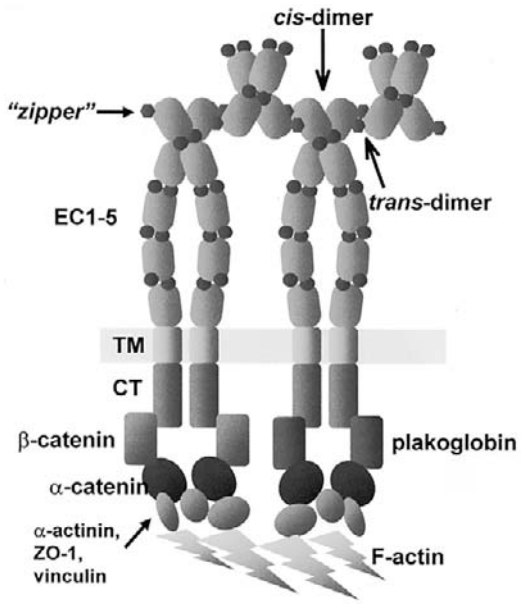

Figure 1 Schematic representation of the cadherin adhesion complex. Two complete cadherin *cis* dimers side by side, forming *trans* dimers with cadherins on the opposing side (partially represented), are illustrated. Each cadherin is shown with its five extracellular domains (EC1–5), as well as their transmembrane (TM) and cytoplasmic (CT) domains. Small circles between the EC domains symbolize calcium ions, and the small hexagons in EC1 represent Trp2, thought to be critical for *trans* dimerization. The alignment of EC1 domains forms the so-called "zipper" structure of the adhesion complex.

Cadherins in Skeletal Development

Molecular cloning from osteoblastic cells of different species has established that Ncad and cad11 are the major cadherins present in bone-forming cells (Okazaki *et al.,* 1994; Cheng *et al.,* 1998; Ferrari *et al.,* 2000), although the

degree of expression and their distribution are not identical in cartilage and bone. Ncad is abundant in mesenchymal cells undergoing cartilage nodule condensation (Tsonis *et al.,* 1994) and it is required for chondrogenesis in the early phases of embryonic limb bud development (Oberlender *et al.,* 1994; Tavella *et al.,* 1994). In fact, perturbation of cadherin-mediated interactions disrupts mesenchymal cell condensation and chondrogenic differentiation (Woodward *et al.,* 1999; Haas *et al.,* 1999). Therefore, Ncad provides a molecular cue for development of the cartilaginous scaffolding of bone rudiments, and while not present in mature cartilage, Ncad expression clearly persists in mature and adult bone (Ferrari *et al.,* 2000). Conversely, cad11 is found primarily in mesenchymal cells and has been considered crucial for mesenchymal organization (Hoffmann *et al.,* 1995; Simonneau *et al.,* 1995). Cad11 is expressed transiently in the cephalic mesoderm and then in the paraxial mesoderm of the trunk during early development, where it participates in cell condensation and segregation in the head, somites, and limb buds (Kimura *et al.,* 1995; Hoffmann *et al.,* 1995). However, at later developmental stages, a wide variety of mesenchymal tissues in both mesodermal and neural crest derivatives express cad11 (Simonneau *et al.,* 1995). Thus, both Ncad and cad11 are present in mesenchymal cells, but with distinct expression patterns; Ncad is less abundant than cad11 in the head and it is absent in branchial arches, in sharp contrast with the abundant presence of cad11 (Kimura *et al.,* 1995) Furthermore, while Ncad is present in the perichondrium (Oberlender *et al.,* 1994; Kimura *et al.,* 1995), cad11 appears only in the primary spongiosa but not in condensing or proliferating chondrocytes of the growth plate, where Ncad is abundant (unpublished observations).

Role of Cadherins in Osteogenic Cells

N-Cadherin and Cadherin-11 Define the Osteogenic Lineage

The presence of multiple cadherins in the same cell type is not an uncommon finding. In general, expression of a certain cadherin is linked to differentiation or commitment to a specific cell phenotype, but terminal differentiation of many specialized tissues is associated with coexpression of others, usually type II cadherins. For example, while Ncad is present in most neural cells, cad6 expression is restricted to synaptic connections between neurons driving their formation (Inoue *et al.,* 1998); similarly, whereas N-cadherin is important for myogenic commitment, it is not required for myoblast fusion (Charlton *et al.,* 1997). In uncommitted mesenchymal cells, such as the embryonic mouse cell line C3H10T1/2, expression of Ncad is increased by bone morphogenetic protein-2 (BMP-2), presumably reflecting the transition to a chondroosteogenic phenotype (Shin *et al.,* 2000). Likewise, cad11 is upregulated in immature mesenchymal cells under stimulation by osteogenic factors, such as BMP-2, whereas it is downregulated when cells undergo adipogenic or myogenic differentiation (Shin *et al.,* 2000; Kawaguchi *et al.,* 2001b) and it disappears in adipocytes (Shin *et al.,* 2000; Kawaguchi *et al.,* 2001b). Similarly, the uncommitted C2C12 cells lose Rcad/cad4 upon osteogenic differentiation, whereas cad11 abundance increases. These cells also express Mcad, indicative of their myogenic potential, but transdifferentiation from myogenic to osteogenic cell phenotypes is associated with a Mcad to cad11 transition (Kawaguchi *et al.,* 2001b). Therefore, it is conceivable that coexpression of cad11 and Ncad may allow sorting and segregation of mesenchymal progenitors committing to osteogenic differentiation from those entering the adipogenic pathway. Interestingly, cad11 abundance decreases with aging in rat bone marrow stromal cells (Goomer *et al.,* 1998), raising the possibility that loss of cad11 and Ncad may be involved in determining a reduced osteogenic potential in the aging skeleton. Based on the accumulated results, a model of "cadherin switch" during mesenchymal cell differentiation has been proposed (Fig. 2). This model predicts that uncommitted precursors express low levels of all mesenchymal cadherins (Ncad, cad11, cad4/Rcad, and perhaps others), and upon commitment to a certain pathway, both Ncad and a second "tissue-defining" cadherin (cad11 for osteoblasts, Mcad for myoblasts) are upregulated, whereas cad4/Rcad is shut off. Whether a second cadherin exists for chondrocytes or adipocytes remains unknown. In their terminally differentiated state, adipocytes, myotubes, and chondrocytes lose their cadherin fingerprinting, whereas osteoblasts do not, perhaps reflecting their active role in a high remodeling tissue. The chondrocyte-to-osteoblast transition during endochondral ossification is marked by cad11 induction.

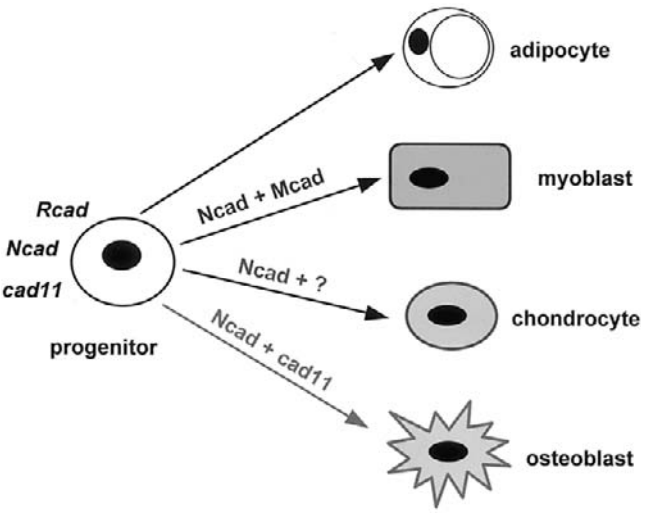

Figure 2 The "cadherin switch" model during mesenchymal cell differentiation. Uncommitted precursors express low levels of all mesenchymal cadherins (Ncad, cad11, and Rcad/cad4). Upon commitment to a certain lineage, both Ncad and a "tissue-defining," usually type II cadherin (cad11 for osteoblasts, M-cad for myoblasts, an unknown cadherin for chondrocytes) are upregulated, whereas Rcad/cad4 is shut off. In their terminally differentiated state, adipocytes, myotubes, and chondrocytes lose their cadherin fingerprinting, whereas osteoblasts do not, perhaps reflecting their active role in a high remodeling tissue.

Regulation of Cadherins in Bone-Forming Cells

In cells already committed to the osteogenic pathway, expression of cad11 does not change substantially with differentiation (Kawaguchi *et al.,* 1999; Tsutsumimoto *et al.,* 1999). A splice variant of cad11 lacking the IC domain has also been described on the surface of osteoblasts, but its function remains uncertain (Kawaguchi *et al.,* 1999). Aside from a modest downregulation by dexamethasone (Lecanda *et al.,* 2000a), cad11 does not appear to be heavily affected by BMP-2 (Cheng *et al.,* 1998). However, Ncad mRNA is sharply downregulated by dexamethasone (Lecanda *et al.,* 2000a) and by IL-1 or TNF-α (Tsutsumimoto *et al.,* 1999), whereas it is upregulated by a constitutively active, mutated FGFR-2 receptor (Lemonnier *et al.,* 1998). Ecad reactivity has been observed in UMR 106–01 cells using an anti-Ecad antibody (Babich *et al.,* 1994). Because both Ncad and Ecad are stimulated rapidly by BMP-2 in calvaria cells and neutralizing antibodies against either Ncad or Ecad prevent BMP-2 stimulation of alkaline phosphatase (Hay *et al.,* 2000), Ecad may also be functionally important in bone. However, one must consider that antibodies are not absolutely specific for a single cadherin isotype, and the presence of Ecad in calvaria cells may reflect their heterogeneous nature, encompassing neural, vascular endothelial and perhaps epithelial cells, in addition to osteoblasts. In fact, a low abundance of Pcad, VEcad, and cad8 has also been detected in mouse calvaria cells (Kawaguchi *et al.,* 2001b), whereas osteoblastic cells isolated from human trabecular bone do not express any of these cadherins (Cheng *et al.,* 1998). Whether the cadherin repertoire changes with terminal differentiation into osteocytes has not been investigated thoroughly, although neither cad11 nor other cadherins have been detected in the osteocyte-like cell line MLO-Y4 (Kawaguchi *et al.,* 2001b).

Function of Cadherins in Bone-Forming Cells

Synthetic peptides containing the HAV adhesion recognition motif (see earlier discussion) have been used to inhibit cadherin-mediated adhesion. Such inhibitory peptides prevent the development of alkaline phosphatase activity in both human bone marrow stromal cells under BMP-2 stimulation (Cheng *et al.,* 1998) and the osteogenic sarcoma cell line SaOS-2 (Ferrari *et al.,* 2000). Exposure to HAV peptides also decreases osteocalcin and immediate early gene *zif* 268 expression by SaOS-2 cells and inhibits bone nodule formation by calvaria cell cultures (Ferrari *et al.,* 2000). A more selective and controlled antagonism of cadherin-mediated adhesion can be obtained by overexpressing cadherin mutants with dominant-negative action. One such molecule, NCadΔC, has been used successfully to disrupt cadherin-mediated cell–cell adhesion (Kintner, 1992; Hermiston *et al.,* 1995; Haas *et al.,* 1999). Accordingly, NcadΔC transfection significantly reduces calcium-dependent cell–cell adhesion between MC3T3-E1 cells, altering their development into fully mature osteoblasts (Cheng *et al.,* 2000), and it inhibits cell–cell aggregation and morphological differentiation by SaOS-2 cells (Ferrari *et al.,* 2000).

Although these data strongly suggest that cadherin-mediated adhesion is required for osteoblast function, peptide inhibitors and dominant-negative cadherin mutants are not specific inhibitors of individual cadherins. Targeted gene deletion should afford a more precise definition of the function of each cadherin isotype in bone. Unfortunately, homozygous loss of the N-cad gene is lethal at early stages of embryogenesis (Radice *et al.,* 1997), making this model unsuitable for studies on bone cell differentiation. However, mice genetically deficient in cad11 are viable and have mildly decreased trabecular bone density and skull abnormalities with reduced diploic space. In addition, calvaria cells isolated from cad11–null mice exhibit stunted mineralization potential *in vitro* (Kawaguchi *et al.,* 2001a). This mild but clear skeletal phenotype demonstrates that cadherins are indeed important for the skeletal system *in vivo.* It also suggests that other cadherins, possibly Ncad, may compensate for the loss of cad11, thus serving partially overlapping roles.

Other Cell Adhesion Molecules in Bone and Cartilage

Neural cell adhesion molecule (N-CAM), a member of the immunoglobulin superfamily, is present in chick limb buds before mesenchymal cell condensation and its abundance increases during cell aggregation in a pattern similar to that of Ncad (Tavella *et al.,* 1994). Both adhesion molecules are undetectable in hypertrophic chondrocytes, but are reexpressed in preosteoblastic cells (Lee *et al.,* 1992; Tavella *et al.,* 1994). Although N-CAM and Ncad are present during chondrogenesis, subtle differences in the timing of expression suggest that Ncad may initiate cell–cell aggregation while N-CAM stabilizes the aggregates, although such hypothesis has not been proven in a more mechanistic fashion. While disappearing in differentiated cartilage, N-CAM persists in the perichondrium of long bones and sclerotomes, and it is present in the calvarium during mesenchymal cell aggregation (Lee *et al.,* 1992). N-CAM is not present homogeneously in osteoblasts, its expression apparently declining with osteogenic differentiation (Lee *et al.,* 1992), and is not regulated by BMP-2 (Hay *et al.,* 2000).

Cell Adhesion Molecules in Osteoclast Development and Function

The physiologic importance of direct cell–cell contact is not limited to homotypic interactions among osteoblasts. Two critical steps of osteoclastogenesis, i.e., heterotypic interactions between hematopoietic osteoclast precursors and stromal/osteoblastic cells and osteoclast precursor fusion, are both dependent on cell–cell adhesion. Osteoblast/stromal cell support of osteoclastogenesis is mediated by the interaction of RANKL on the surface of osteoblasts and stromal cells and its receptor, RANK, present on osteoclast precursors (see Chapter 7). Although soluble RANKL is sufficient to induce osteoclastogenesis *in vitro,* direct contact between

cells of the two lineages seems to be required *in vivo* (Suda *et al.,* 1999). A type II cadherin, cad6 (the murine homologue of human Kcad), and a splice variant of cad6, named cad6/2, are present on the surface of both hematopoietic osteoclast precursors and stromal or osteoblastic cells, and inhibition of cad6/2 expression severely impairs the support of osteoclast differentiation by ST2 cells (Mbalaviele *et al.,* 1998), indicating that cad6 isoforms may be key mediators of heterotypic contact between cells of the two lineages. In contrast, Ecad seems to be important in the fusion of mononuclear precursors, as interference with Ecad adhesion prevents the formation of multinucleated bone-resorbing osteoclasts (Mbalaviele *et al.,* 1995). Interestingly, colocalization of cadherins with vinculin at the sealing zone of the osteoclast suggests that cadherins may be involved in the formation or maintenance of the actin ring in the sealing zone and thus osteoclast attachment to the matrix (Ilvesaro *et al.,* 1998). In any case, cadherin participation to osteoclast–matrix adhesion is likely to be indirect, as cadherins do not bind matrix components, whereas cell–cell and cell–matrix adhesion are coordinated by shared signaling mechanisms (Monier-Gavelle *et al.,* 1997).

Other cell adhesion molecules, particularly, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), members of the immunoglobulin superfamily, play a role in osteoclastogenesis by stimulating cytokine production upon homophilic cell–cell adhesion (Tanaka *et al.,* 1995). Intriguingly, ICAM-1-positive stromal cells are able to support osteoclastogenesis in a far larger degree than ICAM-1-negative cells, and interruption of ICAM-1 or VCAM-1 adhesion to cognate receptors (leukocyte function-dependent antigen-1; LFA-1) dramatically decreases human osteoblast support of osteoclastic cell formation with or without hormonal stimulation (Tanaka *et al.,* 2000). As one would expect, anti-ICAM-1 or anti-VCAM-1 antibodies inhibit osteoblast adhesion to peripheral monocytes, whereas anti-RANKL antibodies do not, implying that RANKL–RANK interaction does not provide cell–cell adhesion (Tanaka *et al.,* 2000). Thus, it is likely that higher affinity cell–cell adhesion and anchorage are required to allow efficient presentation and engagement of membrane-bound RANKL to its receptor and attendant generation of osteoclastogenic signals. These new and intriguing data greatly expand the physiologic role of cadherins and other cell adhesion molecules from morphogenic regulators during organism development to essential regulators of bone cell function during both phases of the bone-remodeling cycle in the adult skeleton.

Direct Cell–Cell Communication via Gap Junctions

Gap Junctions as Intercellular Channels

Gap junctions are transcellular channels that provide aqueous continuity between two cytoplasms. They are composed by juxtaposition of two hemichannels (Li *et al.,* 1996), called

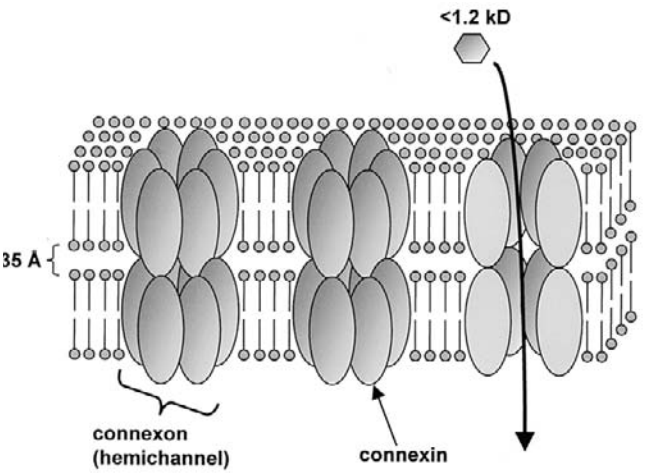

Figure 3 Schematic diagram of gap junctions. One connexon, or hemichannel, is a transmembrane hexamer formed by gap junction proteins, connexins. Two connexons in register on opposing membranes (represented as lipid bilayers) form a gap junction channel, leaving a 35-Å gap between the two cell membranes. The gap junction pore provides aqueous continuity between the two cytoplasms, allowing small molecules of up to 1.2 kDa and ions to pass from one cell to the other.

connexons, to form a complete intercellular channel (Fig. 3). Each connexon is formed by a hexameric array of protein subunits, called connexins (Revel *et al.,* 1967; Goodenough *et al.,* 1996; Kumar *et al.,* 1996). The connexin family is composed of at least 13 genes in rodents, with many homologues in other species (White *et al.,* 1995; Goodenough *et al.,* 1996; Kumar *et al.,* 1996). A widely used nomenclature identifies each connexin by their predicted molecular mass. Thus, the most common connexin in the heart and bone is a 43,036 Da protein, called connexin43 (Cx43) (Beyer *et al.,* 1987). Connexins are integral membrane proteins with four transmembrane-spanning domains, two extracellular loops, one intracellular loop, and both carboxyl and amino termini inside the cell (Fig. 4). The intracellular loop and the

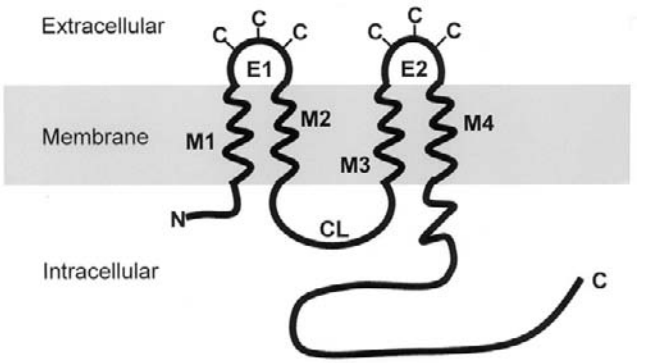

Figure 4 Structure of a connexin. Connexins are integral membrane proteins, with four transmembrane-spanning domains (M1–M4), two extracellular loops, one intracellular loop and both carboxyl and amino-termini inside the cell. The intracellular loop and the long carboxyl-terminal intracellular tail differ widely between the various connexins, both in sequence and in length. Each of the two extracellular loops contains three conserved cysteines, which are required for proper orientation of the extracellular loops and docking to connexins on the adjoining membrane.

long carboxyl-terminal intracellular tail differ widely between the various connexins, both in sequence and in length. Each of the two extracellular loops contains three conserved cysteines, which are required for proper orientation of the extracellular loops and docking to connexins on the adjoining membrane. Although in most circumstances, connexons formed by a certain connexin pair with like connexons on the opposing membrane, heterotypic channels are possible, depending on the compatibility of the extracellular loops (White *et al.,* 1994). Likewise, co-oligomerization into heteromeric connexons may occur when more than one connexin is present in the same cell (Kumar *et al.,* 1996). The phosphorylation state of connexins controls the assembly and degradation of the protein, as well as the functionality of the gap junction pore (Musil *et al.,* 1991; Sàez *et al.,* 1993; Laird *et al.,* 1995). Three-dimensional maps of a recombinant gap junction formed by a truncated Cx43 offer a spectacular confirmation of the predicted hexameric structure of the gap junction channel, with rings of α helices delimiting the pore crossing two plasma membranes and the intercellular gap (Unger *et al.,* 1997; Unger *et al.,* 1999).

The gap junction channel is permeable to ions as well as small molecules, and the size and charge selectivity depend on the connexin isotype that forms the channel. For example, Cx43 assembles in gap junctions with relatively large pores, allowing molecules of up to ~1200 Da of molecular mass and of negative charge to pass through the pore. In contrast, gap junctions formed by Cx45 have smaller pores and favor positively charged ions (Elfgang *et al.,* 1995; White *et al.,* 1995). Thus, signaling molecules such as cAMP, inositol derivatives, nucleotides, and ions such as Ca^{2+}, Zn^{2+}, and Mg^{2+} can travel across the intercellular channels, although the nature of the molecules that are exchanged by cells through gap junctions in physiologic conditions has not been fully established. The functionality of the channels is assessed by two types of tests: those that monitor cell-to-cell diffusion of molecules between cells (chemical coupling) and those that measure electrical currents carried by intracellular ions (electric coupling). The former methods are based on monitoring cell-to-cell diffusion of a membrane-impermeant fluorescent dye that is either microinjected into single cells (Stewart, 1978) or preloaded in "donor" cells that are put in direct contact with "acceptor" cells (Goldberg *et al.,* 1995; Ziambaras *et al.,* 1998). Transjunctional ion currents and unitary channel conductance can be measured using electrophysiological methods based on a double whole cell configuration of the patch-clamp technique (Veenstra and Brink, 1992).

Diversity of Connexin Expression and Distribution in Bone and Cartilage

Multiple Connexins in Osteoblastic Cells

Gap junctions were first identified in bone by electron microscopy in the early 1970s (Doty *et al.,* 1972; Stanka, 1975). In these studies, gap junctions were consistently observed among adjacent osteoblasts, osteocytes, and periosteal fibroblasts (Doty, 1981). Such abundant distribution of gap junctions among cells of the osteoblastic lineage has since been confirmed by a number of ultrastructural studies in histological sections of bone (Palumbo *et al.,* 1990; Jones *et al.,* 1993; Shapiro, 1997). Numerous *in vitro* studies have demonstrated the presence of functional gap junctions among murine calvaria osteoblasts (Jeasonne *et al.,* 1979), odontoblasts (Ushiyama, 1989), human bone cells (Civitelli *et al.,* 1993) and a variety of cell lines (Schiller *et al.,* 1992; Yamaguchi *et al.,* 1994; Donahue *et al.,* 1995b). Heterotypic communication between osteoblasts and epithelial cells has also been demonstrated (Melchiore *et al.,* 1994). The most abundant gap junction protein expressed in primary cultures of osteoblastic cells and in immortalized cell lines, i.e., MC3T3-E1 and hFOB, is Cx43. Although less abundant than Cx43 in these cell models, Cx45 is also present at appositional membranes and occasionally in cytoplasmic areas, suggesting that both connexins may interact in forming gap junctions among osteoblasts (Civitelli *et al.,* 1993; Donahue *et al.,* 2000).

In contrast, the relative abundance of the two connexins, and the resulting gap junctional communication, is highly heterogeneous among transformed cell lines. For instance, human SaOS-2 and rat UMR 106 cell lines express primarily Cx45, whereas ROS 17/2.8 cells only express Cx43 (Steinberg *et al.,* 1994; Donahue *et al.,* 1995b). Consistent with the different molecular permabilities of gap junctions formed by Cx43 and Cx45 (Veenstra *et al.,* 1992), cells that express primarily Cx45 exhibit poor cell-to-cell diffusion of negatively charged molecules the size of Lucifer yellow or calcein (~600 Da), but they are coupled electrically (Steinberg *et al.,* 1994). In contrast, cells that express abundant Cx43 are coupled chemically and electrically, and overexpression of Cx43 in UMR 106–01 cells increases dye coupling, indicating that Cx45 and Cx43 interact in forming gap junctions when coexpressed in the same cells (Steinberg *et al.,* 1994). Conversely, transfection of Cx45 in ROS 17/2.8 cells reduces both intercellular diffusion of Lucifer yellow and transjunctional conductance compared to parent ROS 17/2.8 cells (Koval *et al.,* 1995). However, transfer of smaller fluorescent molecules, such as hydroxycoumarin (~350 Da) is reduced only slightly, demonstrating that Cx45 reduces the pore size of gap junctions in a mixed Cx43/Cx45 background and that the gating properties of Cx45 prevail in the resulting channels (Koval *et al.,* 1995).

While several investigators have consistently failed to detect other connexins, such as Cx26, Cx32, Cx40, or Cx47 (Schirrmacher *et al.,* 1992; Schiller *et al.,* 1992; Civitelli *et al.,* 1993), Cx46 is present in murine osteoblastic cells. However, this connexin is never found on the cell surface, is localized exclusively within intracellular compartments, and does not oligomerize to form gap junctions in these cells (Koval *et al.,* 1997), thus the function of Cx46 in bone remains elusive.

Gap Junctions in Other Skeletal Cells

Although most of the progress on gap junctional communication in bone has been made using osteoblasts as cell

models, chondrocytes, osteocytes, and cells of the osteoclast lineage also express connexins. As mentioned previously, Cx43 is present in osteoblasts, osteocytes, and chondrocytes in rat calvaria (Jones *et al.,* 1993; Shapiro, 1997). In principle, direct communication via gap junctions is particularly important for osteocytes, as it may provide a mechanism of rapid diffusion of signals generated by mechanical forces or chemical stimuli through the osteocytic network and to the cells on the endosteal and periosteal surface. Support for this hypothesis comes from data in the mouse osteocytic cell line MLO-Y4, which expresses abundant Cx43, can diffuse negatively charged dyes, and can engage in heterotypic coupling with osteoblastic cells (Yellowley *et al.,* 2000). Similarly, the presence of Cx43 in osteoclasts at sites of active bone resorption (Jones *et al.,* 1993; Su *et al.,* 1997), primarily at contact sites between osteoclasts and overlying marrow mononuclear cells (Jones *et al.,* 1993; Ilvesaro *et al.,* 2000), suggests that functional coupling between the two cell lineages may occur *in vivo.* These observations are consistent with the presence of Cx43 in macrophages (Beyer *et al.,* 1991), although the exact role of gap junction proteins in cells, i.e., macrophages and osteoclasts, that can function effectively without contact with other cells remains unclear.

Cartilage may seem an unlikely tissue for the presence of intercellular junctions, simply because chondrocytes in mature cartilage are isolated and embedded in the matrix. However, during development, mesenchymal chondrogenic precursors must condense, and adherens and gap junctions appear between adjacent cells (Minkoff *et al.,* 1994; Langille, 1994). Therefore, cell–cell adhesion and direct intercellular communication are important during these early steps of cartilage development, when recruitment, proliferation, and differentiation of precursors occur. Importantly, Cx43 is also expressed and is functional in adult bovine articular chondrocytes when these cells are grown in tissue cultures (Donahue *et al.,* 1995a), suggesting that gap junctional communication can be reëstablished in mature cartilage in conditions that lead to cell proliferation and tissue repair, as it occurs, for example, in osteoarthritis (Hamerman, 1989).

Connexins in Skeletal Development

Earlier immunohistochemical studies in chick embryos demonstrated the presence of connexins in developing teeth and bone. Cx43 is expressed in tooth germs of neonatal rats (Pinero *et al.,* 1994) and is concentrated on mesenchymal cells at early stages of intramembranous bone formation in chick mandible, preceding the appearance of osteogenic cells (Minkoff *et al.,* 1994). In this model, Cx43 is present throughout the entire bone development process and its expression is not altered appreciably with differentiation. Conversely, Cx45 distribution seems to be more restricted to areas of active bone formation, gradually increasing in abundance at successive stages of development (Minkoff *et al.,* 1994). More mechanistic information on the physiologic role of Cx43 in skeletal development has emerged from the analysis of mice genetically deficient of Cx43. Underlining its importance in the heart, targeted deletion of the Cx43 gene in the mouse causes severe conotruncal malformations incompatible with postnatal life (Reaume *et al.,* 1995). The skeleton of homozygous Cx43-null mutants at birth reveals delayed intramembranous and endochondral ossification and clear skull abnormalities, with brittle, misshapen ribs and hypoplastic skull (Lecanda *et al.,* 2000b). As a consequence of the delayed development of all the cranial vault elements, an open foramen remains in the roof of the skull at birth (Lecanda *et al.,* 2000b). Similar abnormalities in craniofacial development have been produced by Cx43 "knock down" using antisense oligonucleotides in developing chick embryos (Becker *et al.,* 1999). Although the precise cellular bases of these defects remain to be elucidated, osteoblasts lacking Cx43 are dysfunctional as bone-forming cells, and it is likely that the delayed ossification of most skeletal elements in Cx43-null mice is related to this cell autonomous defect, regardless of the ontogeny or mode of ossification of each bone (Lecanda *et al.,* 2000b). Thus, Cx43 is functionally involved in skeletogenesis, while the contribution of Cx45 to skeletal development and its potential compensatory function in the absence of Cx43 are still unknown. Unfortunately, ablation of Cx45 is embryonically lethal, precluding the analysis of even the early stages of skeletal development (Kruger *et al.,* 2000).

Regulation of Connexin Expression and Function in Bone Cells

Regulation by Hormones and Local Factors

Prostaglandin E_2 enhances cell coupling in rat calvaria cells (Shen *et al.,* 1986) and in osteosarcoma cell lines, probably by interference with the posttranslational processing of Cx43, resulting in an increased assembly of preformed connexins into gap junction channels (Civitelli *et al.,* 1998). Parathyroid hormone (PTH) also stimulates gap junctional communication among osteoblasts, although this action seems to be dependent on the cell type (Schiller *et al.,* 1992; Donahue *et al.,* 1995b; Civitelli *et al.,* 1998). The hormonal effect on cell coupling is paralleled by a time- and dose-dependent increase of steady-state Cx43 mRNA (Civitelli *et al.,* 1998), is mediated by cAMP production (Schiller *et al.,* 1992; Civitelli *et al.,* 1998), and is prevented by PTH antagonists (Donahue *et al.,* 1995b). Thus, both PTH and prostaglandin E_2, important regulators of bone remodeling, increase gap junctional communication between osteoblasts by modulating Cx43 expression or function via different mechanisms. Both BMP-2 and TGF-β can also enhance gap junctional communication (Rudkin *et al.,* 1996). The effect of BMP-2 is associated with increased cell coupling and Cx43 abundance. In contrast, retinoic acid (Chiba *et al.,* 1994) and cytoplasmic acidification (Yamaguchi *et al.,* 1995) decrease gap junctional communication and Cx43 expression, whereas alkalinization increases gap junctional communication. Changes in cytoplasmic pH have rapid effects on channel permeability, and

prolonged exposures to a low ambient pH decrease Cx43 expression (Yamaguchi *et al.,* 1995).

Regulation by Physical Factors

Intercellular communication is important for mechanotransduction, not only because mechanical and physical factors can modulate gap junctional communication, but also because gap junctions may provide the means by which osteocytes, embedded within the calcified tissue, can transmit mechanical signals to cells on the surface, thus regulating their activity (Donahue, 2000). The number of gap junctions declines in weightlessness conditions (Doty *et al.,* 1982), and application of cyclical stretch increases the number of gap junctions in osteocytes (Lozupone *et al.,* 1996). Furthermore, Cx43 expression is increased in periodontal ligament after experimental tooth movement and in osteocytes after tooth extraction (Su *et al.,* 1997). Thus, cell-cell communication via gap junction is modulated by mechanical load to skeletal structures. At the cellular level, application of cyclical stretch by deformation of the tissue culture substrate leads to a rapid and prolonged increase of intercellular communication among osteoblastic cells, associated with increased abundance of Cx43 on the cell surface, presumably the result of decreased Cx43 turnover (Ziambaras *et al.,* 1998). Data in osteocytic MLO-Y4 cells demonstrate that gap junctional communication is also stimulated by fluid flow-induced shear stress, which causes rapid redistribution of Cx43 to the osteocytic dendritic processes and delayed stimulation of Cx43 expression (Cheng *et al.,* 2001), lending support to the notion that gap junction channels may propagate signals generated by osteocytes in response to mechanical stimuli.

Role of Gap Junctions in Bone Remodeling

Role of Connexin43 Gap Junctions in Bone-Forming Cells

While data on gap junctions and connexin expression in osteoblasts are abundant, evidence for their role in physiologic regulation of bone formation has accumulated only recently. Cx43 abundance increases upon osteoblast differentiation (Chiba *et al.,* 1993; Donahue *et al.,* 2000; Schiller *et al.,* 2001), and this increase correlates with enhanced cell–cell communication (Donahue *et al.,* 2000; Schiller *et al.,* 2001). Conversely, Cx45 abundance does not change during osteoblast differentiation (Donahue *et al.,* 2000). The increased Cx43 expression appears to be related inversely to cell proliferation, reinforcing the idea that gap junctional communication is a feature of postproliferative osteoblasts (Donahue *et al.,* 2000). Importantly, chemical inhibition of gap junctional communication leads to delayed bone nodule formation and disruption of osteoblast gene expression in human osteoblasts (Donahue *et al.,* 2000) and MC3T3-E1 cells (Schiller *et al.,* 2001), and osteoblastic cells isolated from either calvaria or bone marrow of Cx43-null mice are dysfunctional, exhibiting a severely impaired capacity to form mineralized nodules *in vitro* and reduced expression of a differentiated osteoblastic phenotype (Lecanda *et al.,* 2000b). Interestingly, Cx45 abundance is increased in Cx43-null osteoblasts, perhaps reflecting a compensatory mechanism (Lecanda *et al.,* 2000b), however, the increased Cx45 does not effectively compensate for the lack of Cx43, neither in terms of cell coupling nor of differentiation potential.

The presence of Cx43 gap junctions is also permissive for normal cell responsiveness to hormonal and physical stimuli. Rat osteogenic sarcoma cells rendered communication deficient by expression of a Cx43 antisense construct display a reduced cAMP response to parathyroid hormone (Van der Molen *et al.,* 1996), impaired contraction of osteoblast populated collagen lattices (Bowman *et al.,* 1998), and reduced alkaline phosphatase induction in response to electromagnetic fields (Van der Molen *et al.,* 2000). The reduced hormonal response of communication-deficient cells occurs despite a normal adenylate cyclase system, indicating that Cx43 gap junctions amplify the signals generated by local receptor activation, perhaps by allowing diffusion of signaling molecules or ions from responsive to nonresponsive cells, thus equalizing differences in receptor distribution and hormonal responses in osteooblastic populations (Civitelli *et al.,* 1994).

Modulation of Osteoblast Gene Expression by Gap Junctional Communication

The finding that Cx43 and Cx45 interact in forming gap junctions when expressed in the same cells (Koval *et al.,* 1995) offered a powerful strategy to study the consequences of changing intercellular communication on the phenotypic profile and gene expression in osteoblastic cell lines. Steady-state levels of osteocalcin, bone sialoprotein, alkaline phosphatase, and type I collagen are reduced significantly in ROS 17/2.8 cells transfected with Cx45, whereas osteopontin and osteonectin are not appreciably altered (Lecanda *et al.,* 1998). Reduced osteocalcin and bone sialoprotein expression in either ROS 17/2.8 or MC3T3–E1 cells transfected with Cx45 is associated with transcriptional downregulation of the respective gene promoters relative to parent cells and decreased dye coupling. In contrast, transfection of Cx43 in UMR 106–01 (they express more Cx45 than Cx43 and are poorly coupled) is followed by upregulation of both osteocalcin and bone sialoprotein mRNA abundance and promoter activity, in parallel with increased gap junctional permeability (Lecanda *et al.,* 1998). Thus, the relative expression of Cx43 and Cx45 regulates the transcriptional activity of osteoblasts-specific genes in a reciprocal fashion (Fig. 5). This new regulatory function of gap junctional communication is not restricted to bone, as gap junction-dependent gene expression and regulation have been observed in insulin-producing cells (Vozzi *et al.,* 1995), thyrocytes (Statuto *et al.,* 1997), and chromaffin cells (Munari-Silem *et al.,* 1995).

Interestingly, suppression of Cx43 expression by antisense strategies also causes reduced expression of alkaline phosphatase and osteocalcin (Li *et al.,* 1999). Such observations would imply that it is the loss of Cx43 more than the change to a Cx45-mediated intercellular communication that affects osteoblast gene expression. In fact, calvaria or bone marrow stromal cells genetically deficient of Cx43 exhibit

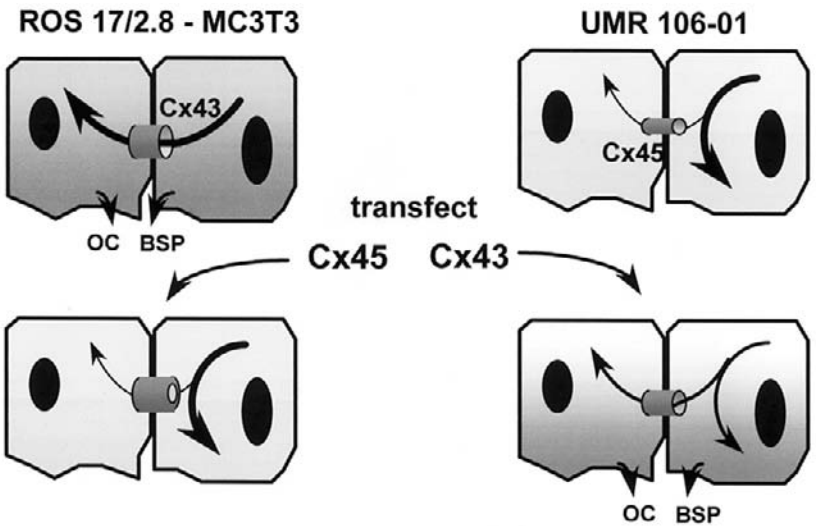

Figure 5 Model of connexin-43 (Cx43)/connexin-45 (Cx45) interactions and osteoblast gene expression regulation. The highly coupled ROS 17/2.8 and the MC3T3-E1 cells (as well as primary murine and human osteoblastic cells) express primarily endogenous Cx43 and are able to constitutively produce matrix proteins, including bone sialoprotein (BSP) and osteocalcin (OC). The poorly coupled UMR 106–01 cells express prevalently Cx45 and are poor producers of BSP or OC. Upon transfection of Cx45 in ROS 17/2.8 and MC3T3–E1 cells, gap junctions are formed by a mix of Cx45 and endogenous Cx43, resulting in channels of lower molecular permeability relative to the parent clones. This is associated with a dramatic reduction of BSP and OC gene transcription. Conversely, transfection of Cx43 in UMR 106–01 cells results in an increased Cx43/Cx45 ratio, increased gap junctional communication, and increased transcription of BSP and OC genes. Presumably, the transfected connexin can form either heteromeric connexons or homomeric, heterotypic gap junctional channels (Kumar *et al.*, 1996). In either case, Cx45 prevails in determining the permeability of the resulting channel (Koval *et al.,* 1995).

reduced osteocalcin and collagen type I expression during *in vitro* differentiation (Lecanda *et al.,* 2000b). The mechanism that links gap junctional communication to gene expression remains elusive, but it certainly depends on the type of signals that permeate the junctional channel. Based on the pore size selectivity of Cx43 and Cx45 gap junctions (Veenstra *et al.,* 1992), one could predict that the intercellular diffusion of signaling molecules, such as cyclic nucleotides or inositol phosphates, may be impaired when Cx43 permeability is decreased by interaction with Cx45. Alternatively, oscillations in intracellular-free calcium concentration or in membrane polarity may be affected by a changed connexin environment. Thus, the type of gap junctional communication provided by Cx43 is necessary for the full development of a differentiated osteoblast phenotype.

CONNEXIN43 IN OSTEOCLASTOGENESIS

As already noted, Cx43 is present in osteoclasts both *in vivo* and in cell cultures (Jones *et al.,* 1993; Su *et al.,* 1997; Ilvesaro *et al.,* 2000). Importantly, chemical inhibition of gap junctional communication leads to decreased number of bone-resorbing, multinucleated, TRAP-positive cells, but the average size of resorption pits may actually increase (Ilvesaro *et al.,* 2000). These intriguing observations would suggest that Cx43 gap junctions may serve a permissive role in mononuclear cell fusion, but once active osteoclasts are formed, Cx43 may function as an inhibitor. One possible scenario is that Cx43 forms hemichannels, which, if open for a long time, would permeabilize the cells with negative consequences, such as an increased rate of apoptosis (Ilvesaro *et al.,* 2000). In support of a role for Cx43 in osteoclastogenesis, we found that the ability of osteoblastic cells derived from Cx43-null mice has a reduced potential to support osteoclast differentiation when cocultured with wild-type mononuclear osteoclast precursors (Furlan *et al.,* 2000). Although these data are still preliminary, they are consistent with the reported failure of Cx43-null marrow stromal cells to support hematopoietic cell differentiation (Cancelas *et al.,* 2000) and indicate that loss of Cx43 impairs both arms of bone remodeling.

Intercellular Communication via Short-Range Calcium Signals

Intercellular Calcium Signaling

Transient and oscillatory elevations of cytosolic-free calcium concentration ($[Ca^{2+}]_i$) initiate or modulate a large number of cellular activities, including cell growth, motility, and secretion. Many studies have helped define cellular calcium homeostasis and the mechanisms by which extracellular signals are translated into intracellular calcium transients, but relatively scant attention has been paid to the mechanisms by

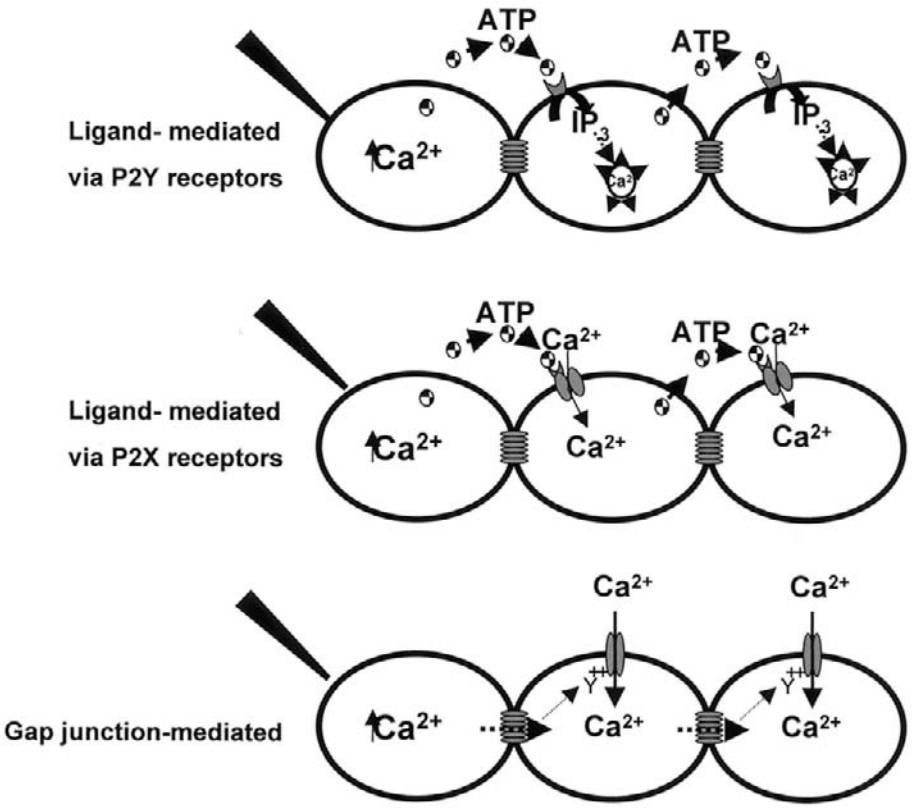

Figure 6 Mechanisms for the propagation of intercellular calcium signals. Ligand-mediated calcium waves: mechanical stimulation increases intracellular-free calcium concentration in the stimulated cell. As a consequence, ATP or a related nucleotide is released to the extracellular space and binds to surface receptors on neighboring cells. If ATP binds to P2Y receptors (top), inositol triphosphate (IP_3) is generated, inducing the release of calcium from IP_3-sensitive intracellular calcium stores. If ATP binds to P2X receptors (middle), conformational changes of the receptor/channel are induced, resulting in the opening of the channel, with a subsequent influx of extracellular calcium. In both cases, a calcium wave is generated by successive activation of P2 receptors in neighboring cells. Gap junction-mediated calcium waves (bottom): the increase in intracellular-free calcium concentration caused by mechanical stimulation produces a signaling molecule (IP_3?) that passes through the gap junction channel into adjacent cells where it induces depolarization of the plasma membrane and the subsequent opening of voltage-operated calcium channels with an influx of calcium from the extracellular space. The intracellular calcium increase is then propagated to the next cell through the same mechanism, thus producing a calcium wave.

which groups of cells propagate calcium signals among themselves and coordinate calcium responses. Two mechanisms of intercellular calcium signaling have been identified: gap junctional communication and release of soluble mediators that act on nearby cells (Fig. 6). Gap junctional communication propagates calcium signals either by allowing the passage of inositol trisphosphate and potentially other small soluble messengers between cells or by allowing electrical coupling of cells and subsequent activation of voltage-sensitive calcium channels (Sanderson *et al.,* 1994). Intercellular calcium signaling by released soluble mediators frequently involves activation of P2 ("purinergic") receptors by extracellular nucleotides such as adenosine triphosphate (ATP) (Osipchuk *et al.,* 1992; Schlosser *et al.,* 1996; Brake *et al.,* 1996).

Purinergic Receptors in Bone

Specific receptors for extracellular ATP that recognize the nucleoside triphosphate, but not adenosine, are termed P2 purinoceptors and are distinct from the P1 purinoceptors that bind adenosine. Two different families of P2 purinergic receptors exist, P2X and P2Y, which differ in structure and sensitivity to nucleotides (Brake *et al.,* 1996). Receptors of the P2X type are ligand-gated ion channel receptors. Binding of the ligand induces a rapid depolarization of the target cell followed by a rapid increase in cytosolic calcium concentration via an influx of calcium ions through the channel. Conversely, P2Y receptors belong to the seven transmembrane domain, G protein-coupled receptor superfamily (Dubyak *et al.,* 1993) and are distributed more diffusely than P2X receptors. Binding of a ligand to a P2Y receptor activates the phospholipase C (PLC) system, with production of IP_3 and subsequent release of intracellular calcium from IP_3-sensitive stores.

It has been known for at least a decade that ATP and ADP can induce transient increases in intracellular calcium concentrations in osteoblastic cells (Kumagai *et al.,* 1991), and there are now many functional studies indicating the presence of P2 receptors in cell lines (Reimer *et al.,* 1992;

Table I P2 Receptors in Bone Cells[a]

P2 receptor subtype	Cell type	Relative agonist potency
P2X2	OC	ATP = 2-MeSATP = ATP-γ-S
P2X4	OC	ATP > 2-MeSATP >> α,β-MeATP
P2X5	OB	ATP > 2-MeSATP > ADP
P2X7	OC	BzATP >> ATP > 2-MeSATP > ATP-γ-S >> ADP
P2Y1	OB/OC	2-MeSATP > ATP = ADP
P2Y2	OB/OC	ATP = UTP > ATP-γ-S >> 2-MeSATP
P2Y4	OB	ATP = UTP = ADP = ATP-γ-S = 2-MeSATP > UDP
P2Y6	OB	UDP > UTP > ADP > 2-MeSATP >> ATP

[a]OC, osteoclasts; OB, osteoblasts; ATP, adenosine 5′-triphosphate; 2-MeSATP, 2-methylthio-ATP; ATP-γ-S, adenosine 5′-*O*-(3-thiotriphosphate); α,β-MeATP, α,β-methyl-ATP; ADP, adenosine 5′-diphosphate; BzATP, 2′,3′-*O*-(4-benzoylbenzoyl)ATP; UTP, uridine 5′-triphosphate; UDP, uridine 5′-diphosphate.

Schofl *et al.,* 1992; Yu *et al.,* 1993), rat primary osteoblasts (Gallinaro *et al.,* 1995), and primary cultures of human osteoblasts (Schofl *et al.,* 1992; Dixon *et al.,* 1997). In human osteoblasts, responses to ATP and ADP may be heterogeneous, perhaps reflecting regulated P2 gene expression during osteoblast differentiation or specific roles of different receptors at various stages of differentiation (Dixon *et al.,* 1997). Indeed, several members of both P2X and P2Y classes are present on the surface of human and rat osteoblastic cells, particularly P2Y2, P2X2, and P2X5 (Bowler *et al.,* 1995; Hoebertz *et al.,* 2000; Jørgensen *et al.,* 1997). Osteoclasts also respond to extracellular nucleotides by increasing intracellular calcium concentrations. Because both extracellular calcium influx and calcium release from intracellular stores are involved in the osteoclast response to ATP (Yu *et al.,* 1993; Weidema *et al.,* 1997; Wiebe *et al.,* 1999), both the P2X and P2Y families of receptors seem to be functional in these cells. However, while P2X2 (Hoebertz *et al.,* 2000), P2X4 (Naemsch *et al.,* 1999; Hoebertz *et al.,* 2000), and P2X7 (Hoebertz *et al.,* 2000) have been identified in rat osteoclasts, P2Y2 is expressed but it does not localize to the cell surface (Bowler *et al.,* 1995). Table I summarizes the currently know P2 receptors identified in bone cells [see also Dixon *et al.* (2000) for a review].

Intercellular Calcium Signaling in Skeletal Tissue

Intercellular Calcium Signaling among Osteoblasts and Osteoclasts

As it occurs in other tissues, one of the functions of P2 receptors in bone cells is to propagate calcium signals from cell to cell. Intercellular calcium waves in response to mechanical stimulation of a single cell can be generated in osteoblastic cell cultures. These calcium waves were first observed in the osteogenic sarcoma cell line ROS 17/2.8 and in osteoblasts derived from rat calvaria (Xia *et al.,* 1992). Propagation of intercellular calcium waves was found to be dependent on the passage of an unknown signaling molecule(s) through gap junctions and regeneration of the calcium transient in neighboring cells by calcium-induced calcium release. In striking contrast with the ROS 17/2.8, cells of another osteoblastic cell line, UMR 106–01, respond to mechanical perturbation with a similar rapid calcium transient, which is followed by a calcium wave of very different kinetics. While ROS 17/2.8 cells propagate a slow wave extending to 5–15 cells over several minutes, calcium waves in UMR 106–01 cultures spread very rapidly, extending to more than 50 cells in less than 30 sec (Jørgensen *et al.,* 1997). The difference in kinetics reflects different mechanisms of intercellular signal diffusion, as ROS 17/2.8 and UMR 106–01 cells follow the two fundamental mechanisms of calcium wave propagation (see Fig. 6): signal diffusion through gap junctional communication among ROS 17/2.8 cells and autocrine release of nucleotides (most probably ATP) with activation of P2 receptors in UMR 106–01 cells.

The strikingly different mechanisms of wave propagation observed in two distinct but phenotypically similar cell lines raise the question as to the physiologic meaning of these short-range intercellular calcium signals. Studies in human bone marrow-derived stromal cells demonstrated that both mechanisms of intercellular wave diffusion are present in normal cells. Upon mechanical stimulation, human osteoblast-like cells propagate a fast wave mediated by P2 receptor activation, thus very similar to that observed in UMR 106–01 cells. However, after desensitization with ATP, the fast nucleotide-mediated wave disappears, and a slow wave is uncovered. This slow wave is very similar to a "ROS 17/2.8 wave" and is likewise mediated by gap junctional communication (Jørgensen *et al.,* 2000). In addition to secretion of paracrine factors and direct cell–cell contact, short-range calcium signals offer alternative mechanisms for the cross-talk between osteogenic and osteoclastogenic lineages in local control of bone remodeling. Because P2 receptors are present in both osteoblasts and osteoclasts, it is logical to ask whether calcium signals can

be transmitted from one cell type to another. Preliminary results obtained in our laboratory show that mechanical stimulation of a single osteoblast generates a signal that is propagated not only to surrounding osteoblasts, but also to nearby osteoclasts. Intriguingly, the signal can go both ways, as mechanical perturbation of an osteoclast induces a calcium wave that propagates to both osteoblasts and other osteoclasts. This type of heterotypic intercellular signaling seems to be entirely dependent on P2 receptor activation, with P2Y receptors responsible for signaling in osteoblasts and P2X receptors mediating calcium signaling in osteoclasts (Jørgensen *et al.,* 1999).

Intercellular Calcium Signaling in Chondrocyte Cultures

As noted earlier, articular chondrocytes can establish direct contact and gap junctional communication when they are allowed to proliferate and grow in tissue cultures. Exposure to extracellular ATP induces trains of repetitive short-lasting calcium spikes in articular chondrocytes followed by initiation of calcium waves affecting neighboring cells (D'Andrea and Vittur, 1996a,b). Likewise, mechanical stimulation of one chondrocyte induces calcium transients that propagate from cell to cell (Guilak *et al.,* 1999; D'Andrea *et al.,* 2000). Wave propagation in these articular chondrocyte cultures seems to be highly dependent on signal diffusion through gap junctions (D'Andrea and Vittur, 1996a; D'Andrea *et al.,* 2000; Donahue *et al.,* 1995a). However, chondrocytes are also responsive to ATP, and it is quite conceivable that a P2-mediated mechanism of intercellular calcium signal diffusion exists in these cells but that in resting conditions the alternative mechanism prevails, in contrast to human osteoblasts. Interestingly, mechanically induced calcium waves can propagate from chondrocytes to sinovial cells in culture via mechanisms involving both extracellular ATP release and gap junctions (D'Andrea *et al.,* 1998; Grandolfo *et al.,* 1998). Thus, heterotypic intercellular communication is possible in cartilage via cell–cell propagation of locally generated calcium signals.

Cell–Cell Adhesion and Intercellular Communication: An Integrated View

A hierarchical relationship links cell–cell adhesion and direct intercellular communication via gap junctions. Gap junctional channels form by weak, non-covalent interactions between connexins on opposite cell membranes, thus the integrity of the channel and its functionality require a stable contact between the two cells. However, connexins assembled into gap junctions are not linked directly to any cytoskeletal structure that may stabilize the transjunctional pores (Goodenough *et al.,* 1996). Therefore, it is commonly believed that cells must first adhere to each other via anchoring junctions, then they can form gap junctions (Singer, 1992); in fact, cell–cell communication and neural differentiation can be inhibited by neutralizing antibodies against N-CAM (Keane *et al.,* 1988), and anti-Ncad antibodies prevent gap junction formation (Meyer *et al.,* 1992; Frenzel *et al.,* 1996). Conversely, transfection of Ecad in communication-deficient cell lines restores gap junctional communication and alters the pattern of Cx43 expression (Musil *et al.,* 1990; Jongen *et al.,* 1991). In addition, by providing specificity for cell sorting, different cadherins may compartmentalize gap junctional communication within separate domains of different cells or allow hetorologous communication between cells on different compartments (Prowse *et al.,* 1997; Woodward *et al.,* 1998). There is virtually no information about the relationships between cadherins and connexins in bone cells, except the observation of Ncad and Cx43 colocalization in developing avian mandibles (Minkoff *et al.,* 1994). It is highly likely that cell–cell adhesion precedes and initiates intercellular communication, perhaps by modulating the synthesis and/or assembly of connexins in gap junctions in osteoblasts and osteoclastic cells. Cell–cell contact and communication represent two aspects of an integrated mechanism that allow cells to develop and work in a social context.

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CHAPTER 19

Histomorphometric Analysis of Bone Remodeling

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Introduction

Purpose of Remodeling

The skeleton has two major functions. It serves as a reservoir for minerals and as a structural framework to support the muscles and protect vital organs. Once growth and modeling of the skeleton have been completed, the bones continually alter their internal structure by remodeling, which is the localized removal of old bone and replacement with newly formed bone. The process is complex, requiring interactive cellular activity, and is regulated by a variety of biochemical and mechanical factors. It is likely that the major reason for remodeling is to enable the bones to adapt to mechanical stresses. Remodeling also allows the bone to repair microdamage and thus maintain its strength. Finally, remodeling is an important component of mineral metabolism. Alterations in remodeling are responsible for most metabolic bone diseases, and interpretation of pharmacological interventions must be done in the context of the remodeling sequence.

Bone as a material compares poorly with other engineering materials. At repetitive loading equal to 100 miles of running, fatigue damage will occur (Marcus, 1987). However, unlike the other materials, bone can repair itself by directing remodeling to the damaged site. In some situations, such as military training, the rate of repair cannot keep up with the rate of damage and the material fractures (Casez *et al.,* 1995; Margulies *et al.,* 1986). In addition to directed remodeling, which repairs cracks and fatigue damage, there is apparently random (Parfitt terms this stochastic) remodeling, which may act to continually renew bone. This prevents accumulation of older, densely mineralized bone, which is more brittle.

Although bone remodeling affects the serum levels of minerals, the minute-to-minute regulation of minerals does not depend on bone remodeling. The strongest evidence for this comes from studies of potent bisphosphonates, which can reduce bone remodeling by up to 98% when evaluated by histologic endpoints, but which have only transient, asymptomatic effects on the serum calcium concentration (Vasikaran *et al.,* 1995; Chavassieux *et al.,* 1997). The time course of remodeling also argues against its function to acutely control calcium concentration. If serum calcium drops, the parathyroid hormone (PTH) increases and serum calcium rises immediately. It takes more time, however, for origination of a basic multicellular unit (BMU) and activation of osteoclasts. The acute release of calcium is a function that is probably performed by lining cells in response to changes in endocrine hormones.

Bone remodeling activity does affect chronic serum mineral levels. For example, when there is a pathological increase in bone resorption, as seen in hyperparathyroidism or malignancy, the serum calcium is increased. In patients with renal failure and adynamic bone disease, decreased bone remodeling results in more brittle control of serum calcium: an increase in calcium intake causes greater increases in serum calcium and chelation or dialysis with low calcium causes a greater fall than in patients with normal bone remodeling rates (Kurz *et al.,* 1998).

Dynamics of Bone Physiology

Bone is not usually considered in classic physiological terms because the events are so slow. Whereas the heart contracts every second and the kidney filters 180 liters each day,

it takes 4 or 5 years for an area on the bone surface to complete one bone remodeling cycle. A time-lapse movie of the trabecular bone, played sufficiently fast, would show quivering at the surfaces, while entire trabeculae would drift, enlarge, or dissolve. An animation demonstrating this movement is available on the internet ("Osteoporosis and Bone Physiology," http://courses.washington.edu/bonephys).

Although the dynamics of bone physiology are slower than other organs, many of the same principles apply. Bone senses and then responds to external forces or stimuli, and it adjusts to different environments. An inability to perform these functions results in diseases, and the knowledge of this physiology is important so that it can be manipulated to treat or prevent the disease.

A fundamental property of bone remodeling is that it occurs in discrete locations and involves a group of different kinds of cells. This secondary level of organization, analogous to the nephron, was named the basic multicellular unit by Frost (1969). Unlike the nephron, the BMU is not a permanent structure. It forms in response to signal or stimulus, performs its function, and disbands, leaving a few residual lining cells and osteocytes. Each BMU undergoes its functions in the same sequence: origination and organization of the BMU, activation of osteoclasts, resorption of old bone, recruitment of osteoblasts, formation of new bone matrix, and mineralization [Figs. 1 and 2 (Fig. 2, see also color plate)]. A major goal of histomorphometric research has been to determine the dynamics of these sequences of bone events.

Methodology

Most of the physiology of bone remodeling has been defined from undecalcified tetracycline-labeled bone biopsies (Fig. 3, see also color plate). Details of bone biopsy technique and histomorphometric measurements are covered in Chapter 94. Insights by Frost and Parfitt are responsible for much of our current interpretation of these measurements (Frost, 1969, 1989; Parfitt *et al.,* 1996). They observed that bone remodeling activity occured in localized areas on the bone surface. The spatial relationships between osteoclastic bone resorption and osteoblastic bone formation gave clues to the temporal sequence of these events. Measuring the distance between two tetracycline labels given over a known interval allows conversion of distance measurements to time intervals. The proportion of the bone surface that is covered with tetracycline labels is the same as the proportion of time spent forming bone at a point on the surface. Newly formed bone has a different orientation from older bone; at the nonconformity is a cement line (Fig. 4, see also color plate). If the surface is quiescent, the distance between the cement line and the bone surface (wall thickness) represents the total cross-sectional thickness of bone formed by that BMU. In osteoid-covered surfaces, those sites closest to a cement line are younger than those farther away. Measurements from the cement lines to the surface can be used to determine the duration of formation at that location. Eriksen *et al.* (1984) made extensive measurements on individual BMUs and reconstructed the entire formation sequence.

Other methods have aided the study of bone remodeling. Stains for acid phosphatase (Fig. 5, see also color plate) identify osteoclasts, and stains for alkaline phosphatase identify active osteoblasts (Bradbeer *et al.,* 1992). The TUNEL stain, which identifies apoptotic cells, has been applied to bone (Weinstein *et al.,* 2000; Verborgt *et al.,* 2000). Immunohistochemical techniques (Derkx *et al.,* 1998) are emerging; these are important in understanding how new *in vitro* molecular biological findings actually work in the bone.

Back-scattered electron photomicrographs and radiodensitometry are the only methods that show the differential mineralization as bone ages (Fig. 6) (Boyde *et al.,* 1993; Jowsey, 1960; Reid and Boyde, 1987)

Other techniques, such as scanning electron microscopy, allow further examination of the three-dimensional structure of bone (Fig. 7) (Dempster *et al.,* 1986; Hahn *et al.,* 1995; Jayasinghe *et al.,* 1993; Moskilde, 1993). Hahn *et al.* (1995) have developed a technique of simultaneously examining the surface and structure of trabecular bone. They cut thick sections and stain only the surface; using reflective light, the surface looks like an ordinary thin section and can be measured. However, using back lighting, the three-dimensional connections can be appreciated. A similar approach by Aaron (2000) was applied to bone biopsies from patients with osteoporosis, allowing differentiation between real and apparent free ends. Another new technique for examining the three-dimensional structure is microcomputed tomorgraphy (Chapter 93).

Reeve *et al.* (1987) used whole body ^{85}Sr kinetics to measure mineral retention and accretion. These showed correlations with histological techniques that used tetracycline labeling. Similarly, Charles *et al.* (1987) and Eastell *et al.* (1988) have shown correlations between histomorphometry and kinetics using ^{47}Ca.

Changes in bone mass and in biochemical markers have added to the understanding of bone remodeling. Bone densitometry, using methods such as dual-energy X-ray absorptiometry, allows an integrated view of remodeling effects, although it cannot predict the microstructure of the bone or differentiate between increased formation and decreased resorption. Because resorption rates cannot be measured accurately on bone biopsies, the combination of bone formation rates and bone mass changes can provide an estimate of net resorption. The role of biochemical markers is discussed in Chapter 90.

Remodeling at the BMU Level

Origination

At any one time, ~20% of the cancellous bone surface is undergoing remodeling, and at any one surface location, remodeling will occur on average every 2–years. This is known as the activation frequency (Parfitt *et al.,* 1987). The skeleton contains millions of BMUs, all at different stages.

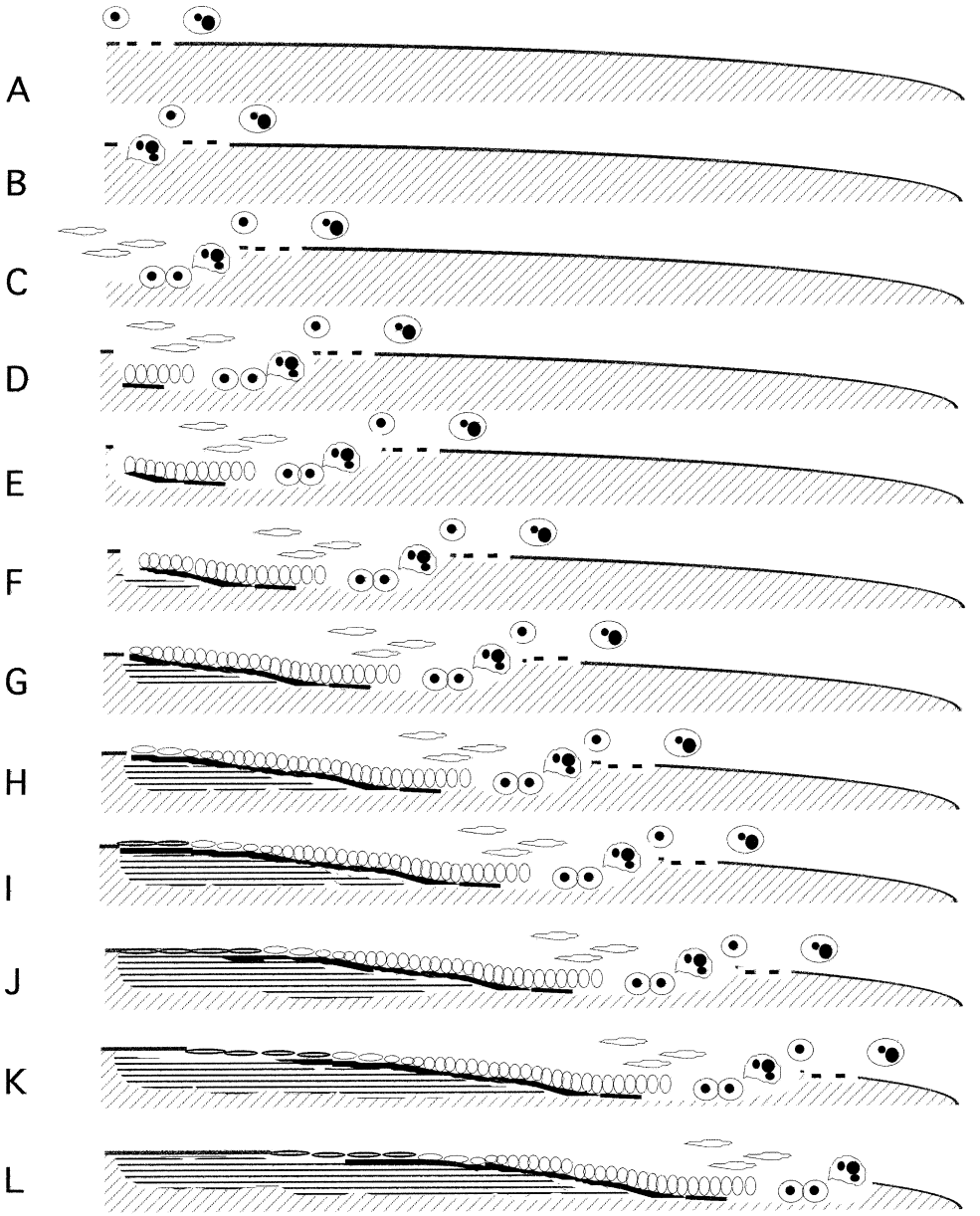

Figure 1 The basic multicellular unit moving along a cancellous surface. Each step represents ~10 days, and the BMU moves at about 10 μg each day. (A) Origination of BMU; lining cells contract to expose collagen and attract preosteoclasts. (B) Osteoclasts fuse into multinucleated cells, which resorb cavity. (C) Mononuclear cells continue resorption, and preosteoblasts are stimulated to proliferate. (D) The osteoblast team forms at the bottom of the cavity and starts forming osteoid. (E) Osteoblasts continue forming osteoid (black) and previous osteoid starts to mineralize (horizontal lines). (F–H) Osteoblasts continue formation and mineralization. (I and J) Osteoblasts begin to flatten. (K and L) Osteoblasts turn into lining cells; bone remodeling at initial surface (left of drawing) is now complete, but BMU is still advancing (to the right).

What initiates the organization of a new BMU? This question has not yet been answered, but evidence shows that mechanical stress can be sensed by osteocytes that can signal lining cells to form a new BMU at either cortical or cancellous surfaces. The osteocytes excrete paracrine factors when subjected to mechanical stimuli, e.g., IGF I expression increases 6 hr after mechanical loading (Turner and Forwood, 1995). Following fatigue loading, osteocyte apoptosis is seen in association with microdamage as well as resorption (Verborgt *et al.,* 2000). Martin (2000) hypothesized that the osteocytes chronically inhibit lining cells so that the origination of a BMU is caused by release of the inhibition. Lining cells themselves may also detect the mechanical strains; when grown *in vitro,* these cells respond to either str in or estrogen via an estrogen receptor (Zaman *et al.,* 2000). Local and circulating hormones cytokines, and growth factors certainly influence the origination of BMUs but it is not clear which ones, if any, actually set one off. Mori and Burr (1993)

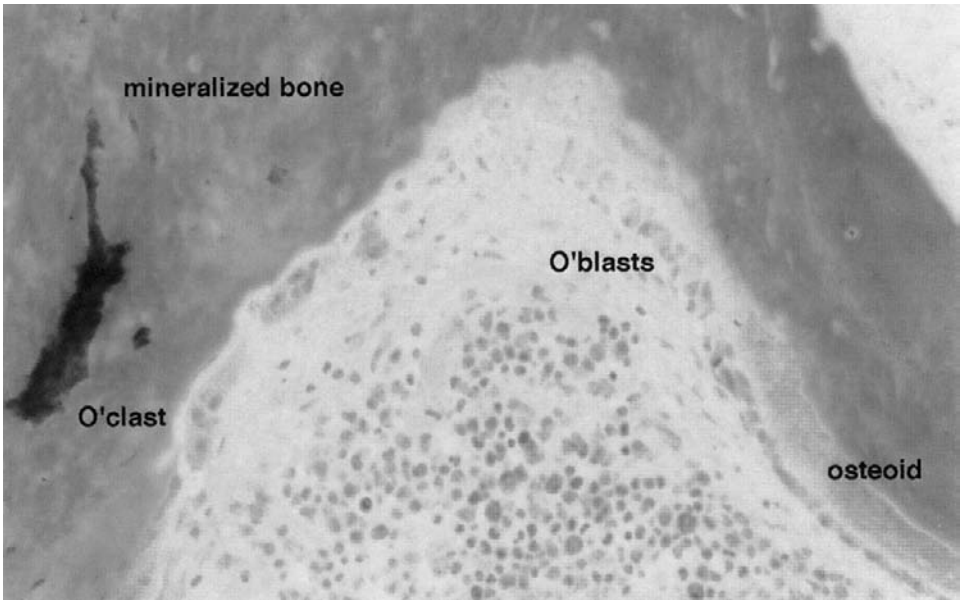

Figure 2 Photomicrograph showing BMU along a trabecular surface from a patient with renal failure. At left is osteoclastic resorption ("cutting edge") and at the right are osteoblasts and osteoid. (See also color plate.)

demonstrated an association between fatigue damage and intracortical remodeling. They anesthetized mature dogs and applied a cyclic load to the radius, which did not cause the animals to limp but did cause microscopic cracks. Eight days later an identical load was applied to the opposite radius and the animals were sacrificed. Histologic sections showed an equal number of cracks on each side. However, at the site with the earlier load, there was a significant increase in resorption cavities that were adjacent to the cracks. The temporal design of the study demonstrated that the resorption occurred after the fatigue damage.

Origination is the first step in organizing a BMU, and thus it must involve gathering of the initial cells that will form the new BMU. Precursor cells must proliferate and be available. A host of hormones and cytokines that have attracted the attention of molecular biologists probably exert most of their influence at this step. These include parathyroid hormone, 1,25-dihydroxyvitamin D, interleukins 6 and

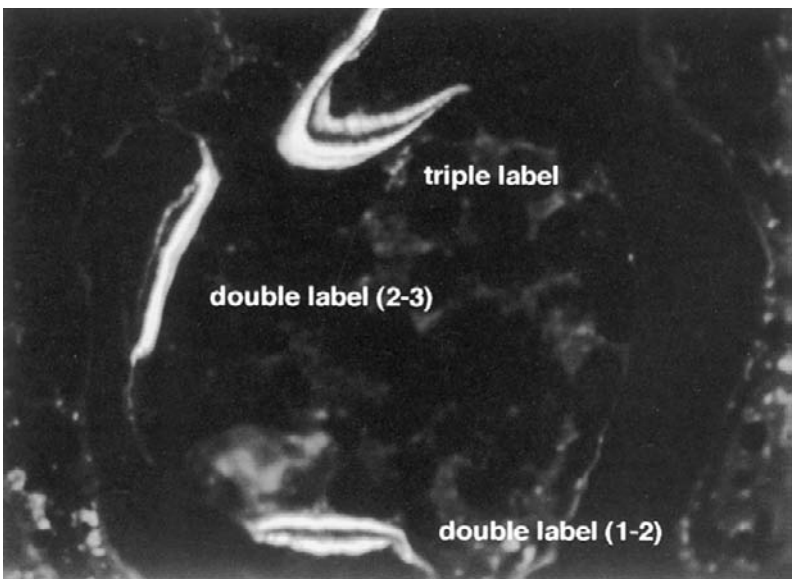

Figure 3 Photomicrograph of tetracycline labeling in an unstained section under fluorescent light. The patient was given three labels, and some BMUs show all three; others demonstrate label escape, and only the first two or last two are seen. (See also color plate.)

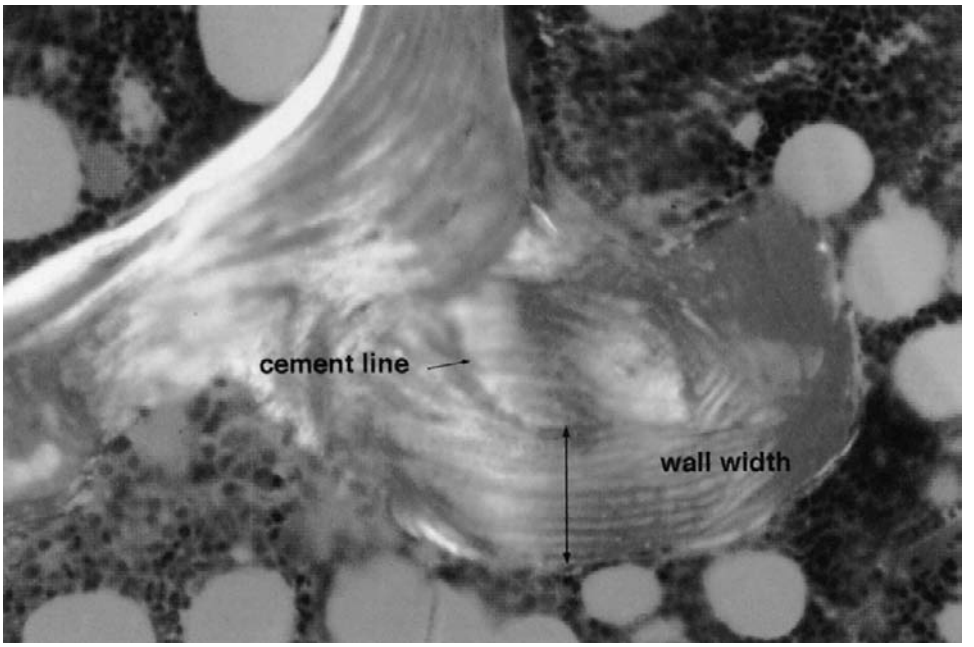

Figure 4 Photomicrograph demonstrating wall thickness; the stained section viewed under polarized light shows a change in the orientation of bone lamella. (See also color plate.)

11, estrogens, androgens, and prostaglandins. To complicate matters, factors that can originate BMUs during disease or in tissue culture are not necessarily important regulators in normal physiology. This is an exciting area of current research.

The life span of a BMU is not well defined. Cortical BMUs can wander for months, usually in a straight line. Parfitt (1994) estimates that the duration is 2–8 months. This is harder to measure in cancellous bone because the two-dimensional sections do not capture the entire serpentine course of the BMU. More research is needed to understand the life span of a BMU and what controls its journey.

Activation

The life span of individual cells in a BMU is much shorter than that of a BMU. As the BMU progresses, new cells must be recruited, continually, which is the essence of activation. Activation frequency can be calculated from two-dimensional histomorphometric measurements, and it represents the probability that remodeling activity will begin at any point on the surface (Parfitt *et al.,* 1987). It is important to distinguish between origination and activation. The former occurs only once for each BMU at a quiescent surface of the cancellous bone or on a surface nearest a crack in the cortical bone. Activation is a continuing process that occurs at the cutting edge of the BMU, and as the BMU spreads, new surfaces undergo activation. The BMU "front" travels at a rate of about 10 μm/day. In the cortical bone, the BMU progresses into solid bone so there are no lining cells to participate in activation or recruitment. The signal comes from existing BMU cells, although nearby osteocytes could play a role. Replacement osteoclasts must come from the capillaries that are formed within the BMU. By analogy, the cancellous bone may also rely on signals from BMU cells and osteoclasts may interact with the new capillaries. In addition, lining cells of osteoblastic lineage are available on the cancellous surfaces.

When exposed to hormones, lining cells change their shape from flat epithelial-like cells to rounded cells, thereby exposing some of the collagen matrix. They also secrete collagenase to expose the bone mineral. These activated cells then produce RANK ligand, which binds to receptors on preosteoclasts and causes them to fuse and become mature osteoclasts. Where do the preosteoclasts come from?

Systemic hormones, growth factors, and interleukins play a role at this step, helping to recruit new osteoclasts by enlarging the precursor pool. Some of these may also have played a role in origination of the BMU, but systemic factors cannot localize the osteoclasts to the cutting edge. As the BMU progresses, new osteoclasts are required at shifting locations. Most research on osteoclast recruitment has focused on differentiation and proliferation and not on localization to the precise site of resorption, but as Parfitt (1996) said, "the manufacture and packaging of a product is of little use if it is not delivered to the right address." This function is probably the responsibility of cells within the BMU.

Resorption

There are two phases of resorption. The first is the most rapid, carried out by multinucleated osteoclasts, and lasts ~8 days. Then comes a slower phase, involving mononuclear cells, which lasts ~34 days (Ericksen *et al.,* 1984).

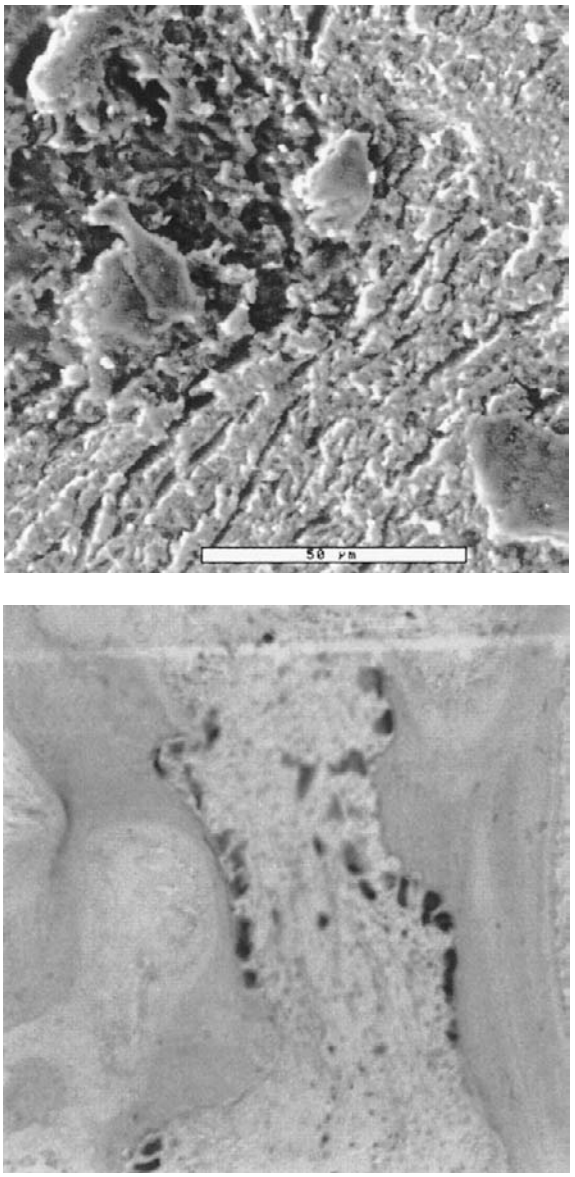

Figure 5 (Top) Environmental scanning electron micrograph of osteoclasts from the same patient shown in the bottom half of the figure. This new technique allows scanning of unstained bone. This sample was embedded in methacrylate. Photograph courtesy of Lara Touryan. (Bottom) Acid phosphatase stain of osteoclasts, which appear red, from a patient with secondary hyperparathyroidism and renal failure. (See also color plate.)

In cortical bone, two types of eroded surfaces are seen (Jaworski *et al.,* 1972). One is a cutting cone, with osteoclasts at the surface; the other is sausage-shaped, more shallow, and lacking in osteoclasts. These might represent surfaces in which resorption had been aborted (Parfitt, 1994). Croucher *et al.* (1995) have measured resorption depths in cancellous bone with analogous findings. The frequency distribution of depths shows greater numbers of shallow cavities than would be expected for a model of continuous resorption. They concluded that bone resorption is interrupted or permanently arrested.

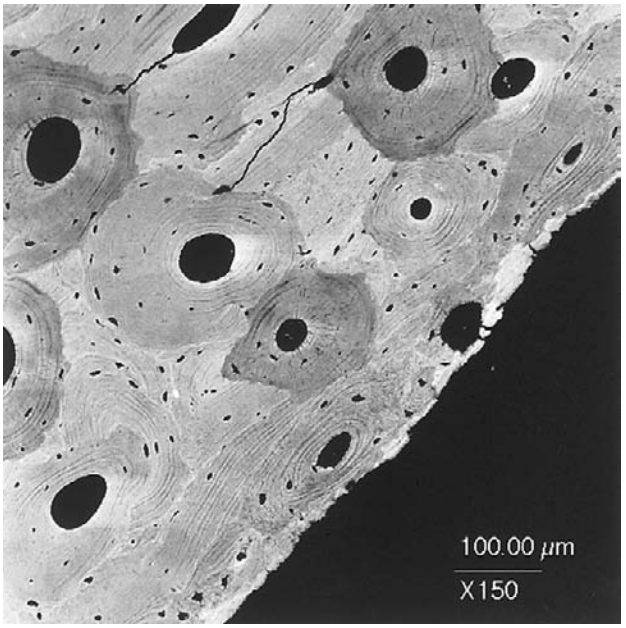

Figure 6 Back-scattered electron micrograph of bone from a 72-year-old woman showing different densities of BMUs. The crack, which occurred during preparation, is characteristically through older, interstitial bone. Courtesy of Barbara Carter.

Multinucleated osteoclasts are active for ~12 day (Parfitt *et al.,* 1996) and then undergo apoptosis. This process may be promoted by TGFβ. Cells undergoing apoptosis have been located at the junction between the resorbing surface and the reversal surface, which suggests that the process might also be involved in signalling new osteoblasts. Osteoclasts have been shown to excrete interleukin 6 and annexin-II, both of which could signal new osteoblasts. The depth of the eroded cavity is also linked to the life span of the active osteoclasts so that early apoptosis would result in a more shallow eroded cavity.

During resorption, bone-derived growth factors are released. These include transforming growth factor-β (TGFβ), insulin-like growth factor (IGF), and fibroblastic growth factor (FGF), which were deposited into the matrix by the previous generation of osteoblasts. Some, like TGFβ, may be activated by the acid environment caused by osteoclastic proton secretion. These growth factors (delayed autocrine factors) might account for the coupling between resorption and formation that is seen in normal situations. Direct evidence for this theory is lacking; possibly the proteolytic milieu in the resorption cavity inactivates these factors before they can recruit osteoblasts (Parfitt, 2000). Certain pathological conditions, such as Paget's disease and postmenopausal osteoporosis, also demonstrate coupling. High correlations between total skeletal resorption and formation could be seen if biochemical stimuli (e.g., interleukin 6) for origination of BMUs also participated in the recruitment of osteoblasts. Which mechanisms predominate in normal physiology and which are involved in pathophysiology are not yet known. Parfitt (2000) has proposed that vascular

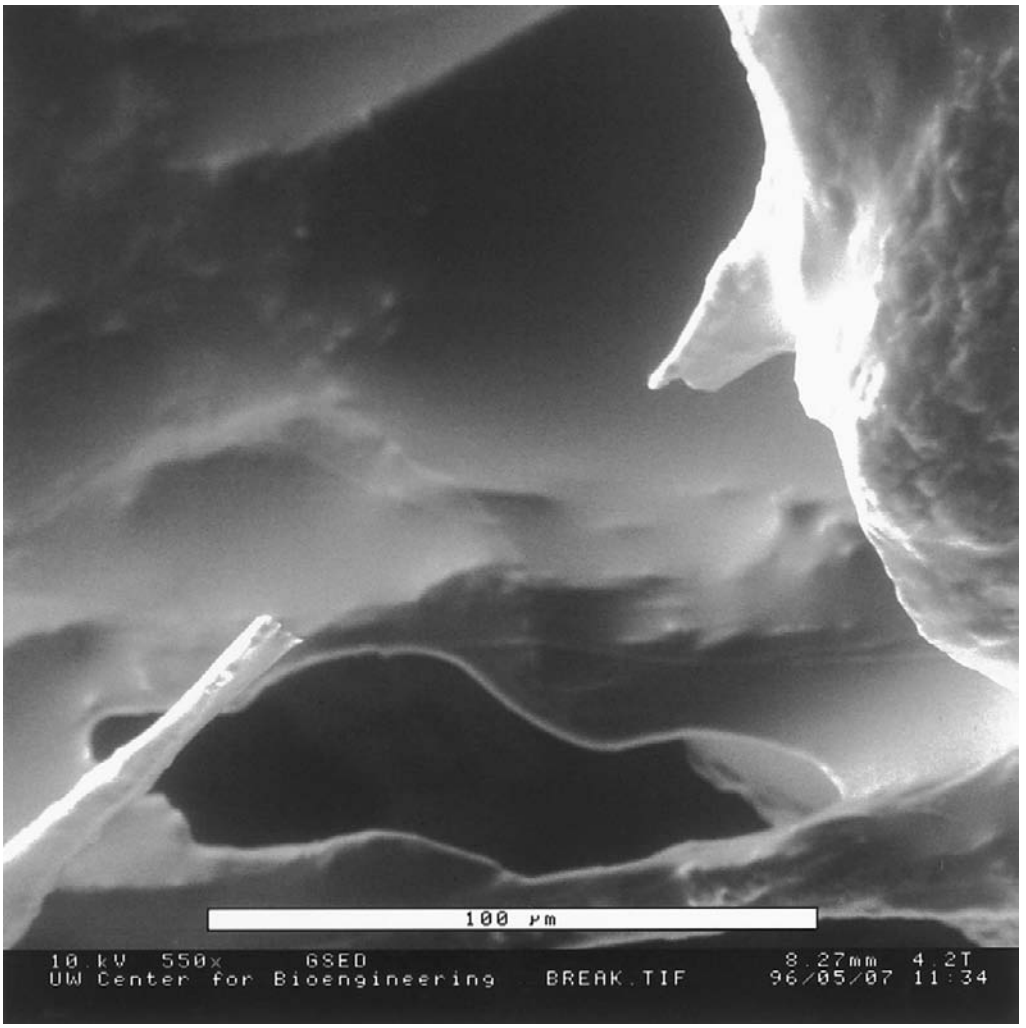

Figure 7 Environmental scanning electron micrograph of trabecula. The sample had been removed from a patient in the operating room minutes before the photograph was taken; the only preparation was rinsing with water. Broken trabecular bridging can be seen. Photograph courtesy of Lara Touryan.

endothelial cells are responsible for the coupling between bone formation and resorption (discussed later).

During resorption, collagen is digested. Some fragments can be used as biochemical markers for overall bone resorption as discussed in Chapter 90.

Formation

After the maximum eroded depth has been achieved, there is a reversal phase that lasts ~9 days (Eriksen *et al.,* 1984). During this phase, the osteoblasts converge at the bottom of the cavity. The team of osteoblasts then begins to form the osteoid. After 15 days, the osteoid begins to mineralize. The osteoblasts continue to form and to mineralize the osteoid until the cavity is filled or nearly filled. The time to fill in the cavity at any given point on the surface is 124–168 days in normal individuals (Erickson *et al.,* 1984).

The apposition rate of matrix and of mineral is most rapid initially, as determined by measurements of osteoid seams that are very close to the cement lines. The initial rate is 1.2 μm/day, which gradually decreases to zero as the cavity is filled. The delay between osteoid formation and mineralization is 15 days initially, with an increase to 27 days, then a gradual decrease (Erikson *et al.,* 1984). These rates were determined using reconstructive techniques. It is easier to measure the average mineral apposition rate and calculate the average osteoid maturation time, which is the mean time interval between the onset of matrix depostion and the onset of mineralization (Parfitt *et al.,* 1987). The average normal adult osteoid maturation time is 17–20 days (Parfitt *et al.,* 1997; Recker *et al.,* 1988; Vedi, *et al.,* 1983).

At the bottom of the cavity, the new osteoblasts are plump and vigorous, they have tall nuclei, and they make a thick layer of osteoid. The cells then gradually flatten as they slow production, and finally they become quiescent lining cells. Some of the osteoblasts differentiate into osteocytes and remain in the matrix. The osteocytes may secrete inhibitory factors that slow the rate of bone formation as the resorbed cavity is nearly filled (Martin, 2000). Adjacent osteoblasts appear to be the same age; plump and flat cells

are not intermixed. This suggests that there are no replacement osteoblasts that join a team that is already filling the resorption space. Autoradiographic studies showing osteoblastic cells in teams of the same age confirm these observations (Parfitt *et al.,* 1996). As the BMU progresses, new osteoblasts are added, but only at the edge of the formation site.

The density of the osteoblasts at the formation site may vary. When the cells are more crowded they are taller and narrower, and they collectively can make more osteoid than when there are fewer cells. Parfitt *et al.* (1995) have shown that osteoporotic patients have the same rate of osteoid production per cell, but overall the wall thickness is decreased and the amount of newly formed bone is inadequate to fill the resorbed cavity. They suggests that when there are not enough osteoblasts, they must flatten out sooner to cover the bone.

The percentage of the cancellous bone as measured in surface that is mineralizing (shows tetracycline labels), different studies, is shown in Fig. 8.

A high proportion (40–60%) of osteoid surfaces do not have associated tetracycline labels. Some osteoid that does not take tetracycline is newly formed and has not yet begun mineralization; this accounts for only ~7% of the osteoid (Ott, 1993). Two theories have been proposed to account for the unlabeled osteoid surface. One explanation is that the bone formation is not continuous; in other words, there are active "on" periods where osteoid is made and mineralized followed by inactive "off" periods where the osteoblasts rest (Frost 1980). The other theory is that tetracycline-free osteoid represents terminal osteoid, which persists at the end of formation, mineralizing too slowly to be measured. It is possible that both mechanisms are operative.

An indirect way to assess the presence of "off" periods is to calculate the label escape, which is the amount of single tetracycline label seen when two labels were given. During the interval between the two tetracycline labels, some osteoid surfaces will have finished formation and others will have started. A single first label is seen when the osteoid finished mineralization before the next label was given, and a single second label is seen when the osteoid starts after the first label was given. The proportion of double labels to single labels depends on the days in the labeling interval as well as the formation period. The shorter the interval or the longer the formation period, the higher the proportion of double labels compared to single labels (Keshawarz and Recker, 1986; Martin, 1989). Therefore, if the formation period and label interval are known, the amount of expected label escape can be calculated. When the observed measurements are different from the expected ones, it suggests that there could be off periods during the formation. Several studies in humans and animals have shown too much label escape, which supports the idea of discontinuous formation.

Other findings are more consistent with the theory of slow terminal mineralization. Keshawarz and Recker (1986)

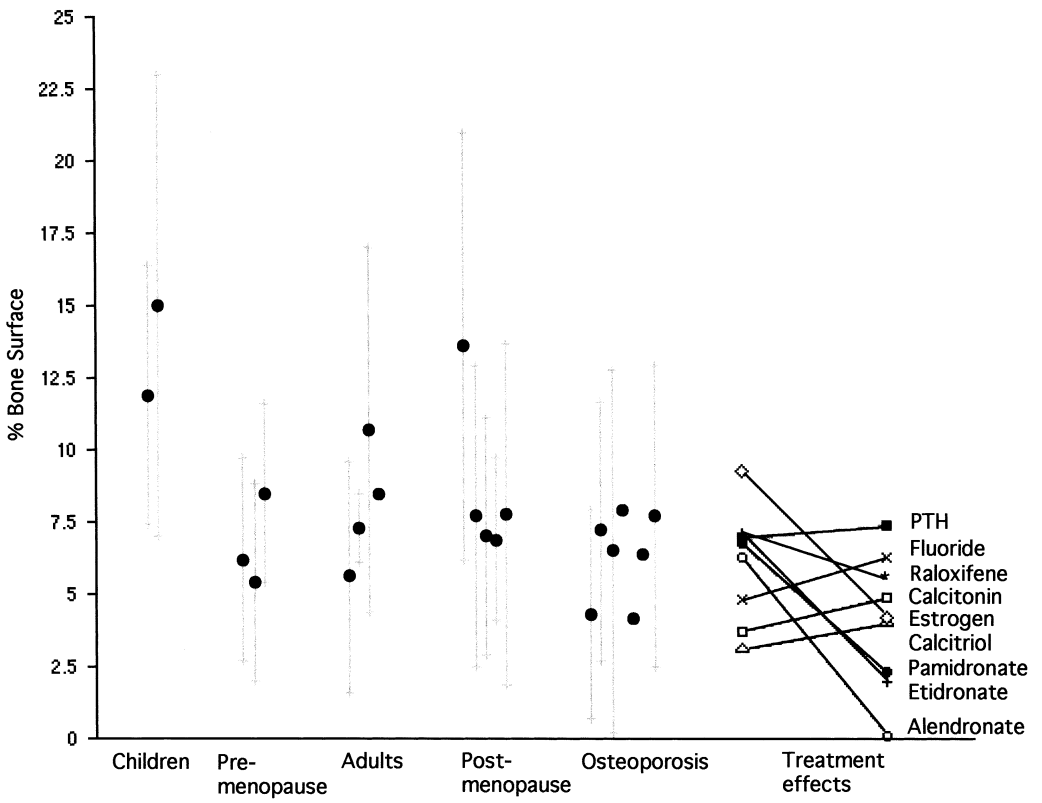

Figure 8 Mineralizing surface per bone surface in normal individuals and patients with osteoporosis. Also shown are effects of treatment in women with osteoporosis. Error bars are 1 SD. Points represent double plus 1/2 single label, and points without error bars were calculated from available data.

found less label escape in women with osteoporosis than in normal controls. Ott (1993) found that osteoporotic women had somewhat higher label escape than expected from calculations of the formation period (which involve measurements of wall thickness that may not be current). However, use of triple tetracycline labeling failed to demonstrated any discontinuations in bone formation between 3 and 35 days of observation. There were no first and third labels without a second label. Patel and colleagues (1999) administered quadruple tetracycline labels to postmenopausal women before and after commencing estrogen therapy; they did not see any BMUs with a missing label. Parfitt (1997) studied differences between ethnic groups and found that 40% of osteoid surface in blacks had labels compared to 53% in whites. The blacks appeared to have slower terminal mineralization, and the authors suggested that this could be due to lower bone blood flow. More studies in normals and patients with other diseases need to be done to clarify this issue.

During formation, osteoblasts make osteocalcin and bone-specific alkaline phosphatase, which can be used as serum biochemical markers of formation (see Chapter 90).

Mineralization

Mineralization begins ~15 days after osteoid has been formed. In most situations (except osteomalacia), the average rate of osteoid formation and the rate of mineralization are the same and are measured by tetracycline labels.

After the BMU has completely restored the bone volume, mineralization continues to increase. Older bone has more densely packed crystals, and microradiographic studies have shown that newly formed bone may be 25% less dense than older bone (Jowsey, 1960). The length of time for this increased mineralization is uncertain. If the bone turnover is decreased, then gradually the mean age of the remodeled bone will increase, and the bone will become denser. As individuals age, the interstitial bone (bone between newly remodeled osteons) becomes older and more densely mineralized (Boyde *et al.,* 1993; Reid and Boyde, 1987). The increased mineralization seen with more densely packed crystals or with change in the crystal structure itself may have several consequences. Schaffler *et al.* (1995) reported observations of microcracks, which increased exponentially with aging. The overwhelming majority of microcracks (87%) were observed within the interstitial bone. Another consequence of aging mineral is that it has less water and the minerals are less able to exchange with extracellular fluid (Parfitt, 1994).

Potential Roles of Local Environmental Factors in Bone Remodeling

Vasculature

Each BMU is associated with a capillary. In cortical bone, the capillary grows along the excavated tunnel. On trabecular surfaces, small capillaries are frequently seen adjacent to osteoblasts. It is interesting to note that ^{85}Sr kinetic studies have shown correlations between the blood flow and the work rate of osteoblasts, as well as biochemical indices of bone formation and resorption (Reeve *et al.,* 1988).

These have not been well characterized, and most investigators, if they considered the capillaries at all, thought their role was to provide nutrients and a source of precursor bone cells. Parfitt (2000) theorized that vascular endothelial cells could also provide a mechanism for the coupling of formation to resorption. These cells are stategically located to sense growth factors derived from the resorption of bone; in turn they secrete several types of growth factors that can be mitogenic for osteoblasts

Nervous system

Anatomic studies have documented a dense and intimate innervation of bone tissue, but the function of the nervous system is not clear. Serre and colleagues (1999) have demonstrated the presence of fibers that run along vessels adjacent to bone trabeculae.

Immunocytochemical studies showed that the fibers contained three different markers for neural tissue and that some were sensory fibers and other sympathetic fibers. Nerve endings were seen in contact with bone cells. Glutamate was expressed in fibers that were in proximity to bone cells, suggesting a potential role of glutamatergic innervation in the bone remodeling process.

Evidence has shown a role for the central nervous system in the control of bone formation rates. Leptin injected into brains of mice causes a substantial decrease in bone formation rates at doses that do not affect the body weight. The leptin does not have this effect when administrered intravascularly. The mechanism of this effect remains unknown (Ducy *et al.,* 2000).

Bone Marrow Cells

Bone stromal cells secrete a variety of cytokines that can stimulate the proliferation of osteoblasts and osteoclasts. These are discussed in detail in other chapters of this text. Bone remodeling is higher in areas with more red marrow, possibly because these areas have more cytokine and interleukin activity.

Adipocytes

Adipocytes and osteoblasts derive from the same precursors, which are multipotential stromal cells. Studies have suggested that oxidized lipids promote the adipogenic differentiation of these precursors (Parhami *et al.,* 2000). Histologic studies have shown that the area occupied by fat cells increases as bone volume decreases (Meunier *et al.,* 1971). *In vitro* experiments have shown that mature adipocytes inhibit osteoblast proliferation (Maurin *et al.,* 2000).

How Remodeling Affects the Three-Dimensional Structure of Bone

Conversion of Plates to Rods

Young healthy cancellous bone forms a well-connected lattice, with plate-shaped trabeculae. Remodeling does not necessarily change this overall structure, but when resorption exceeds formation, the bone loss leads to thinning of the trabecular plates and/or to perforations (Fig. 9). For the same amount of bone loss, the mechanical strength of the bone is impaired to a greater extent if the plates are perforated.

Plates can perforate if the resorption depth of one BMU is deeper than the thickness of the plate or if two BMUs on opposite sides of the plate resorb bone at the same time. In any case, once the bone has perforated, further bone loss will be amplified (Fig. 10) because there is no more scaffolding to support a team of osteoblasts. Instead of a cement line, there is empty marrow space. By this process, the plate-shaped trabecular bone is converted into a rod-shaped structure with loss of surface available for formation (Parfitt *et al.,* 1983).

Once the plates have become rods, they may become disconnected. This has been demonstrated by scanning electon micrographs (Dempster, 2000) and by examination of thick, superficially stained bone sections (Aaron *et al.,* 2000). The isolated trabecular rods have no mechanical stimuli and are severed from the osteocyte network and are resorbed rapidly (Mosekilde, 1993).

Several kinds of measurements from bone biopsies can estimate the degree of connectivity, the thickness of trabeculae, the volume of the marrow spaces, or the extent of perforated or isolated rods (Croucher *et al.,* 1996). Patients with osteoporotic fractures have more structural abnormalities than those without fractures, even when the total bone volume is taken into account. Bone biopsies from patients with similar bone mass (Kleerekoper *et al.,* 1985) show that patients with fractures have fewer trabecula but greater trabecular width than those without fracture. In subjects with and without fractures, Recker (1993) matched bone biopsies according to bone volumes: those from women with fracture had poor measures of connectivity. Legrand *et al.* (2000) found increased values of interconnectivity index, free end to free end stuts, and trabecular spacing were greater in men with fractures than in men without fractures after adjusting for age and bone density. Oleksik *et al.* (2000) found that structural measurements indicating disruption of the trabecular lattice were different in postmenopausal osteoporotic women with fractures than in those without fractures after adjustment for bone mass.

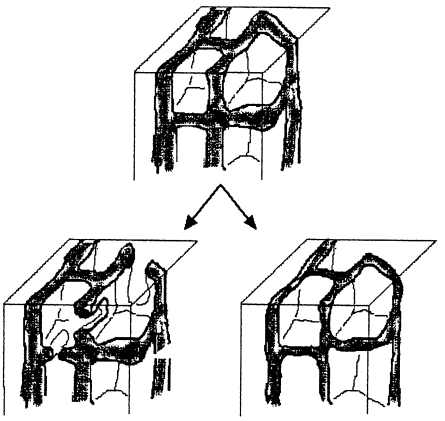

Figure 9 Diagram depicting two possible methods of losing bone with aging. Trabeculae could thin without loss of structure or could be perforated with structural damage.

Measurements from usual bone sections are two dimensional and may not reflect the three-dimensional reality of the bone accurately. A group of postmenopausal women with or without vertebral fractures was matched for trabecular bone mass. The two-dimensional measurements of bone histology and trabecular width, including plate density, star volume, and node:terminus ratio, were not significantly different between those with and without a fracture (Hordon *et al.,* 2000).

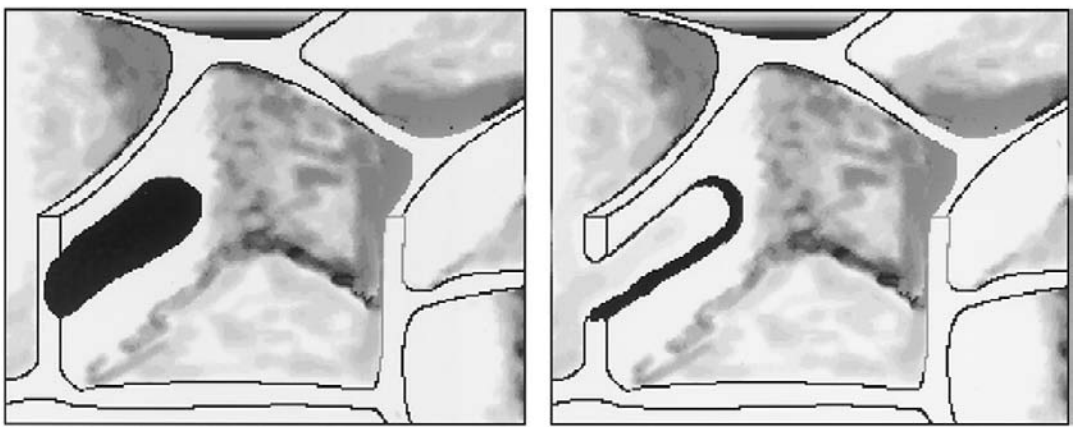

Figure 10 Diagram of loss of surface space by deep resorption. (Left) Cancellous surfaces just after resorption; available surface for formation of new bone is shown in black. (Right) If resorption is slightly deeper, the plate will be perforated, and the only available surface is around the edge of the hole. The plate is being converted to a rod.

Three-dimensional analysis, however, showed that the fracture group had almost four times as many "real" trabecular termini as the group without fractures (Aaron *et al.,* 2000).

Cortical versus Cancellous Bone

The overall surface of cancellous bone is much higher than cortical bone. In the entire skeleton, however, ~80% of bone is composed of cortical bone and only 20% is cancellous bone. The surface/volume relationships are much greater in the cancellous bone. Thus it is metabolically more active. This is the usual explanation for differences between remodeling activity in the two types of bone, but other important differences are also seen. Cortical bone and cancellous bone share many features of remodeling, particularly in the sequence of origination, activation, resorption, formation, and mineralization. Whereas BMUs in the cancellous bone lie along the surface, those in the cortex burrow through the bone. Intracortical BMUs form a tunnel, but cancellous BMUs either form a trench (half-tunnel) or spread out over an area (Hahn *et al.,* 1995; Jayasinghe *et al.,* 1993; Parfitt, 1994) (Fig. 11). Thus, intracortical bone remodeling could not possibly result in increased bone volume, whereas remodeling on exterior or cancellous surfaces could potentially increase bone volume.

Endocortical bone is a special surface that may react differently than either cortical or cancellous. In postmenopausal osteoporosis, for example, this surface shows more formation (Arlot *et al.,* 1990; Brown *et al.,* 1987; Parfitt *et al.,* 1996) and resorption (Keshawarz and Recker, 1984) than other areas, with a net result of "trabecularization" of the interior surface of the cortical bone.

The cortical bone of the femoral neck has been studied using sections that contain the entire cross-sectional area in a group of patients who fractured the femoral neck. Compared to cadaveric control samples, cortical bone had greater porosity in the inferoanterior and superoposterior regions, there were more giant Haversian canals in those regions, and those canals were more likely to be composite osteons. Although most osteons had remodeling measurements that were similar in controls and fracture cases, the giant, composite osteons had significantly lower wall thickness. The authors suggest that in some cortical BMUs, there is failure to recruit osteoblasts or that osteoblasts cease bone deposition prematurely (Bell *et al.,* 1999, 2000).

Cancellous bone can also form microcalluses, for which there is no room in cortical bone. However, the periosteal surface of cortical bone can add woven bone in response to mechanical loading; this is not observed in cancellous bone. Another difference is that cancellous bone BMUs proceed along a surface lined with lining cells, which could participate in remodeling, whereas there are no lining bone cells within the cortex. Cancellous bone has also a more ready access to marrow cells than cortical bone.

Sometimes in cancellous bone a trabecula can become isolated, and the lack of continuity will remove both the ability to sense mechanical forces and the structural function of the segment of bone. These fragments are then prey to rapid resorption and virtually disappear, enhancing loss of bone mass. This does not occur in cortical bone.

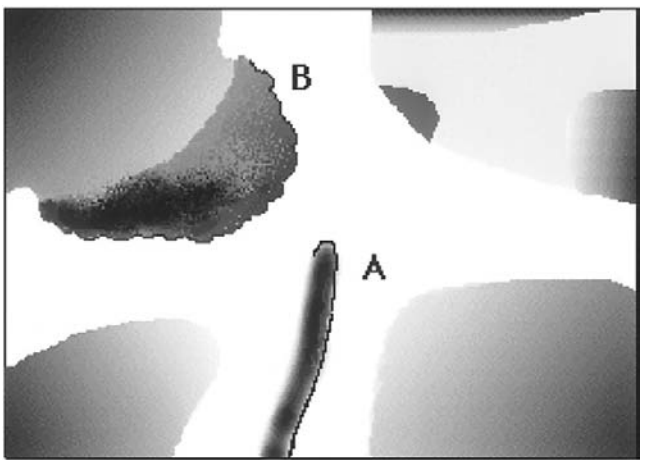

Figure 11 Remodeling on cancellous surfaces showing resorption in the trench (A) or along the surface (B).

Remodeling in Osteoporosis

The Trouble with "Turnover"

Frequently, metabolic bone diseases are described using the term "turnover." The increased use of biochemical markers has resulted in an even greater use of the term, which is often ambiguous or misleading. Turnover is an appropriate description only when both bone formation and bone resorption rates are similar. Thus, "high turnover" is seen in hyperparathyroidism and acute estrogen deficiency; "low turnover" is seen in hypoparathyroidism. In growing children, both bone formation and resorption rates are high, but the bone is not really turning over, it is modeling. In many conditions, the formation rate is different from the resorption rate. For example, in corticosteroid-induced osteoporosis, early pregnancy, or space travel, the bone formation rate is decreased while the bone resorption rate is increased. These situations have been called "low turnover" based on the markers of bone formation; others have called them "high turnover" based on the bone resorption markers. Furthermore, "high turnover" is used frequently as a synonymn for "negative bone balance." This is not always true; e.g., intermittent injections of PTH increase bone formation and resorption but result in a net gain of bone volume.

Biochemical markers in serum and urine correlate to tetracycline-measured bone formation rates and to calcium kineteics determined using radioactive calcium isotopes. These methods show correlations with each other, but the slopes of the regression lines between accretion and biochemical markers change with age (Eastell *et al.,* 1988). The markers also do not always reflect the changes in the bone formation rates with therapy. For example, after treatment with

alendronate, biochemical markers decrease by about 50%, but the tetracycline-based measurements show decreases of 95 to 98% (Chavassieux *et al.,* 1997).

Changes Seen in Osteoporosis

Postmenopausal osteoporosis is a heterogeneous disorder, and several kinds of bone remodeling abnormalities have been described. Menopause itself is associated with an increase in the origination of BMUs, possibly because estrogen deficiency results in increases of interleukin 6 and other cytokines, which are potent factors involved in the proliferation of both osteoclasts and osteoblasts (Manolagas and Jilka, 1995). This alone would not necessarily cause loss of bone mass, but each BMU does not completely replace as much bone as was resorbed. Therefore, if there are more BMUs, there will be greater bone loss. In the cortical bone, more BMUs will result in more cement lines, which can lead to decreased strength of the bone because cement lines are weaker than other bone.

In addition, there may be abnormalities in the BMUs themselves. Menopause causes an increased depth of resorption, perhaps because of a longer osteoclast life span (Eriksen *et al.,* 1999) or decreased osteoclast apoptosis (Kameda *et al.,* 1997) These changes can result in loss of structural integrity as discussed earlier.

With aging, osteoblasts lose their ability to fill the resorbed spaces. This is shown by age-related decreases in the wall thickness (Arlot *et al.,* 1990; Lips and Meunier, 1978; Parfitt *et al.,* 1995). This results in a gradual loss of bone volume and senile osteoporosis. Parfitt *et al.* (1995) have shown that women with postmenopausal osteoporosis have a lower ratio of osteoblastic surface to osteoid surface than normal postmenopausal women. They also have a lower mineral apposition rate (Arlot *et al.,* 1990). These findings can be explained by insufficient numbers of osteoblasts. In addition, the findings suggested that women with osteoporosis had fewer active BMUs than normal postmenopausal women. Scanning electron micrographs of patients with osteoporosis show a high percentage of surface with unmineralized matrix (Jayasinghe *et al.,* 1993), suggesting that formation at these sites is incomplete. This may account for the findings of a low percentage of osteoid that has tetracycline labels (Ott, 1993).

When bone porosity increases, the remaining bone accumulates microdamage at an exponential rate, whereas osteocyte lacunar density decreases (Vashishth *et al.,* 2000). A viscious cycle is begun; bone mass decreases, so the remaining bone is subject to more fatigue damage, which increases bone resorption, which may further weaken the bone and disrupt the osteocyte network.

Corticosteroid-Induced Osteoporosis

The worst case scenario is when increased resorption is combined with decreased formation, which is seen with high doses of corticosteroids. The resorption surfaces are increased and wall thickness is decreased (Dempster, 1989; Dempster *et al.,* 1983). The effect is more prominent in cancellous bone. In addition to an imbalance at each BMU, steroids increase activation frequency, which further enhances bone loss.

Corticosteroids also increase apoptosis of osteocytes. In femoral heads of patients with osteonecrosis of the hip, Weinstein *et al.* (2000) demonstrated many apoptotic cells; thus, the bone was not really necrotic, but was suffering the consequences of apoptosis and the disruption of the osteocyte network. Apoptotic osteoblasts were also seen in rats treated with high doses of glucocorticoids (Silvestrini *et al.,* 2000).

Consequences of Pharmacological Agents That Affect Remodeling

Understanding bone remodeling is important for prediction of the response to therapeutic agents used in osteoporosis. The effects on bone formation, as measured by tetracycline-labeled surfaces on bone biopsies, are shown in Fig. 8. Currently approved therapies (estrogen, bisphosphonates, risedronate, calcitonin) all decrease the bone formation. Most of these drugs inhibit BMU origination or activation, but because they increase bone mass, many physicians believe they promote bone formation. However, antiresorptive drugs eventually inhibit bone formation, although this is not necessarily a direct effect. Bone volume will increase as long as the formation period if the drug blocked activation or as long as the BMU life span if the drug blocked only origination (Fig. 12). Thus, some increase in bone volume could occur for 8 months or even longer if there were inactive formation phases that resumed activity. Eventually a new steady-state bone volume is reached. The total amount of gain in bone mass depends on the remodeling rate, and once the resorption cavities are filled, there can be no further gain in bone volume. The plateau reached could be termed the "remodeling barrier." Any further increases in bone volume would have to occur by nonremodeling mechanisms or by agents that had direct anabolic effects on the osteoblasts.

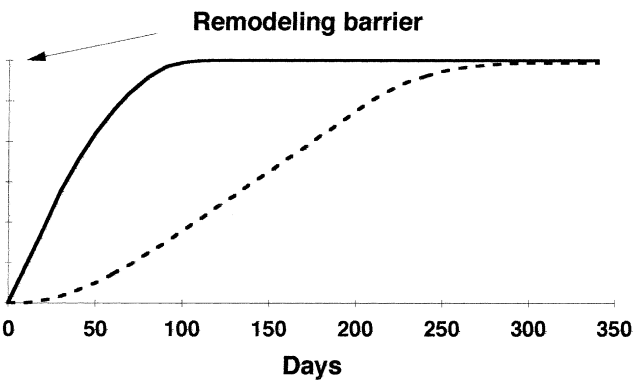

Figure 12 Plot from a computer model of bone loss following a sudden block in origination (dashed line) or activation (solid line), assuming no direct effect on formation.

Bone *density* will increase even after bone *volume* has reached a steady state, as the newly formed bone becomes more mineralized. The duration of mineralization is not precisely known. In a report of 7 years continuous treatment with alendronate, hip bone density measured by dual-energy X-ray absorptiometry rose rapidly during the first 6 months (corresponding to increases in bone volume from refilling resorption cavities), rose more gradually until 36 months (corresponding to increasing mineralization), and did not change significantly thereafter. This suggests that mineralization reaches maximal levels after 3 years (Tonino *et al.,* 2000). At the spine, bone density showed slight increases even during the sixth and seventh years, but arthritic changes and soft tissue calcifications may falsely increase the spine measurements.

Bisphosphonates have been reported to affect various steps of the remodeling process (Parfitt *et al.,* 1996). They could affect lining cells, inhibit IL-6 production, affect osteoclast action directly, or promote early apoposis. Aminobisphosphonates inhibit an enzyme in the cholesterol synthesis pathway resulting in low levels of geranylgeranyl diphosphate. This results in inhibition of rho21, a GTP-binding protein, which results in an inability of osteoclasts to form ruffled borders. Thus, osteoclastic resorption is directly inhibited. With no prior resorption to signal osteoblasts, there is a secondary inhibition of bone formation. The histology of human subjects treated with these agents documents very low tetracycline-labeled bone formation rates and decreased activation frequency (Ott *et al.,* 1994)(Chavassieux *et al.,* 1997). Backscattered electron images demonstrate higher mineralization levels with alendronate treatment (Roschger *et al.,* 1997). Some studies have found increased wall thickness, leading to speculation that bisphosphonates can increase bone formation in the few remaining BMUs (Balena *et al.,* 1993). It is not clear, however, that walls with increased thickness represent those formed at the time of measurement.

With inhibition of origination or of activation, the initial gain in bone volume will increase bone strength, which appears to be the predominant effect of many pharmacological agents. Furthermore, the decrease in bone resorption will prevent some of the architectural deterioration that would have occurred without therapy. Clinical studies of up to 3 years duration in women with established osteoporosis have shown significant reductions in fracture rates in those treated with bisphosphonates compared to those treated with placebo (Black *et al.,* 1996; McClung *et al.,* 2001.)

Newer bisphosphonates are deposited in bone with a half-life of greater than 10 years (Gertz *et al.,* 1993) so the drugs will accumulate with continuing use. There has been concern that long-term 95% suppression of bone formation could result in adverse effects on bone strength due to a failure to repair microdamage or to increased brittleness from hypermineralized bone (Boyce and Bloemaum, 1993). Beagles were treated with high doses of risedronate or alendronate for a year, resulting in suppressed bone formation rates and increased microdamage accumulation. Biomechanical studies showed no significant effect on bone strength, but there was reduced toughness of the ribs (Mashiba *et al.,* 2000). It is not yet clear if prolonged use of usual doses of these drugs will reduce bone strength. A 7-year study of continuous alendronate has been reported (Tonino *et al.,* 2000), but the fracture rates during years 6 and 7 were not reported using the same criteria as during the first 3 years. Investigations on the long-term safety of potent inhibitors of bone formation are needed, especially since they are prescribed to women in their 50's for the prevention of osteoporotic fractures, which are not expected to occur for at least 15 years.

Estrogen also decreases activation frequency, which is consistent with the theory that estrogen prevents increased IL-6 production. The decrease in bone formation rate is not as great as with aminobisphosphonates. (Steiniche *et al.,* 1989). Raloxifene also decreases bone formation, similar to estrogen (Ott *et al.,* 2000).

Calcitonin acts directly on osteoclasts, and bone biopsies from patients treated with the drug show no effects on bone formation rate or activation frequencies (Thamsborg *et al.,* 1996).

Parathyroid hormone (1-34) has been investigated in clinical trials but is not available for clinical use. Daily injections result in large increases of bone density. Bone histomorphometric studies document increased bone resorption and bone formation rates. The bone volume does not increase, and trabecular architecture does not show reconnection. Wall thickness is increased, suggesting an anabolic effect. Of interest, increases in the bone formation rate are largely due to increases in the mineral apposition rate, with only modest increases in the percentage of surface undergoing mineralization. (Hodsman *et al.,* 2000).

Fluoride treatment appears to bypass the remodelling system, causing markedly enhanced bone formation without previous bone resorption (Balena *et al.,* 1998). This is discussed further in the next section.

Other Mechanisms of Altering Bone Structure

Microcallus Formation

Hahn *et al.* (1995) carefully examined sections of human spine from normal men and women and from patients with osteoporosis. They observed that microcallus formations in spine sections increase after age 50. There are more in females than in males and even more in osteoporotic persons. These are mostly seen in the lower thoracic and lumbar spine near the end plates. They are found in only 1.4% of iliac crest biopsies. In those with osteoporosis, the number of microcallus formations correlates with the trabecular bone pattern factor, which measures intertrabecular connection ($r = 0.75$) and trabecular thickness ($r = 0.45$) but not bone volume or age.

Microcallus formations can allow formation of new trabeculae by forming bridges between existing trabecula. These formations can account for up to 10% of the trabecular bone volume. They undergo resorption and modeling,

eventually becoming mineralized and indistinguishable from trabecular bone. Hansson and Roos (1981) also described microcallus formation in the spine, which increased as bone mineral content decreased. This mechanism of altering trabecular structure could be relatively important in patients with osteoporosis.

Back-scattered electron images also show microcallus formation. These are less well mineralized than normal bone, and a gap remains between the microcallus and the original trabecular bone (Boyce, 1993)

Direct Activation

Bone formation also can, in some situations, take place along surfaces in the absence of previous resorption. With fluoride therapy, bone formation surfaces increase markedly, but the bone is woven and not normal lamellar bone (Kleerekoper and Balena, 1991). After 5 years of fluoride, there is little residual woven bone, but mineralization defects are seen despite calcium and vitamin D treatment; the wall thickness is greater than the resorption depth, and the formation period is prolonged (Eriksen *et al.,* 1985). Beagles treated with aluminum show new bone formation in some, but not all, experimental conditions (Galceran *et al.,* 1987; Quarles *et al.,* 1988), but this phenomenon is not seen in humans, who develop osteomalacia when exposed to parenteral aluminum (Ott *et al.,* 1983).

Bone Arising from Marrow Spaces

Metastatic prostate cancer causes several types of bone lesions. Unlike most metastatic lesions, which are osteolytic, prostate cancer can form blastic lesions. In some areas that have prostate cancer cells in the marrow, spindle-shaped cells in the marrow spaces are adjacent to extracellular tissue, which shows early mineralization. In other areas, woven bone is found inside the marrow spaces, suggesting progression. Osteosclerotic lesions appear to be the end result of this process (Roudier *et al.,* 2000).

Woven versus Remodeled Bone

Woven bone, seen in response to injury, may be an important adaptation, which may involve positive feedback loops with osteoblasts stimulating further osteoblast action (Turner, 1992). Severe repetitive stress on cortical bone can lead to rapid increases in bone formation that cannot be accounted for by the remodeling process. Animal studies of increased load frequency show that the periosteal surface makes new woven bone (Burr *et al.,* 1989; Lanyon, 1989). If the loads are placed so that the endosteal surface is not bent, then there will be periosteal new woven bone without a change in endosteal surfaces (Turner and Forsood, 1995). Studies in military recruits have documented a 7.5% increase in tibial bone mineral density after only 4 months. This was probably also woven bone on the periosteum because the tibial cross-sectional area also increases. Of interest, these same recruits showed a decreased vertebral bone mass, which suggests that the trabecular bone was subject to increased origination and developed an excessive resorption space. After 2 years the vertebral bone density was slightly (nonsignificantly) higher than at baseline (Casez *et al.,* 1995).

The periosteal surfaces of cortical bone can form without previous resorption, and this process may occur throughout life. Boyce *et al.* (1993) observed a zone of hypermineralized bone on the periosteal surface of femoral bone from aged humans. This zone does not have osteonal remodeling, which suggests that it was woven bone that had become highly mineralized. Because this phenomenon was seen only in older subjects who also had decreased cortical thickness, one can speculate that the weakened osteoporotic bone results in excess mechanical stress on these cortical surfaces. Perhaps aged bone can no longer rely on remodeling to increase bone mass and must resort to a secondary method of forming woven bone in calluses and on the periosteum.

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CHAPTER 20

Phosphorus Homeostasis and Related Disorders

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Phosphorus plays an important role in cellular physiology and skeletal mineralization, serving as a constituent of nucleic acids and hydroxyapatite, a source of the high-energy phosphate in adenosine triphosphate, an essential element of the phospholipids in cell membranes, and a factor influencing a variety of enzymatic reactions (e.g., glycolysis) and protein functions (e.g., the oxygen-carrying capacity of hemoglobin by regulation of 2,3-diphosphoglycerate synthesis). Indeed, phosphorus is one of the most abundant components of all tissues, and disturbances in phosphate homeostasis can affect almost any organ system. Most phosphorus within the body is in bone (600–700 g), while the remainder is largely distributed in soft tissue (100–200 g). As a consequence, less than 1% of the total is in extracellular fluids. The plasma contains about 12 mg/dl of phosphorus, of which approximately 8 mg is organic and contained in phospholipids, a trace is an anion of pyrophosphoric acid, and the remainder is inorganic phosphate (P_i) (Yanagawa *et al.,* 1994). Inorganic phosphate is present in the circulation as monohyrogen phosphate, which is divalent, and dihydrogen phosphate, which is monovalent. At normal pH, the relative concentrations of monohydrogen and dihydrogen phosphate are 4:1.

The critical role that phosphorus plays in cell physiology has resulted in the development of elaborate mechanisms designed to maintain phosphate balance. These adaptive changes are manifest by a constellation of measurable responses, the severity of which is modified by the difference between metabolic P_i need and exogenous P_i supply. Such regulation maintains plasma and extracellular fluid phosphorus within a relatively narrow range and depends primarily on gastrointestinal absorption and renal excretion as mechanisms to affect homeostasis. Although investigators have recognized a variety of hormones that influence these various processes, in concert with associated changes in other metabolic pathways, the sensory system, the messenger, and the mechanisms underlying discriminant regulation of P_i balance remain incompletely understood.

While long-term changes in phosphate balance depend on these variables, short-term changes in phosphate concentrations can occur due to redistribution of phosphate between the extracellular fluid and either bone or cell constituents. Such redistribution results secondary to various mechanisms, including elevated levels of insulin and/or glucose; increased concentrations of circulating catecholamines; respiratory alkalosis; enhanced cell production or anabolism; and rapid bone remineralization. In many of these circumstances, hypophosphatemia manifests in the absence of phosphorus depletion or deprivation.

Regulation of Phosphate Homeostasis

Phosphate is sufficiently abundant in natural foods that phosphate deficiency is unlikely to develop except under conditions of extreme starvation, as a consequence of administration of phosphate binders, or secondary to renal phosphate wasting. Indeed, the major proportion of ingested phosphate is absorbed in the small intestine, and hormonal regulation of this process plays only a minor role in normal phosphate homeostasis. In contrast, absorbed phosphate, in response to complex regulatory mechanisms, is eliminated by the kidney, incorporated into organic forms in proliferating cells, or deposited as a component of bone mineral (hydroxyapatite). The vast majority of the absorbed phosphate, however, is excreted in the urine. Thus, under

usual conditions, phosphate homeostasis depends for the most part on the renal mechanisms that regulate tubular phosphate transport. Alternatively, during times of severe phosphate deprivation, the phosphate contained in bone mineral provides a source of phosphate for the metabolic needs of the organism. The specific role that the intestine and kidney play in this complex process is discussed below.

Gastrointestinal Absorption of Phosphorus

The average dietary phosphate intake in humans, derived largely from dairy products, meat, and cereals, is 800 to 1600 mg/day, one and one-half to threefold greater than the estimated minimum requirement. This phosphate is in both organic and inorganic forms, but the organic forms, except for phytates, are degraded in the intestinal lumen to inorganic phosphate, which is the form absorbed. Absorption occurs throughout the small intestine with transport greatest in the jejunum and ileum and less in the duodenum. Essentially no absorption occurs in the colon (Walling, 1977).

In normal subjects, net P absorption is a linear function of dietary P intake. Indeed, for a dietary P range of 4 to 30 mg/kg/day, the net P absorption averages 60 to 65% of the intake (Lee *et al.,* 1986). Intestinal P absorption occurs via two routes: a cellularly mediated active transport mechanism and diffusional flux, largely through a paracellular shunt pathway (Cross *et al.,* 1990). Active P transport requires entry of P across the luminal membrane of the intestinal cells, a process mediated by a vitamin D- and Na^+-dependent mechanism. The effects of $1,25(OH)_2D$ on this process are modulated by the calcitriol-induced transcription of messenger RNA. In this regard, much work has variably identified several vitamin D responsive Na^+-dependent phosphate cotransporters in intestinal brush border membranes, which have a high affinity for P binding (Debiec and Lorenc, 1988; Katai *et al.,* 1999; Hilfiker *et al.,* 1998; Bai *et al.,* 2000). Phosphate incorporated into intestinal cells by this mechanism is ferried from the apical pole to the basolateral pole likely through restricted channels such as microtubules. At the basolateral membrane, phosphate is released from intestinal cells by a passive mechanism, which is carrier mediated and occurs in accord with the electrochemical gradient. Although such active transport systems are responsive to 25(OH)D and $1,25(OH)_2D$ (Lee *et al.,* 1986; Rizzoli *et al.,* 1977), these hormones and systems play a relatively minor role in normal phosphate homeostasis. Indeed, during vitamin D deficiency, the percentage of P absorbed from the diet is reduced by only 15%. Moreover, a substantial portion of this decline is secondary to the failure to absorb calcium, which results from vitamin D deficiency and the resultant formation of calcium phosphate that reduces the free phosphate concentration.

The vast majority of phosphate absorption occurs via the process of diffusional absorption. This results as a consequence of the relatively low K_m of the active transport process (2 m*M*) and the luminal P content during feeding, which generally exceeds 5 m*M* throughout the intestine and the occurrence of net diffusional absorption of P whenever luminal P concentration exceeds 1.8 m*M* (a concentration generally exceeded even when fasting) (Karr and Abbott, 1935; Walton and Gray, 1979; Wilkinson, 1976). Given these conditions, the active component of transport becomes important only under unusual circumstances, such as when dietary P is extremely low. Under these conditions, studies suggest that an activator protein for sodium-dependent phosphate transport (P_iUS) and the type III Na/P_i cotransporter P_iT-2 may be important components in the regulation of the intestinal phosphate transport system (Katai *et al.,* 1999). Regardless, the bulk of intestinal P absorption is mediated by a diffusional process, presumably through the paracellular space, and therefore is primarily a function of P intake. Because most diets contain an abundance of P, the quantity of phosphate absorbed always exceeds the need both under normal circumstances and disease states such as uremia. Thus, active, transcellular P absorption becomes predominant only under conditions of low luminal P availability, such as dietary P deprivation and/or excessive luminal P binding (Lee *et al.,* 1979; Kurnik and Hruska, 1984). Factors that may influence the diffusional process adversely are the formation of nonabsorbable calcium, aluminum, or magnesium phosphate salts in the intestine and age, which reduces P absorption by as much as 50%.

Renal Excretion of Phosphorus

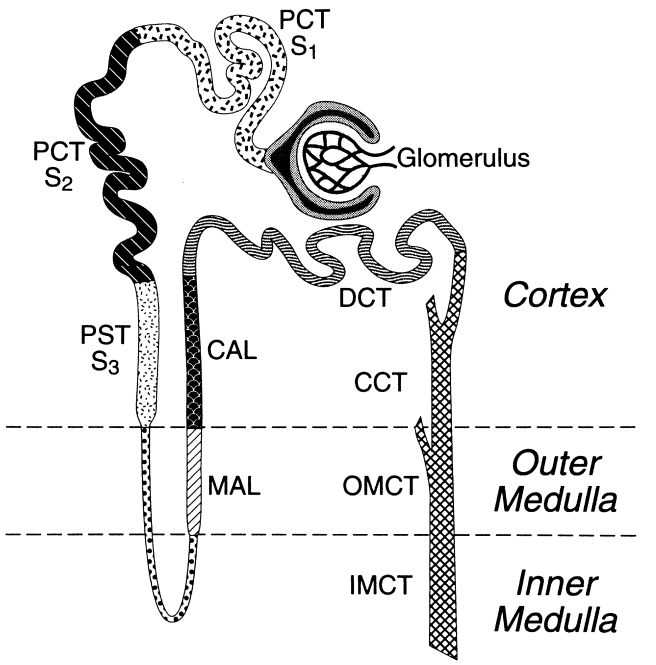
The kidney is immediately responsive to changes in serum levels or dietary intake of phosphate. Renal adaptation is determined by the balance between the rates of glomerular filtration and tubular reabsorption (Mizgala and Quamme, 1985).

The concentration of phosphate in the glomerular ultrafiltrate is approximately 90% of that in plasma because not all of the phosphate in plasma is ultrafilterable (Harris *et al.,* 1977). Nondiffusable phosphorus includes plasma P that is protein bound and a small fraction of plasma P that complexes with calcium and magnesium. With increasing serum calcium levels, the calcium–phosphate–protein colloid complex increases, reducing the ultrafilterable plasma P to as little as 75% (Rasmussen and Tenenhouse, 1995). Because the product of the serum phosphorus concentration and the glomerular filtration rate (GFR) approximates the filtered load of phosphate, a change in the GFR may influence phosphate homeostasis if uncompensated by commensurate changes in tubular reabsorption.

Normally, 80 to 90% of the filtered phosphate load is reabsorbed, primarily in proximal tubules, with higher rates at early segments (S_1/S_2 vs S_3) and in deep nephrons (Agus, 1983; Cheng and Jacktor, 1981; Dousa and Kempson, 1982; Suki and Rouse, 1996). The transcellular transport of phosphate is a carrier-mediated, saturable process limited by a transfer maximum or T_{max}. The T_{max} varies considerably as dietary phosphorus changes, and the best method to

approximate this variable is to measure maximum phosphate reabsorption per unit volume of glomerular filtrate (T_mP/GFR) during acute phosphate infusions. Alternatively, the nomogram developed by Bijvoet allows estimation of the T_mP/GFR with measurement of phosphate and creatinine excretion and plasma phosphate concentration (Walton and Bijvoet, 1975).

The major site of phosphate reabsorption is the proximal convoluted tubule, at which 60 to 70% of reabsorption occurs (Fig. 1). Along the proximal convoluted tubule the transport is heterogeneous. In the most proximal portions, the S_1 segment, phosphate reabsorption exceeds that of sodium and water, whereas, more distally, phosphate reabsorption parallels that of fluid and sodium. Additional reabsorption in the proximal straight tubule accounts for 15–20% of phosphate reclamation. In contrast, there is little evidence to suggest net P transport in the thin and thick ascending loops of Henle. However, increasing, but not conclusive, data support the existence of a P reabsorptive mechanism in the distal tubule. Currently, however, definitive proof for tubular secretion of phosphate in humans is lacking (Knox and Haramati, 1981).



	Phosphate Reabsorption	PTH Sensitive Adenylate Cyclase	PTH Inhibited Phosphate Reabsorption	Calcitonin Sensitive Adenylate Cyclase	Calcitonin Inhibited Phosphate Reabsorption
PCT	60–70%	++	++	–	++
PST	15–20%	++	++	–	++
DCT	5–10%	++	++	++	–
CAL	0%	++	–	++	–
MAL	0%	–	–	++	–

Figure 1 Model of the renal tubule and distribution of phosphate reabsorption and hormone-dependent adenylate cyclase activity throughout the structure. The renal tubule consists of a proximal convoluted tubule (PCT), composed of an S_1 and S_2 segment, a proximal straight tubule (PST), also known as the S_3 segment, the loop of Henle, the medullary ascending limb (MAL), the cortical ascending limb (CAL), the distal convoluted tubule (DCT), and three segments of the collecting tubule: the cortical collecting tubule (CCT), the outer medullary collecting tubule (OMCT), and the inner medullary collecting tubule (IMCT). Phosphate reabsorption occurs primarily in the PCT but is maintained in the PST and DCT as well. In general, parathyroid hormone (PTH) influences phosphate reabsorption at sites where PTH-dependent adenylate cyclase is localized. In contrast, calcitonin alters phosphate transport at sites distinct from those where calcitonin-dependent adenylate cyclase is present, suggesting that response to this hormone occurs by a distinctly different mechanism.

At all three sites of phosphate reabsorption—the proximal convoluted tubule, proximal straight tubule, and distal tubule—several investigators have mapped PTH-sensitive adenylate cyclase (Fig. 1) (Knox and Haramati, 1981; Morel, 1981). Not surprisingly, there is clear evidence that PTH decreases phosphate reabsorption at these loci by a cAMP-dependent process, as well as a cAMP-independent signaling mechanism. In contrast, calcitonin-sensitive adenylate cyclase maps to the medullary and cortical thick ascending limbs and the distal tubule (Fig. 1) (Berndt and Knox, 1984). Nevertheless, calcitonin inhibits phosphate reabsorption in the proximal convoluted and proximal straight tubule, certainly by a cAMP-independent mechanism that may be mediated by a rise in intracellular calcium (Murer *et al.,* 2000). An action of calcitonin on the distal tubule is uncertain, despite the abundant calcitonin-sensitive adenylate cyclase.

MECHANISM OF PHOSPHATE TRANSPORT

The most detailed studies of the cellular events involved in P movement from the luminal fluid to the peritubular capillary blood have been performed in proximal tubules and cultured cells derived from them. These investigations indicate that P reabsorption occurs principally by a unidirectional process that proceeds transcellularly with minimal intercellular backflux from the plasma to the lumen. Entry of phosphate into the tubular cell across the luminal membrane proceeds by way of a saturable active transport system that is sodium dependent (analogous to the sodium-dependent cotransport in the intestine) (Fig. 2). With each phosphate transported, two Na^+ ions enter the proximal tubule cell. Because transport of HPO_4^{2-} is electroneutral and $H_2PO_4^-$ electrogenic, the rate of phosphate transport is dependent on the magnitude of the Na^+ gradient maintained across the luminal membrane, which depends on the Na^+/ATPase or sodium pump on the basolateral membrane. Further, the rate-limiting step in transcellular transport is likely the Na^+-dependent entry of phosphate across the luminal membrane, a process with a low K_m for luminal phosphate (~0.43 M), which permits highly efficient phosphate transport. However, several studies in proximal convoluted tubules from various species indicate that there are two such Na^+-dependent phosphate transport systems: one of low affinity and high capacity, responsible for the majority of phosphate reabsorption, and a high-affinity, low-capacity system, accountable for the remainder (Brunette *et al.,* 1984). In contrast, a single high-affinity system operates in the proximal straight tubule.

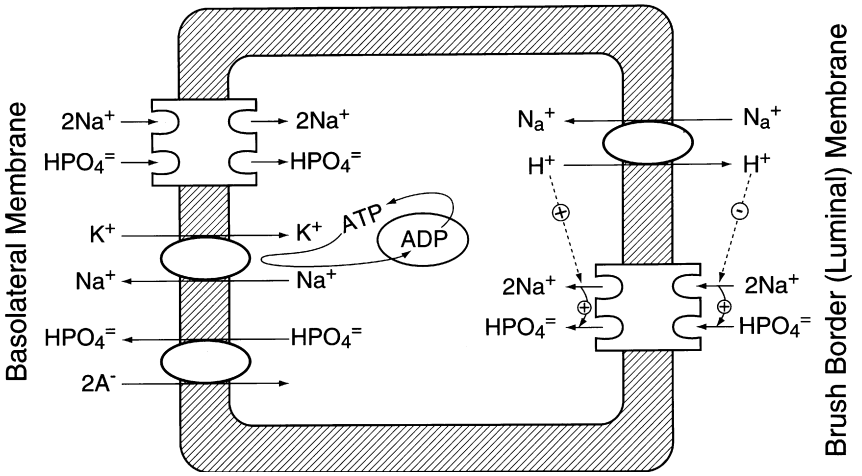


Figure 2 Model of inorganic phosphate ($HPO_4^=$) transcellular transport in the proximal convoluted tubule of the mammalian kidney. On the brush border or luminal membrane, a Na^+/H^+ exchanger and a $2Na/HPO_4^=$ cotransporter operate. $HPO_4^=$ that enters the cell across the luminal surface mixes with the intracellular metabolic pool of phosphate and is eventually transported out of the cell across the basolateral membrane via an anion (A-) exchange mechanism. On the basolateral membrane there is also a $2Na/HPO_4^=$ cotransporter and a Na^+/K^+-ATPase system. The ATPase transports the Na^+ out of the cell, maintaining the Na^+ gradient-driving force for luminal phosphate entry.

The phosphate that enters the tubule cell plays a major role in governing various aspects of cell metabolism and function and is in rapid exchange with intracellular phosphate. Under these conditions, the relatively stable-free P_i concentration in the cytosol implies that P_i entry into the cell across the brush border membrane must be tightly coupled with its exit across the basolateral membrane (Fig. 2). The transport of phosphate across the basolateral membrane is apparently a passive process driven by an electrical gradient secondary to an anion-exchange mechanism. However, several P_i transport pathways have been postulated, including Na^+-P_i cotransport and an unspecific P_i leak, as well as anion exchange. In any case, basolateral P_i transport serves at least two functions: (1) complete transcellular P_i reabsorption when luminal P_i entry exceeds the cellular P_i requirments and (2) guaranteed basolateral P_i influx if apical P_i entry is insufficient to satisfy cellular requirements (Schwab *et al.,* 1984).

Until recently there was very little information about the molecular structure of the phosphate transporters. However, the cellular scheme for proximal tubular P_i reabsorption currently includes three Na^+-P_i cotransporters (Helps *et al.,* 1995; Verri *et al.,* 1995; Sorribas *et al.,* 1994; Werner *et al.,* 1994; Magagnin *et al.,* 1993), which have been identified molecularly and named type I, type II, and type III Na^+-P_i cotransporters. The three families of Na^+-P_i cotransporters share no significant homology in their primary amino acid sequence and exhibit substantial variability in substrate affinity, pH dependence, and tissue expression (Table I). Tissue expression, relative renal abundance, and overall transport characteristics of type I, II (IIa), and III $Na-P_i$ cotransporters suggest that the type IIa transporter plays a key role in brush border membrane P_i flux. Indeed, changes in expression of the type IIa Na^+-P_i cotransporter protein parallel alterations in proximal tubular P_i handling, documenting its physiological imporance (Murer *et al.,* 1998, 1999). In addition, molecular and/or genetic suppression of the type IIa Na^+-P_i cotransporter support its role in mediating brush border membrane Na^+-P_i cotransport. Thus, intravenous injection of specific antisense oligonucleotides reduces brush border membrane Na^+-P_i cotransport activity in accord with a decrease in type IIa cotransporter protein (Oberbauer *et al.,* 1996). In addition, disruption of the type IIa Na^+-P_i cotransporter gene (*Npt2*) in mice leads to a 70% reduction in brush border Na^+-P_i cotransport rate and complete loss of the protein (Beck *et al.,* 1998; Hoag *et al.,* 1999). Although the molecular basis for the brush border membrane Na^+-P_i cotransport remaining after *Npt2* gene disruption is unclear, residual transport activity may depend on the type I transporter protein or another yet unidentified Na^+-P_i cotransporter.

HORMONAL/METABOLIC REGULATION OF PHOSPHATE TRANSPORT

Several hormones and metabolic pertubations modulate phosphate reabsorption by the kidney. Among these, PTH, PTHrP, calcitonin, transforming growth factor-α (TGFα), glucocorticoids, and phosphate loading inhibit renal phosphate reclamation. In contrast, IGF-1, insulin, thyroid hormone, $1,25(OH)_2D$, epidermal growth factor (EGF), and phosphate deprivation (depletion) stimulate renal phosphate

Table I Characteristics of Na–P_i Cotransporters

	Type I	Type II		Type III
		Type IIa	Type IIb	
Chromosomal location (human)	6	5	4	2 (P_iT-1) 8 (P_iT-2)
Amino acids	~465	~640	~690	679 656
Function (in *Xenopus* oocytes)	Na–P_i cotransport; Cl channel activity; organic anion interaction	Na–P_i cotransport; electrogenic, pH dependent	Na–P_i cotransport; electrogenic	Na–P_i cotransport; electrogenic
Substrate	P_i; organic anions	P_i	P_i	P_i
Affinity for P_i	~1.0 m*M*	0.1–0.2 m*M*	0.05 m*M*	0.025 m*M*
Affinity for Na	50–60 m*M*	50–70 m*M*	33 m*M*	40–50 m*M*
Na^+–P_i Coupling	>1	3	3	3
Tissue expression (mRNA, protein)	Kidney cortex, parathyroid, liver, brain	Kidney, parathyroid	Small intestine, lung, other tissues	Ubiquitous
PTH regulation	No	Yes	No	No
Dietary Pi regulation	No	Yes	Yes	Yes

reabsorption. The diversity of these factors indicates that the mechanisms by which modulation of phosphate transport occurs are widely varied. However, the common target for regulation is the renal proximal tubular cell.

Insight to the molecular mechanisms that regulate phosphate transport has resulted predominantly from studies of PTH effects on this physiological process. These investigations indicate that both the cAMP–protein kinase A and the phospholipase C–protein kinase C signal transduction pathways modulate proximal tubule phosphate transport. In this regard, PTH-mediated inhibition of phosphate reabsorption operates through the protein kinase C system at low hormone concentrations (10^{-8} to 10^{-10} *M*) and via protein kinase A at higher concentrations. Consistent with this hypothesis is the demonstration that the pattern of apical membrane protein phosphorylation in response to PMA, an activator of protein kinase C, resembles that obtained with low concentrations of PTH, whereas the phosphorylation pattern in response to 8-bromo-cAMP resembles that obtained with high concentrations of PTH. More recently, the mechanism by which these second messenger systems alter phosphate transport has become apparent. In this regard, several investigators have shown that endocytosis and subsequent lysosomal degradation of the phosphate transporters is central to PTH effects on phosphate reabsorption. Thus, interference with the endocytotic pathway, either by the microtubule-disrupting agent colchicine or high-medium osmolarity, reduces PTH inhibition of phosphate transport in OK cells. Further, recovery of Na^+–P_i cotransport activity following PTH inhibition requires protein synthesis, consistent with degradation of the receptors. In any case, the reputed PTH effect on membrane recycling is consistent with the change in the V_{max} for P transport observed secondary to hormonal stimulation. In concert with these findings, studies indicate that expression of the NPT-2 protein at renal tubular sites is increased in parathyroidectomized rats and decreased after PTH treatment. In addition, Northern blot analysis of total RNA shows that the abundance of NPT-2-specific mRNA is not changed by parathyroidectomy, but is decreased minimally in response to the administration of parathyroid hormone. These data indicate that parathyroid hormone regulation of renal Na^+–P_i cotransport is determined by changes in expression of NPT-2 protein in the renal brush border membrane (Kempson *et al.,* 1995).

Although it is generally accepted that PTH is the most important physiologic influence on renal P excretion and is the major determinant of plasma P concentrations through its effect on T_mP/GFR, there is no compelling evidence supporting the importance of this hormone in overall P balance. Indeed, repeated observations have confirmed that the balance between urinary excretion and dietary input of P is maintained not only in normal humans but in patients with hyper- and hypoparathyroidism. In fact, the renal tubule has a seemingly intrinsic ability to adjust the reabsorption rate of P according to dietary P_i intake and the need and availability of P to the body (Levi *et al.,* 1994). Thus P reabsorption is increased under conditions of greater P need, such as rapid growth, pregnancy, lactation, and dietary restriction. Conversely, in times of surfeit, such as slow growth, chronic renal failure, or dietary excess, renal P reabsorption is curtailed. This adaptive response is localized in the proximal convoluted tubule and involves an alteration in the apparent V_{max} of both the high-capacity, low-affinity and the low-capacity, high-affinity Na^+–phosphate cotransport systems independent of any change in affinity. Such changes in response to chronic changes in P_i availability are characterized by parallel changes in Na^+–phosphate

cotransport activity, the NPT-2 mRNA level, and NPT-2 protein abundance. In contrast, the acute adaptation to altered dietary P is marked by parallel changes in Na^+–phosphate cotransporter activity and NPT-2 protein abundance in the absence of a change in NPT-2 mRNA. Thus, in response to chronic conditions, protein synthesis is requisite in the adaptive response, whereas under acute conditions, the number of NPT-2 cotransporters is changed rapidly by mechanisms independent of *de novo* protein synthesis, such as insertion of existing transporters into the apical membrane or internalization of existing transporters.

Although the signal for the adaptive alteration in phosphate transport is not yet known, several possibilities are evident. Studies using MRI spectroscopy indicate a reciprocal relationship between intracellular phosphate concentration and brush border membrane phosphate transport, suggesting that cytosolic phosphate may serve as an important cellular signal mediating the transport response. Alternatively, experiments in OK cell monolayers suggest that apical Na^+-dependent phosphate influx may also play a role in triggering the adaptive response to altered extracellular phosphate. In this regard, depletion of P_i at the apical site is sufficient to provoke an adaptive increase of the apical $Na-P_i$ cotransport rate, whereas removal of P_i only from the basolateral cell surface is without effect. Thus, there is the possibility of a P_i-sensing mechanism at the apical surface or the rate of P_i entry at the apical cell surface contributes to P_i sensing. In addition, alterations in cytosolic Ca^{2+} concentrations may be part of the P_i-sensing mechanism.

Clinical Disorders of Phosphate Homeostasis

The variety of diseases, therapeutic agents, and physiological states that affect phosphate homeostasis are numerous and reflect a diverse pathophysiology. Indeed, rational choice of an appropriate treatment for many of these disorders depends on determining the precise cause for the abnormality. The remainder of this chapter discusses several clinical states that represent primary disorders of phosphate homeostasis. These include X-linked hypophosphatemic rickets/osteomalacia (XLH); autosomal-dominant hypophosphatemic rickets (ADHR); tumor-induced osteomalacia (TIO); hereditary hypophosphatemic rickets with hypercalciuria (HHRH); Dent's disease; Fanconi's syndrome (FS), types I and II; and tumoral calcinosis (TC). Table II documents the full spectrum of diseases in which disordered phosphate homeostasis occurs. Many of these are discussed in other chapters.

Table II Diseases of Disordered Phosphate Homeostasis

- Increased phosphate
  - Reduced renal phosphate excretion
    - Renal failure
    - Hypoparathyroidism
    - Tumoral calcinosis[a]
    - Hyperthyroidism
    - Acromegaly
    - Diphosphonate therapy
  - Increased phosphate load
    - Vitamin D intoxication
    - Rhabdomyolysis
    - Cytotoxic therapy
    - Malignant hyperthermia
- Decreased phosphate
  - Decreased gastrointestinal absorption
    - Phosphate deprivation[a]
    - Gastrointestinal malabsorption
  - Increased renal phosphate excretion
    - Hyperparathyroidism
    - X-linked hypophosphatemic rickets/osteomalacia[a]
    - Fanconi's syndrome, type I[a]
      - Familial idiopathic
      - Cystinosis (Lignac–Fanconi disease)
      - Hereditary fructose intolerance
      - Tyrosinemia
      - Galactosemia
      - Glycogen storage disease
      - Wilson's disease
      - Lowe's syndrome
    - Fanconi's syndrome, type II[a]
    - Vitamin D-dependent rickets
    - Autosomal-dominant hypophosphatemic rickets[a]
    - Dent's disease (X-linked recessive hypophosphatemic rickets)[a]
    - Tumor-induced osteomalacia[a]
    - Hereditary hypophosphatemic rickets with hypercalciuria[a]
  - Transcellular shift
    - Alkalosis
    - Glucose administration
  - Combined mechanisms
    - Alcoholism
    - Burns
    - Nutritional recovery syndrome
    - Diabetic ketoacidosis

[a]Primary disturbance of phosphate homeostasis.

Impaired Renal Tubular Phosphate Reabsorption

X-Linked Hypophosphatemic Rickets

X-linked hypophosphatemic rickets/osteomalacia is the archetypal phosphate-wasting disorder, characterized in general by progressively severe skeletal abnormalities and growth retardation. The syndrome occurs as an X-linked dominant disorder with complete penetrance of a renal tubular abnormality resulting in phosphate wasting and consequent hypophosphaemia (Table III). The clinical expression of the disease is widely variable even in members of the same family, ranging from a mild abnormality, the apparent isolated occurrence of hypophosphatemia, to severe bone disease (Lobaugh *et al.,* 1984). On average, disease severity is similar in males and females, indicating minimal, if any, gene dosage effect (Whyte *et al.,* 1996). The most common clinically evident manifestation is short stature. This height deficiency is a consequence of abnormal lower extremity growth, averaging 15% below normal. In contrast, upper segment growth is not affected. The

Table III Biochemical Abnormalities in Primary Disorders of Phosphorus Homeostasis[a]

	XLH	HHRH	ADHR	Dent's disease	TIO	FS I	FS II	TC
Calcium metabolism								
Serum Ca	N/LN	N/HN	N/LN	N	N/LN	N/LN	N/HN	N/HN
Urine Ca	↓	↑	↓	↓	↓	↓	↑	↑
GI Ca absorption	↓	↑	↓	↓	↓	↓	↑	↑
Serum PTH	N	N/LN	N	N/LN	N	N	N/LN	N
Phosphate metabolism								
Serum P	↓	↓	↓	N/↓	↓	↓	↓	↑
T_mP/GFR	↓	↓	↓	N/↓	↓	↓	↓	↑
GI P absorption	↓		↓	↓	↓	↓		N
Alkaline phosphatase	N/↑	N/↑	N/↑	N/↑	N/↑	N/↑	N/↑	N
Vitamin D metabolism								
Serum 25(OH)D	N	N	N	N	N	N	N	N
Serum $1,25(OH)_2D$	(↓)	↑	(↓)	(↓)	↓	(↓)	↑	↑

[a]Modified from Econs *et al.* (1992). XLH, X-linked hypophosphatemic rickets; HHRH, hereditary hypophosphatemic rickets with hypercalciuria; ADHR, autosomal-dominant hypophosphatemic rickets; TIO, tumor-induced osteomalacia; FS I, Fanconi's syndrome type I; FS II, Fanconi's syndrome type II; TC, tumoral calcinosis. N, normal; LN, low normal; HN, high normal; ↑, increased; ↓, decreased; (↓), decreased relative to the serum phosphorus concentration

majority of children with the disease exhibit enlargement of the wrists and/or knees secondary to rickets, as well as bowing of the lower extremeties. Additional signs of the disease may include late dentition, tooth abscesses secondary to poor mineralization of the interglobular dentine, enthesopathy (calcification of tendons, ligaments, and joint capsules), and premature cranial synostosis. However, many of these features may not become apparent until age 6 to 12 months or older (Harrison *et al.,* 1966). Despite marked variability in the clinical presentation, bone biopsies in affected children and adults invariably reveal osteomalacia, the severity of which has no relationship to sex, the extent of the biochemical abnormalities, or the severity of the clinical disability. In untreated youths and adults, serum 25(OH)D levels are normal and the concentration of $1,25(OH)_2D$ is in the low-normal range (Haddad *et al.,* 1973; Lyles *et al.,* 1982). The paradoxical occurrence of hypophosphatemia and normal serum calcitriol levels is due to the aberrant regulation of renal 25(OH)D-1 α-hydroxylase activity due most likely to abnormal phosphate transport. Indeed, studies in *hyp*-mice, the murine homologue of the human disease, have established that defective regulation is confined to enzyme localized in the proximal convoluted tubule, the site of the abnormal phosphate transport (Lobaugh and Drezner, 1983; Nesbitt *et al.,* 1986, 1987; Nesbitt and Drezner, 1990).

Pathophysiology Investigators generally agree that the primary inborn error in XLH results in an expressed abnormality of the renal proximal tubule that impairs P_i reabsorption. This defect has been indirectly identified in affected patients and directly demonstrated in the brush border membranes of the proximal nephron in *hyp*-mice. Until recently, whether this renal abnormality is primary or secondary to the elaboration of a humoral factor has been controversial. In this regard, demonstration that renal tubule cells from *hyp*-mice maintained in primary culture exhibit a persistent defect in renal P_i transport (Bell *et al.,* 1988; Dobre *et al.,* 1990), likely due to decreased expression of the Na^+–phosphate cotransporter (NPT-2) mRNA and immunoreactive protein (Tenenhouse *et al.,* 1994, 1995; Collins and Ghishan, 1994), supported the presence of a primary renal abnormality. In contrast, transfer of the defect in renal P_i transport to normal and/or parathyroidectomized normal mice parabiosed to *hyp*-mice implicated a humoral factor in the pathogenesis of the disease (Meyer *et al.,* 1989a,b). Current studies, however, have provided compelling evidence that the defect in renal P_i transport in XLH is secondary to the effects of a circulating hormone or metabolic factor. In this regard, immortalized cell cultures from the renal tubules of *hyp*-mice exhibit normal Na^+–phosphate transport, suggesting that the paradoxical effects observed in primary cultures may represent the effects of impressed memory and not an intrinsic abnormality (Nesbitt *et al.,* 1995, 1996). Moreover, the report that cross-transplantation of kidneys in normal and *hyp*-mice results in neither transfer of the mutant phenotype nor its correction unequivocally established the humoral basis for XLH (Nesbitt *et al.,* 1992). Subsequent efforts, which resulted in localization of the gene encoding the Na^+–phosphate cotransportor to chromosome 5, further substantiated the conclusion that the renal defect in brush border membrane phosphate transport is not intrinsic to the kidney (Kos *et al.,* 1994). While these data establish the presence of a humoral abnormality in XLH, the identity of the putative factor, the spectrum of its activity, and the cells producing it have not been definitively elucidated. Indeed, to date, such a hormone has not been isolated or cloned, but several groups have measured phosphaturic and bone mineralization inhibitory activity in the serum and conditioned medium from osteoblasts of affected patients and/or

hyp-mice (Xiao *et al.,* 1998; Lajeunesse *et al.,* 1996; Nesbitt *et al.,* 1999). Moreover, several investigators have identified the presence and partially characterized phosphaturic factors (inhibitors of Na^+–dependent phosphate transport) in patients with tumor-induced osteomalacia (see later) (Cai *et al.,* 1994; Wilkins *et al.,* 1995) and in patients with end-stage renal disease (Kumar *et al.,* 1995). Whether any one of these factors is increased in patients with XLH remains unknown. Regardless, additional investigation is essential to fully understand the precise physiologic derangement underlying this X-linked hypophosphatemic disorder.

Genetic Defect Efforts to better understand XLH have more recently included attempts to identify with certainty the genetic defect underlying this disease. In 1986, Read and co-workers and Machler and colleagues reported linkage of the DNA probes DXS41 and DXS43, which had been previously mapped to Xp22.31–p21.3, to the *HYP* gene locus. In subsequent studies, Thacker *et al.* (1987) and Albersten *et al.* (1987) reported linkage to the *HYP* locus of additional polymorphic DNA, DXS197, and DXS207 and, using multipoint mapping techniques, determined the most likely order of the markers as Xpter-DXS85-(DXS43/-DXS197)-*HYP*-DXS41-Xcen and Xpter-DXS43-*HYP*-(DXS207/DXS41)-Xcen, respectively. The relatively small number of informative pedigrees available for these studies prevented definitive determination of the genetic map along the Xp22–p21 region of the X chromosome and only allowed identification of flanking markers for the *HYP* locus 20 c*M* apart. More recently, the HYP consortium, in a study of some 20 multigenerational pedigrees, used a positional cloning approach to refine mapping of the Xp22.1–p21 region of the X chromosome, identify tightly linked flanking markers for the *HYP* locus, construct a YAC contig spanning the *HYP* gene region, and eventually clone and identify the disease gene as PHEX, a phosphate-regulating gene with homologies to endopeptidases located on the X chromosome. In brief, these studies ascertained a locus order on Xp22.1 of

Xcen-DXS451-(DXS41/DXS92)-DXS274-DXS1052-DXS1683-*HYP*-DXS7474-DXS365-(DXS443/DXS3424)-DXS257-(GLR/DXS43)-DXS315-Xtel.

Moreover, the physical distance between the flanking markers, DXS1683 and DXS7474, was determined as 350 kb and their location on a single YAC ascertained. Subsequently, a cosmid contig spanning the *HYP* gene region was constructed and efforts were directed at discovery of deletions within the *HYP* region. Identification of several such deletions permitted characterization of cDNA clones that mapped to cosmid fragments in the vicinity of the deletions. Database searches with these cDNAs detected homologies at the peptide level to a family of endopeptidase genes, which includes neutral endopeptidase (NEP), endothelin-converting enzymes 1 and 2 (ECE-1 and ECE-2), soluble secreted endopeptidase (SEP), the "orphan" peptidase X chromosome controlling element (XCE), and the Kell blood group antigen (KELL). These efforts clearly established PHEX as the candidate gene responsible for XLH (Econs *et al.,* 1993, 1994a,b; Francis *et al.,* 1994; Rowe *et al.,* 1996; The Hyp Consortium, 1995), a conclusion confirmed by the partial rescue of the phenotype accomplished by using bone marrow transplantation as a means to replace the defective gene product(s) with the normal gene product(s) (Miyamura *et al.,* 2000).

Subsequent studies found that PHEX is expressed predominantly in bones, teeth, and parathyroid glands; mRNA, protein, or both have also been identified in lung, brain, muscles, and gonads. However, the gene is not expressed in kidney and even in bones and teeth PHEX/Phex is a low-abundance transcript. In addition, since discovery of the PHEX gene, approximately 150 different mutations have been identified in patients with XLH (see *http://data.mch.mcgill.ca/phexdb/*), including deletions, frame shifts, exon splice, missense, and nonsense mutations. However, no genotype/phenotype correlations have been recognized, albeit changes in conserved amino acids more often result in clinical disease than changes in nonconserved amino acids (Filisetti *et al.,* 1999).

Unfortunately, the precise role that this gene and its product play in the regulation of phosphate homeostasis and the pathogenesis of the disease remains unknown. However, because NEP, ECE-1, KELL, and SEP are proteolytic enzymes (Florentin *et al.,* 1984; Koehn *et al.,* 1987), it is likely that the PHEX gene product either activates or degrades a peptide hormone (see later). In any case, cloning the human PHEX gene led relatively rapidly to cloning the mouse Phex gene, which has high homology to its human counterpart. Unlike 97% of other known genes, however, neither the human nor the murine PHEX/Phex gene has a classic Kozak sequence, consisting of a purine at the −3 position before the ATG initiation sequence. This suggests that regulation of PHEX gene expression will occur by posttranscriptional mechanisms, as occurs in genes that do not have such Kozak sequences.

Pathogenesis Despite the remarkable advances that have been made in understanding the genetic abnormality and pathophysiology of XLH, the detailed pathogenetic mechanism underlying this disease remains unknown. Nevertheless, several observations suggest the likely cascade of events that result in the primary abnormalities characteristic of the syndrome. In this regard, the X-linked dominant expression of the disorder with little, if any, gene dosage effect likely results from PHEX mutations that result in an haploinsufficiency defect, in which one-half the normal gene product (or null amounts) causes the phenotype. The alternative possibility that the PHEX gene results in a dominant-negative effect is unlikely because, inconsistent with this prospect, several mutations reported in affected humans (Francis *et al.,* 1997) and the murine *Gy* mutation almost certainly result in the lack of message production (Meyer *et al.,* 1998). In any case, it is tempting to speculate that the PHEX gene product acts directly or

indirectly on a phosphaturic factor that regulates renal phosphate handling. Given available data, the PHEX gene product, a putative cell membrane-bound enzyme, may function normally to inactivate "phosphatonin," a phosphaturic hormone. However, data from parabiotic studies of normal and *hyp*-mice argue strongly that extracellular degradation of the phosphaturic factor does not occur. Indeed, such activity would preclude transfer of the *hyp*-mouse phenotype to parabiosed normals. Alternatively, the PHEX gene product may function intracellularly to inactivate "phosphatonin." In this regard, Jalal *et al.* (1991) reported the internalization of neutral endopeptidase and a potential role for this enzyme in intracellular metabolism. In addition, Thompson *et al.* (2001) reported that the PHEX protein in osteoblasts is found predominantly in the Golgi apparatus and the endoplasmic reticulum. Less likely, the PHEX gene product may enzymatically activate a protein that suppresses production of phosphatonin. While this is consistent with all previous data, it is a complex process and requires the production of PHEX, phosphatonin, and the suppressor protein in the same cell in order to accommodate data from the parabiotic studies. Nevertheless, in accord with this possibility, Mari *et al.* (1992) reported that the neutral endopeptidase on human T cells may be involved in the production of lymphokines through the processing of an activating factor at the surface of the lymphocyte. In any of these cases, a defect in the PHEX gene will result in overproduction and circulation of phosphatonin and consequent decreased expression of the renal Na^+–phosphate cotransportor, *Npt2*, the likely scenario in the pathogenesis of XLH. Although such overproduction of phosphatonin is a favored hypothesis based on available data, including identification and isolation of such factors in patients with tumor-induced osteomalacia and detection of biological activity in conditioned medium from *hyp*-mouse osteoblasts (Xiao *et al.,* 1998; Nesbitt *et al.,* 1999), it is possible that XLH results from the inability of mutant PHEX to activate a phosphate-conserving hormone. However, the only known phosphate-conserving hormone, stanniocalcin, is synthesized in active form within the kidney and has little known bioactivity in humans. These features strongly mitigate against a role for stanniocalcin in the pathogenesis of XLH. Nevertheless, it is evident that further information is requisite to enhance our understanding of the pathogenesis of XLH and, in turn, regulation of phosphate homeostasis. Unfortunately, clarifying the role that the PHEX gene product may play in regulating phosphatonin will not completely unravel the role that PHEX has in the genesis of XLH. Indeed, such information will not discriminate if significant elements of the *hyp*-mouse phenotype, including rickets and osteomalacia and aberrantly regulated renal 25(OH)D-1α-hydroxylase activity, are a direct consequence of renal phosphate wasting and hypophosphatemia or result from a separate PHEX gene-mediated pathway(s) of action. At present, however, preliminary studies suggest that PHEX in osteoblasts does play a key role in the regulation of bone mineralization. Such investigations illustrate that *hyp*-mouse osteoblasts *in vitro* exhibit decreased alkaline phosphatase activity, collagen deposition and reduced osteocalcin, bone sialoprotein, and vitronectin at both protein and mRNA levels. Furthermore, conditioned medium from cultures of these cells induce analogous defects in osteoblasts from normal mice. While these data suggest that alterations in the PHEX gene may control bone mineralization, further investigations are critical to understanding the pathogenesis of XLH and the regulation of phosphate and mineral homeostasis, as well as vitamin D metabolism. Such investigations may have significant impact on the determination of optimal treatment strategies for many of the vitamin D-resistant diseases.

Treatment In past years, physicians employed pharmacologic doses of vitamin D as the cornerstone for treatment of XLH. However, long-term observations indicate that this therapy fails to cure the disease and poses the serious problem of recurrent vitamin D intoxication and renal damage. More recently, current treatment strategies for children directly address the combined calcitriol and phosphorus deficiency characteristic of the disease. Generally, the regimen includes a period of titration to achieve a maximum dose of calcitriol, 40–60 ng/kg/day in two divided doses, and phosphorus, 1–2 g/day in four to five divided doses (Friedman *et al.,* 1991, 1993). Such combined therapy often improves growth velocity, normalizes lower extremity deformities, and induces healing of the attendant bone disease. Of course treatment involves a significant risk of toxicity that is generally expressed as abnormalities of calcium homeostasis and/or detrimental effects on renal function secondary to abnormalities such as nephrocalcinosis. In addition, refractoriness to the growth-promoting effects of treatment is often encountered, particularly in youths presenting at $<$5th percentile in height (Friedman *et al.,* 1993). Several studies, however, indicate that the addition of growth hormone to conventional therapy increases growth velocity significantly. Unfortunately, such a benefit is realized more frequently in younger patients, and disproportionate growth of the trunk often continues to manifest. Moreover, the definitive impact of growth hormone treatment on adult height remains unknown (Wilson, 2000)

Therapy in adults is reserved for episodes of intractable bone pain and refractory nonunion bone fractures.

HEREDITARY HYPOPHOSPHATEMIC RICKETS WITH HYPERCALCIURIA

This rare genetic disease is characterized by hypophosphatemic rickets with hypercalciuria (Tieder *et al.,* 1985). The cardinal biochemical features of the disorder include hypophosphatemia due to increased renal phosphate clearance and normocalcemia. In contrast to other diseases in which renal phosphate transport is limited, patients with HHRH exhibit increased $1,25(OH)_2D$ production (Table III). The resultant elevated serum calcitriol levels

enhance gastrointestinal calcium absorption, which in turn increases the filtered renal calcium load and inhibits parathyroid secretion (Tieder *et al.,* 1985). These events cause the hypercalciuria observed in affected patients.

The clinical expression of the disease is heterogeneous, although initial symptoms, evident at 6 months to 7 years of age, generally consist of bone pain and/or deformities of the lower extremities. The bone deformities vary from genu varum or genu valgum to anterior external bowing of the femur and coxa vara. Additional features of the disease include short stature, muscle weakness, and radiographic signs of rickets or osteopenia. These various symptoms and signs may exist separately or in combination and may be present in a mild or severe form. Relatives of patients with evident HHRH may exhibit an additional mode of disease expression. These subjects manifest hypercalciuria and hypophosphatemia, but the abnormalities are less marked and occur in the absence of discernible bone disease (Tieder *et al.,* 1987). Bone biopsies in children with characteristic HHRH exhibit classical osteomalacia, but the mineralization defect appears to vary in severity with the magnitude of the hypophosphatemia. Histological measurements are within the normal range in family members with idiopathic hypercalciuria.

Pathophysiolgy and Genetics Liberman and co-workers (Tieder *et al.,* 1985, 1987; Lieberman, 1988) have presented data that indicate the primary inborn error underlying this disorder is an expressed abnormality in the renal proximal tubule, which impairs phosphate reabsorption. They propose that this pivotal defect results in enhanced renal 25(OH)D-1α-hydroxylase, thus promoting the production of $1,25(OH)_2D$ and increasing its serum and tissue levels. Consequently, intestinal calcium absorption is augmented, resulting in the suppression of parathyroid function and an increase of the renal filtered calcium load. The concomitant prolonged hypophosphatemia diminishes osteoid mineralization and accounts for the ensuing rickets and/or osteomalacia.

The suggestion that abnormal phosphate transport results in increased calcitriol production remains untested. Indeed, the elevation of $1,25(OH)_2D$ in patients with HHRH is a unique phenotypic manifestation of the disease that distinguishes it from other disorders in which abnormal phosphate transport is likewise manifest. Such heterogeneity in the phenotype of these disorders suggests that disease at variable anatomical sites along the proximal convoluted tubule uniformly impairs phosphate transport but not 25(OH)D-1α-hydroxylase activity. Alternatively, the aberrant regulation of vitamin D metabolism in other hypophosphatemic disorders may occur independently (e.g., in XLH secondary to the PHEX gene abnormality) and override the effects of the renal phosphate transport.

Although X-linked transmission of this disease has been ruled out, the mode of genetic transmission for HHRH/hypercalciuria remains uncertain. Indeed, current observations suggest that the mode of inheritance may be heterogenous. However, an autosomal dominant form of disease has been described, with less pronounced clinical and biochemical abnormalities. These observations are consistent with an autosomal codominant pattern of inheritance with high, but incomplete penetrance. Under such circumstances, the variability in this disorder may be explained by assuming that individuals with HHRH or idiopathic hypercalciuria are homozygous and heterozygous, respectively, for the same mutant allele.

Although identification of the gene underlying HHRH has not occurred, a few candidate genes have been proposed and mapped. These include the Na^+-dependent phosphate cotransporter gene 1 (NPT1) and the Na^+-dependent phosphate cotransporter gene 2 (NPT2), which are autosomal and expressed predominantly in the kidney. Interestingly, NPT2 null transgenic mice have a biochemical and physical phenotype that resembles HHRH. In fact, heterozygous mice exhibit urinary phosphate excretion and serum calcium levels intermediate between normal mice and homozygotes, consistent with the supposition that HHRH may represent a codominant disorder. Whether a mutation in NPT2 or inactivating mutations in genes coding for ancillary proteins that regulate NPT2 function underlies HHRH, however, remains unknown.

Treatment In accord with the hypothesis that a singular defect in renal phosphate transport underlies HHRH, affected patients have been treated successfully with high-dose phosphorus (1–2.5 g/day in five divided doses) alone. In response to therapy, bone pain disappears and muscular strength improves substantially. Moreover, the majority of treated subjects exhibit accelerated linear growth and radiologic signs of rickets disappear completely within 4–9 months. Concordantly, serum phosphorus values increase toward normal, the $1,25(OH)_2D$ concentration decreases, and alkaline phosphatase activity declines. Despite this favorable response, limited studies indicate that such treatment does not heal the associated osteomalacia. Therefore, further investigation will be necessary to determine if phosphorus alone is truly sufficient for this disorder.

AUTOSOMAL-DOMINANT HYPOPHOSPHATEMIC RICKETS

Several studies have documented an autosomal-dominant inheritance, with incomplete penetrance, of a hypophosphatemic disorder similar to XLH (Harrison and Harrison, 1979). The phenotypic manifestations of this disorder include lower extremity deformities and rickets/osteomalacia. Indeed, affected patients display biochemical and radiographic abnormalities indistinguishable from those of individuals with XLH. These include hypophosphatemia secondary to renal phosphate wasting and normal levels of parathyroid hormone and 25(OH)D, as well as inappropriately normal (relative to the serum phosphorus concentration) $1,25(OH)_2D$ (Table III). However, unlike patients with XLH, some with ADHR display variable incomplete penetrance and delayed onset of penetrance (Econs and

McEnery, 1997). Thus, long-term studies indicate that a few of the affected female patients exhibit delayed penetrance of clinically apparent disease and an increased tendency for bone fracture, uncommon occurrences in XLH. Moreover, these individuals present in the second through the fourth decade with weakness and bone pain but do not have lower extremity deformities. Further, other patients with the disorder present during childhood with phosphate wasting, rickets and lower extremity deformity but manifest postpubertal loss of the phosphate-wasting defect. Finally, a few apparently unaffected individuals have been identified, who seemingly are carriers for the ADHR mutation.

An apparent forme fruste of this disease, (autosomal-dominant) hypophosphatemic bone disease, has many of the characteristics of XLH and ADHR, but reports indicate that affected children display no evidence of rachitic disease (Scriver *et al.,* 1977, 1981). Because this syndrome is described in only a few small kindreds and radiographically evident rickets is not universal in children with familial hypophosphatemia, these familes may have ADHR. Further observations are necessary to discriminate this possibility.

Pathophysiology and Genetics The primary inborn error in ADHR results in an expressed abnormality of the renal proximal tubule that impairs P_i reabsorption. Until recently, whether this renal abnormality is primary or secondary to the elaboration of a humoral factor has been controversial. However, identification of the genetic defect underlying this disease has established that hormonal dysregulation is the pivotal abnormality in this disorder. In this regard, studies localized the ADHR gene to a 6.5 c*M* interval on chromosome 12p13, flanked distally by D12S1685 and proximally by D12S397 (Econs *et al.,* 1997). Moreover, extending these studies, the ADHR Consortium (2000) used a positional cloning approach to identify 37 genes within 4 Mb of genomic sequence in the 6.5 c*M* interval and identified missense mutations in a gene encoding a new member of the fibroblast growth factor (FGF) family, FGF-23. The FGF-23 gene product not only shares sequence homology with other fibroblast growth factors, but is a secreted protein. Thus, transient transfection of OK-E, COS-7 and HEK293 cells with the plasmid encoding full-length FGF-23 results in secretion of two protein species, 32 and 12 kDA, into the incubation medium, which react with a polyclonal antibody to FGF-23. More recent studies have documented that the biological actions of FGF 23 include induction of renal phosphate wasting, a central defect in the hypophosphatemic rachitic diseases. Nevertheless, the relationship between FGF 23, PHEX, and phosphatonin remains unknown. In this regard, considerable controversy exists regarding whether FGF 23 is a substrate for PHEX and evidence has not been presented to determine if FGF 23 has activating or inactivating mutations. However, further studies will undoubtedly establish the relationship between XLH and ADH.

TUMOR-INDUCED OSTEOMALACIA

Since 1947 there have been reports of approximately 120 patients in whom rickets and/or osteomalacia has been induced by various types of tumors (Drezner, 1996). In at least 58 cases a tumor has been clearly documented as causing the rickets–osteomalacia, as the metabolic disturbances improved or disappeared completely upon removal of the tumor. In the remainder of cases, patients had inoperable lesions, and investigators could not determine the effects of tumor removal on the syndrome or surgery did not result in complete resolution of the evident abnormalities during the period of observation.

Affected patients generally present with bone and muscle pain, muscle weakness, rickets/osteomalacia, and occasionally recurrent fractures of long bones. Additional symptoms common to younger patients are fatigue, gait disturbances, slow growth, and bowing of the lower extremities. Biochemistries include hypophosphatemia secondary to renal phosphate wasting and normal serum levels of calcium and 25(OH)D. Serum $1,25(OH)_2D$ is overtly low in 19/23 patients in whom measurements have been made (Table III). Aminoaciduria, most frequently glycinuria, and glucosuria, is occasionally present. Radiographic abnormalities include generalized osteopenia, pseudofractures, and coarsened trabeculae, as well as widened epiphyseal plates in children. The histologic appearance of trabecular bone in affected subjects most often reflects the presence of low turnover osteomalacia. In contrast, bone biopsies from the few patients who have tumors that secrete a nonparathyroid hormone factor(s), which activates adenylae cyclase, exhibit changes consistent with enhanced bone turnover, including an increase in osteoclast and osteoblast number.

The large majority of patients with this syndrome harbor tumors of mesenchymal origin and include primitive-appearing, mixed connective tissue lesions, osteoblastomas, nonossifying fibromas, and ossifying fibromas. However, the frequent occurrence of Looser zones in the radiographs of moribund patients with carcinomas of epidermal and endodermal derivation indicates that the disease may be secondary to a variety of tumor types. Indeed, the observation of tumor-induced osteomalacia concurrent with breast carcinoma (Dent and Gertner, 1976), prostate carcinoma (Lyles *et al.,* 1980; Murphy *et al.,* 1985; Hosking *et al.,* 1975), oat cell carcinoma (Leehey *et al.,* 1985), small cell carcinoma (Shaker *et al.,* 1995), multiple myeloma, and chronic lymphocytic leukemia (McClure and Smith, 1987) supports this conclusion. In addition, the occurrence of osteomalacia in patients with widespread fibrous dysplasia of bone (Dent and Gertner, 1976; Saville *et al.,* 1955), neurofibromatosis (Weidner and Cruz, 1987; Konishi *et al.,* 1991), and linear nevus sebaceous syndrome (Cary *et al.,* 1986) could also be tumor induced. Although proof of a causal relationship in these disorders has been precluded in general by an inability to excise the multiplicity of lesions surgically, in one case of fibrous dysplasia, removal of virtually all of the abnormal bone did result in appropriate biochemical and radiographic improvement.

Regardless of the tumor cell type, the lesions at fault for the syndrome are often small, difficult to locate, and present in obscure areas, which include the nasopharynx, a sinus, the popliteal region, and the suprapatellar area. In any case, a careful and thorough examination is necessary to document/exclude the presence of such a tumor. Indeed, a CT and/or MRI scan of an clinically suspicious area should be undertaken. In addition, several groups have used octreotide scanning to identify suspected, but nonlocalized, tumors.

Pathophysiology The relatively infrequent occurrence of this disorder has confounded attempts to determine the pathophysiological basis for TIO. Nevertheless, most investigators agree that tumor production of a humoral factor(s) that may affect multiple functions of the proximal renal tubule, particularly phosphate reabsorption, is the probable pathogenesis of the syndrome. This possibility is supported by (1) the presence of phosphaturic activity in tumor extracts from three of four patients with TIO (Aschinberg *et al.,* 1977; Yoshikawa *et al.,* 1977; Lau *et al.,* 1979); (2) the absence of parathyroid hormone and calcitonin from these extracts and the apparent cyclic AMP-independent action of the extracts; (3) the occurrence of hypophosphatemia and increased urinary phosphate excretion in heterotransplanted tumor-bearing athymic nude mice (Miyauchi *et al.,* 1988);. (4) the demonstration that extracts of the heterotransplanted tumor inhibit renal 25-hydroxyvitamin D-1 α-hydroxylase activity in cultured kidney cells (Miyauchi *et al.,* 1988); and (5) the coincidence of aminoaciduria and glycosuria with renal phosphate wasting in some affected subjects, indicative of complex alterations in proxima renal tubular function (Drezner and Feinglos, 1977). Indeed, partial purification of "phosphatonin" from a cell culture derived from a hemangioscleroma causing tumor-induced osteomalacia has reaffirmed this possibility (Cai *et al.,* 1994). These studies reveal that the putative phosphatonin may be a peptide with a molecular mass of 8–2 kDa that does not alter glucose or alanine transport, but inhibits sodium-dependent phosphate transport in a cyclic AMP-independent fashion. However, studies, which document the presence in various disease states of additional phosphate transport inhibitors (and stimulants), indicate that tumor-induced osteomalacia syndrome may be heterogeneous and that "phosphatonin" may be a family of hormones. In this regard, excessive tumor production and secretion of FGF-23 (White *et al.,* 2001) and matrix extracellular phosphoglycoprotein (MEPE) have been identified in large numbers of patients with tumor-induced osteomalacia. In addition, the related finding that PHEX is also present in tumors from patients with tumor-induced osteomalaica adds further complexity to the assumption that overproduction of "phosphatonin" explains this syndrome. Indeed, it seems likely that the putative phosphatonin, underlying TIO, is likely a PHEX substrate. As such, excessive tumor production of phosphatonin must overwhelm the function of normal PHEX in order to alter phosphate homeostasis. Within the context of this model (Quarles and Drezner, 2001), data definitively implicating either FGF-23 or MEPE as the phosphaturic agent in tumor-induced osteomalacia are lacking. Hence, the phosphaturic actions of FGF-23 and/or mutated FGF-23 have not been documented and this factor is not an established substrate for PHEX. Similarly, while several groups have documented unequivocal phosphaturic activity of truncated MEPE, both *in vitro* and *in vivo,* evidence that MEPE is a substrate for PHEX is lacking. The discovery of yet additional hormones that regulate phosphate homeostasis add further doubt to the identity of the phosphaturic factor(s) in TIO. For example, stanniocalcin 1 (STC1) and 2 (STC2), phosphate-regulating hormones cloned from a human osteoblast cDNA library, respectively, stimulate and inhibit renal phosphate reabsorption and are secreted as phosphoproteins from human fibrosarcoma cells. While the existence of these multiple hormonal regulators of phosphate homeostasis cloud the pathophysiology of TIO, the demand that ectopic hormone production by a tumor is commensurate with overproduction of a normally occurring hormone, and the similarities in phenotype between TIO and genetic forms of phosphate wasting, XLH and ADRH, argue for commonality in the pathogenesis of these syndromes. Thus, it remains likely that further advances in our knowledge of the biological function(s) of FGF-23 and MEPE, as well as identification of PHEX substrates, will unravel the pathophysiology of TIO and establish the relationship of this disease with ADHR and XLH.

In contrast to these observations, patients with TIO secondary to hematogenous malignancy manifest abnormalities of the syndrome due to a distinctly different mechanism. In these subjects the nephropathy induced with light chain proteinuria or other immunoglobulin derivatives results in the decreased renal tubular reabsorption of phosphate characteristic of the disease. Thus, light chain nephropathy must be considered a possible mechanism for the TIO syndrome.

Treatment The first and foremost treatment of TIO is complete resection of the tumor. However, recurrence of mesenchymal tumors, such as giant cell tumors of bone, or inability to resect certain malignancies completely, such as prostatic carcinoma, has resulted in the development of alternative therapeutic intervention for the syndrome. In this regard, administration of $1,25(OH)_2D$ alone or in combination with phosphorus supplementation has served as effective therapy for TIO. Doses of calcitriol required range from 1.5 to 3.0 μg/day, whereas those of phosphorus are 2–4 g/day. Although little information is available regarding the long-term consequences of such treatment, the high doses of medicine required raise the possibility that nephrolithiasis, nephrocalcinosis, and hypercalcemia may frequently complicate the therapeutic course. Indeed, hypercalcemia secondary to parathyroid hyperfunction has been documented in at least five treated subjects. All of these patients received phosphorus as part of a combination regimen, which may have stimulated parathyroid hormone secretion and led to parathyroid autonomy. Thus, a careful

assessment of parathyroid function, serum and urinary calcium, and renal function is essential to ensure safe and efficacious therapy.

DENT'S DISEASE (X-LINKED RECESSIVE HYPOPHOSPHATEMIC RICKETS)

In the past several decades, multiple syndromes have been described that are characterized by various combinations of renal proximal tubular dysfunction (including renal phosphate wasting), proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis, renal failure, and rickets; these disorders, referred to as Dent's disease, include X-linked recessive hypophosphatemic rickets, X-linked recessive nephrolithiasis with renal failure, and low molecular weight proteinuria with nephrocalcinosis. The spectrum of phenotypic features in these diseases is remarkably similar, except for differences in the severity of bone deformities and renal impairment. The finding that all of these syndromes are caused by mutations affecting a chloride channel has clarified their relationship to one another and established that they are variants of a single disease (Scheinman, 1998).

Urinary loss of low molecular weight proteins is the most consistent abnormality in the disease: it is present in all affected males and in almost all female carriers of the disorder (Wrong *et al.,* 1994; Reinhart *et al.,* 1995). Other signs of impaired solute reabsorption in the proximal tubule, such as renal glycosuria, aminoaciduria, and phosphate wasting, are variable and often intermittent. Hypercalciuria is an early and common feature while hypokalemia occurs in some patients. Urinary acidification is normal in over 80% of affected subjects, and when it is abnormal, the defect has been attributed to hypercalciuria or nephrocalcinosis (Wrong *et al.,* 1994; Reinhart *et al.,* 1995; Buckalew *et al.,* 1974. The disease apparently does not recur after kidney transplantation (Scheinman, 1998).

These phenotypic features indicate the presence of proximal tubular dysfunction but do not suggest its pathophysiologic basis. However, mapping studies have established linkage to the short arm of the X chromosome (Xp11.22) (Scheinman *et al.,* 1997). Moreover, a chromosomal microdeletion detected in one family led to mapping of the region and identification of a gene encoding a voltage-dependent chloride channel, ClC-5, that is expressed predominantly in the kidney. A total of 35 mutations have been identified to date in 46 families (Scheinman, 1998; Lloyd *et al.,* 1996; Hoopes *et al.,* 1998; Igarashi *et al.,* 1998). Expression studies confirm that these mutations inactivate the chloride channel (Lloyd *et al.,* 1996; Hoopes *et al.,* 1998; Igarashi *et al.,* 1998). The gene is a member of a family of genes encoding voltage-gated chloride channels. Impairment of this channel could limit endosomal acidification, thus causing defective reabsorption of proteins, and might also lead to impaired reabsorption of other solutes if membrane protein recycling were altered.

It is not clear how this process leads to the increased intestinal calcium absorption and high serum $1,25(OH)_2D$ levels in this disorder (Reinhart *et al.,* 1995), as the 25(OH)D-1α-hydroxylase that catalyzes its formation is located in the mitochondria of proximal tubular cells, whereas ClC-5 is expressed in the thick ascending limb of Henle's loop (Devuyst *et al.,* 1999), a major site of renal calcium reabsorption. The role of this channel in the reabsorption of calcium in the thick ascending limb remains unknown.

FANCONI'S SYNDROME

Rickets and osteomalacia are frequently associated with Fanconi's syndrome, a disorder characterized by phosphaturia and consequent hypophosphatemia, aminoaciduria, renal glycosuria, albuminuria, and proximal renal tubular acidosis (De Toni, 1933; McCune *et al.,* 1943; Brewer, 1985; Chan and Alon, 1985; Chesney, 1990). Damage to the renal proximal tubule, secondary to genetic disease (Table II) or environmental toxins, represents the common underlying mechanism of this disease. Resultant dysfunction results in renal wasting of those substances primarily reabsorbed at the proximal tubule. The associated bone disease in this disorder is likely secondary to hypophosphatemia and/or acidosis, abnormalities that occur in association with aberrantly (Fanconi's syndrome, type I) or normally regulated (Fanconi's syndrome, type II) vitamin D metabolism.

Type I The type I disease resembles in many respects the more common genetic disease, X-linked hypophosphatemic rickets (Table III). In this regard, the occurrence of abnormal bone mineralization appears dependent on the prevailing renal phosphate wasting and resultant hypophosphatemia. Indeed, disease subtypes in which isolated wasting of amino acids, glucose, or potassium occur are not associated with rickets and/or osteomalacia. Further, in the majority of patients studied, affected subjects exhibit abnormal vitamin D metabolism, characterized by serum $1,25(OH)_2D$ levels that are overtly decreased or abnormally low relative to the prevailing serum phosphorus concentration (Chesney *et al.,* 1984). Although the aberrantly regulated calcitriol biosynthesis may be due to the abnormal renal phosphate transport, proximal tubule damage and acidosis may play important roles.

A notable difference between this syndrome and XLH is a common prevailing acidosis, which may contribute to the bone disease. In this regard, several studies indicate that acidosis may exert multiple deleterious effects on bone. Such negative sequellae may be related to the loss of bone calcium that occurs secondary to calcium release for use in buffering. Alternatively, several investigators have reported that acidosis may impair bone mineralization secondary to the direct inhibition of renal 25(OH)D-1α-hydroxylase activity. Others dispute these findings and claim that acidosis does not cause rickets or osteomalacia in the absence of hypophosphatemia. Most likely, however, hypophosphatemia and abnormally regulated vitamin D metabolism are the primary factors underlying rickets and osteomalacia in this form of the disease.

Type II Tieder *et al.* (1988) have described two siblings (from a consanguineous mating) who presented with classic characteristics of Fanconi's syndrome, including renal phosphate wasting, glycosuria, generalized aminoaciduria, and increased urinary uric acid excretion. However, these patients had appropriately elevated (relative to the decreased serum phosphorus concentration) serum $1,25(OH)_2D$ levels and consequent hypercalciuria (Table III). Moreover, treatment with phosphate reduced the serum calcitriol in these patients into the normal range and normalized the urinary calcium excretion. In many regards, this syndrome resembles HHRH and represents a variant of Fanconi's syndrome, referred to as type II disease. The bone disease in affected subjects is likely due to the effects of hypophosphatemia. In any case, the existence of this variant form of disease is probably the result of renal damage to a unique segment of the proximal tubule. Further studies will be necessary to confirm this possibility.

Treatment Ideal treatment of the bone disease in this disorder is correction of the pathophysiological defect influencing proximal renal tubular function. In many cases, however, the primary abnormality remains unknown. Moreover, efforts to decrease tissue levels of causal toxic metabolites by dietary (such as in fructose intolerance) or pharmacological means (such as in cystinosis and Wilson's syndrome) have met with variable success. Indeed, no evidence exists that indicates if the proximal tubule damage is reversible upon relief of an acute toxicity. Thus, for the most part, therapy of this disorder must be directed at raising the serum phosphorus concentration, replacing calcitriol (in type I disease) and reversing an associated acidosis. However, use of phosphorus and calcitriol in this disease has been limited. In general, such replacement therapy leads to substantial improvement or resolution of the bone disease (Schneider and Schulman, 1983). Unfortunately, growth and developmental abnormalities, more likely associated with the underlying genetic disease, remain substantially impaired. More efficacious therapy, therefore, is dependent on future research into the causes of the multiple disorders that cause this syndrome.

TUMORAL CALCINOSIS

Tumoral calcinosis is a rare genetic disease characterized by periarticular cystic and solid tumorous calcifications. Biochemical markers of the disorder include hyperphosphatemia and a normal or an elevated serum $1,25(OH)_2D$ concentration (Table III). Using these criteria, evidence has been presented for autosomal recessive inheritance of this syndrome. However, an abnormality of dentition, marked by short bulbous roots, pulp stones, and radicular dentin deposited in swirls, is a phenotypic marker of the disease that is variably expressed (Lyles *et al.,* 1985). Thus, this disorder may have multiple formes frustes that could complicate genetic analysis. Indeed, using the dental lesion, as well as the more classic biochemical and clinical hallmarks of the disease, an autosomal dominant pattern of transmission has been documented.

The hyperphosphatemia characteristic of the disease results from an increase in capacity of renal tubular phosphate reabsorption secondary to an unknown defect. Hypocalcemia is not a consequence of this abnormality, however, and the serum parathyroid hormone concentration is normal. Moreover, the phosphaturic and urinary cAMP responses to parathyroid hormone are not disturbed. Thus, the defect does not represent renal insensitivity to hormone, or hypoparathyroidism. Rather, the basis of the disease is probably an innate or hormone/metabolic factor-mediated abnormality of the renal tubule that enhances phosphate reabsorption. Undoubtedly, calcific tumors result from the elevated calcium–phosphorus product. The observation that long-term phosphorus depletion alone or in association with administration of acetazolamide, a phosphaturic agent, leads to resolution of the tumor masses supports this possibility.

An acquired form of this disease is rarely seen in patients with end-stage renal failure. Affected patients manifest hyperphosphatemia in association with either (1) an inappropriately elevated calcitriol level for the degree of renal failure, hyperparathyroidism, or hyperphosphatemia or (2) long-term treatment with calcium carbonate, calcitriol, or high calcium content dialysates. Calcific tumors again likely result from an elevated calcium–phosphorus product. Indeed, complete remission of the tumors occurs on treatment with vinpocetine, a mineral scavenger drug, dialysis with low calcium content dialysate, and renal transplantation.

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CHAPTER 21

Magnesium Homeostasis

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Magnesium (Mg) is the fourth most abundant cation and the second most abundant intracellular cation in vertebrates. Mg is involved in numerous biological processes and is essential for life (Rude, 2000). This mineral has evolved to become a required cofactor in literally hundreds of enzyme systems (Frausta de Silva and Williams, 1991; Rude, 2000). Examples of the physiological role of Mg are shown in Table I. Mg may be required for enzyme substrate formation. For example, enzymes that utilize ATP do so as the metal chelate, MgATP. Free Mg^{2+} also acts as an allosteric activator of numerous enzyme systems, as well as playing a role in ion currents and for membrane stabilization. Mg is therefore critical for a great number of cellular functions, including oxidative phosphorylation, glycolysis, DNA transcription, and protein synthesis.

Magnesium Metabolism

The normal adult total body Mg content is approximately 25 g (2000 mEq or 1 mol) of which 50–60% resides in bone (Elin, 1987; Wallach, 1988). Mg constitutes 0.5–1% of bone ash (200 mmol/kg ash weight). One-third of skeletal Mg is surface limited and exchangeable, and this fraction may serve as a reservoir for maintaining a normal extracellular Mg concentration (Wallach, 1988; Rude, 2000). The remainder of Mg in bone is an integral component of the hydroxyapatite lattice, which may be released during bone resorption. The rest of body Mg is mainly intracellular. The Mg content of soft tissues varies between 6 and 25 mEq/kg wet weight (Elin, 1987; Rude, 2000). In general, the higher the metabolic activity of the cell, the higher the Mg content. The concentration of Mg within cells is in the order of 5–20 mmol/liter, of which 1–5% is ionized or free (Elin, 1987; Romani and Scarpa, 1992; Romani *et al.,* 1993a). The distribution of Mg in the body is shown in Table II and Fig. 1.

Extracellular Mg accounts for about 1% of total body Mg. Mg concentration or content may be reported as mEq/liter, mg/dl, or mmol/liter. Values reported as mEq/liter can be converted to mg/dl by multiplying by 1.2 and to mmol/liter by dividing by 1/2. The normal serum Mg concentration is 1.5–1.9 mEq/liter (0.7–1.0 mmol/liter)(Elin, 1987; Rude, 2000). About 70–75% of plasma Mg is ultrafilterable, of which the major portion (55% of total serum Mg) is ionized or free and the remainder is complexed to citrate, phosphate, and other anions as represented schematically in Fig. 2. The remainder is protein bound; 25% of total serum Mg is bound to albumin and 8% to globulins.

Intestinal Mg Absorption

Intestinal Mg absorption is proportional to the amount ingested (Fine *et al.,* 1991; Schweigel and Martens, 2000). The mechanism(s) for intestinal Mg absorption is unclear but includes passive diffusion, solvent drag, and active transport (Fine *et al.,* 1991; Kayne and Lee, 1993; Bijvelds *et al,* 1998; Schweigel and Martens, 2000). As shown in Fig. 3, rat studies suggest there may be both a saturable active and an unsaturable passive transport system for Mg absorption (Ross, 1962), which may account for the higher fractional absorption at low dietary Mg intakes (Fine *et al.,* 1991; Kayne and Lee, 1993). Others have concluded that intestinal Mg absorption in humans increases linearly with Mg intake (for review, see Schweigel and Martens, 2000). The report of a patient with primary hypomagnesemia who was shown to malabsorb Mg during low Mg concentration in the intestine suggests an active transport process (Milla *et al.,* 1979). This familial defect has been mapped to chromosome 9q (Walder *et al.,* 1997). Under normal dietary conditions in healthy individuals, approximately 30–50% of ingested Mg is absorbed (Brannan *et al.,* 1976; Hardwick *et al.,* 1990; Fine *et al.,* 1991; Kayne and Lee, 1993).

Table I Examples of the Physiological Role of Magnesium

I.	Enzyme substrate (ATP Mg, GTP Mg)
	A. ATPase or GTPase (Na^+, K^+-ATPase, Ca^{2+}-ATPase)
	B. Cyclases (adenylate cyclase, guanylate cyclase)
	C. Kinase (hexokinase, creatine kinase, protein kinase)
II.	Direct enzyme activation
	A. Adenylate cyclase
	B. Phospholipase C
	C. Na^+, K^+-ATPase
	D. Ca^{2+}-ATPase
	E. K^+,H^+-ATPase
	F. G proteins
	G. 5′-Nucleotidase
	H. Creatine kinase
	I. Phosphofructokinase
	J. 5-Phosphoribosyl-pyrophosphate synthetase
	K. Lipoprotein lipase
III.	Influence membrane properties
	A. K^+ channels
	B. Ca^{2+} channels
	C. Nerve conduction

Table II Distribution of Magnesium in Adult Humans[a]

Tissue	Body mass, kg (wet wt)	Mg concentration, mmol/kg (wet wt)	Mg content (mmol)	% of total body Mg
Serum	3.0	0.85	2.6	0.3
Erythrocyte	2.0	2.5	5.0	0.5
Soft tissue	22.7	8.5	193.0	19.3
Muscle	30.0	9.0	270.0	27.0
Bone	12.3	43.2	530.1	52.9
Total	70.0		1000.7	100.0

[a]Adapted from Elin (1987).

Mg is absorbed along the entire intestinal tract, including the large and small bowel, but the sites of maximal Mg absorption appear to be the ilium and distal jejunum (Brannan *et al.,* 1976; Hardwick *et al.,* 1990; Fine *et al.,* 1991; Kayne and Lee, 1993).

The recommended daily allowance for Mg is 420 mg per day for adult males and 320 mg per day for adult females (Institute of Medicine, 1997). The dietary Mg intake in Western culture, however, appears to fall below that in a large section of the population across all ages, ranging from approximately 150 to 350 mg per day, suggesting that occult Mg depletion may be relatively prevalent (Morgan *et al.,* 1985; Marier, 1986). The major sources of Mg are nuts, cereals, green leafy vegetables, and meats.

A principal factor that regulates intestinal Mg transport has not been described. Vitamin D, as well as its metabolites 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] have been observed in some studies to enhance intestinal Mg absorption but to a much lesser extent than they do calcium absorption (Brannan *et al.,* 1976; Hodgkinson *et al.,* 1979; Krejs *et al.,* 1983). Although net intestinal calcium absorption in humans correlates with plasma 1,25$(OH)_2$D concentrations, Mg does not (Wilz *et al.,* 1979). A low Mg diet has been shown to increase intestinal calbindin-D_{9k}, suggesting that this vitamin D-dependent calcium binding protein may play a role in intestinal Mg absorption (Hemmingsen *et al.,* 1994).

Bioavailability of Mg may also be a factor in Mg intestinal absorption as other nutrients may affect Mg absorption. Although dietary calcium has been reported to both decrease and increase Mg absorption, human studies have

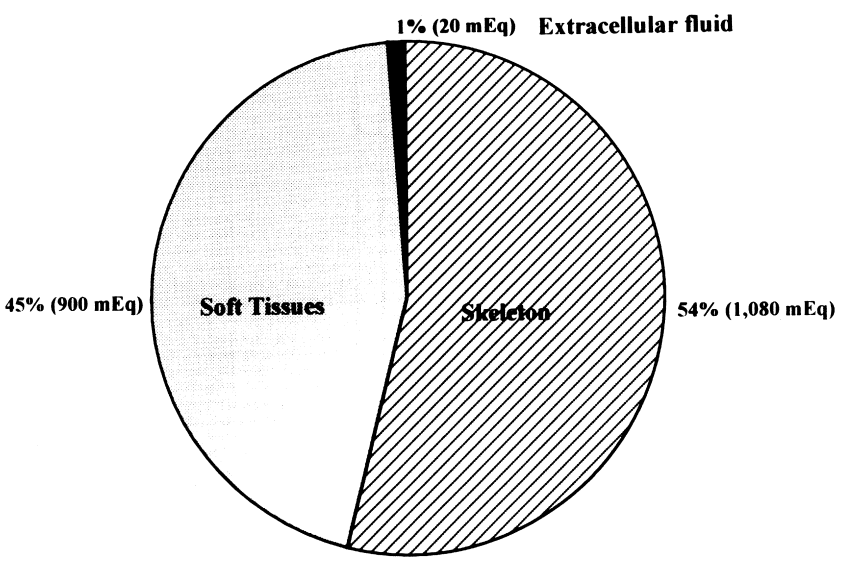

Figure 1 Distribution of magnesium in the body.

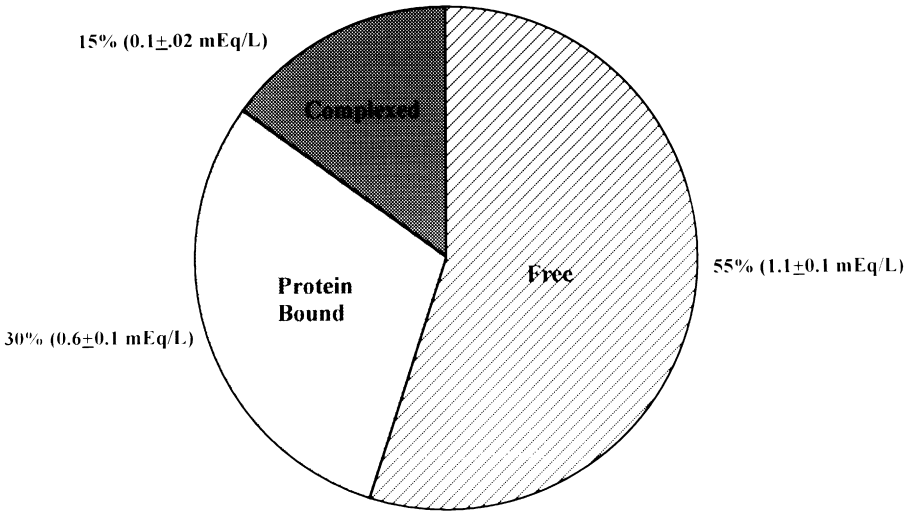

Figure 2 Physicochemical states of magnesium in normal plasma.

shown no effect (Brannan *et al.,* 1976; Fine *et al.,* 1991). The presence of excessive amounts of substances such as free fatty acids, phytate, oxalate, polyphosphates, and fiber may bind Mg and impair absorption (Seelig, 1981; Franz, 1989).

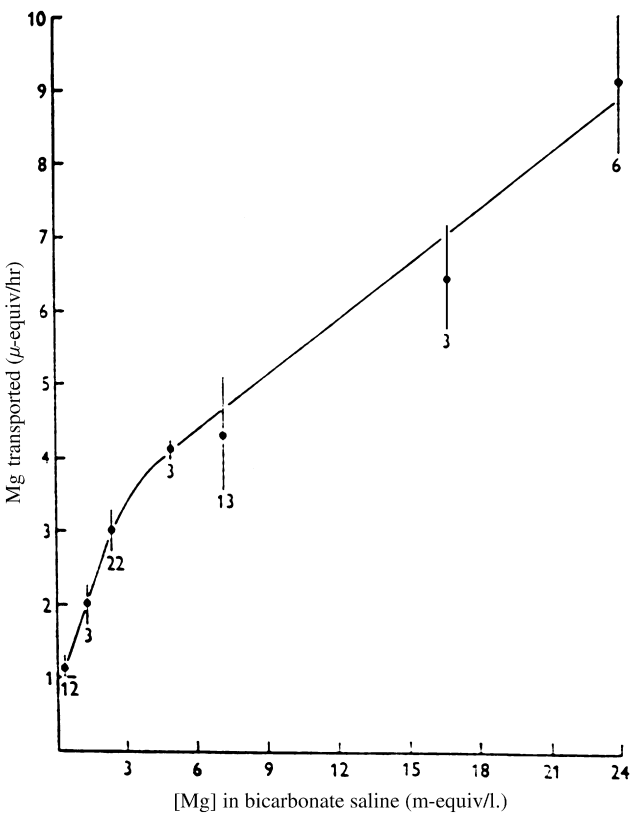

Figure 3 Relation between magnesium transported per hour and circulating luminal fluid magnesium concentration in rats. Values represent mean ± SEM. The number below shows number of experiments in the group (Ross, 1962).

Renal Mg Handling

The kidney is the principal organ involved in Mg homeostasis (Quamme and De Rouffignac, 2000; Cole and Quamme, 2000). During Mg deprivation in normal subjects, the kidney conserves Mg avidly and less than 1–2 mEq is excreted in the urine per day (Barnes *et al.,* 1958). Conversely, when excess Mg is taken, it is excreted into the urine rapidly (Heaton and Parson, 1961). The renal handling of Mg in humans is a filtration–reabsorption process; there appears to be no tubular secretion of Mg. Micropuncture studies of the nephron in several mammalian species have indicated that Mg is absorbed in the proximal tubule, thick ascending limb of Henle, and distal convoluted tubule (Quamme and De Rouffignac, 2000; Cole and Quamme, 2000), as illustrated in Fig. 4.

Approximately 15–20% of filtered Mg is reabsorbed in the proximal convoluted tubule. Current data suggest that Mg transport in this segment is reabsorbed passively through the paracellular pathway (Quamme and De Rouffignac, 2000; Cole and Quamme, 2000). The majority, approximately 65–75%, of filtered Mg is reclaimed in the loop of Henle with the major site at the cortical thick ascending limb. Magnesium transport in this segment appears to be dependent on the transepithelial potential generated by NaCl absorption (Quamme and De Rouffignac, 2000; Cole and Quamme, 2000). Micropuncture studies have also demonstrated that hypermagnesemia or hypercalcemia will decrease Mg reabsorption in this segment independent of NaCl transport (Quamme and De Rouffignac, 2000; Cole and Quamme, 2000). Studies suggest that the concentration of calcium and/or Mg in the extracellular fluid may regulate absorption of Mg in the thick ascending limb of Henle by activation of the Ca^{2+}-sensing receptor in this segment of the nephron (Brown and Hebert, 1995; Quamme and De Rouffignac, 2000; Cole and Quamme, 2000). Approximately 5–10% of Mg is reclaimed in the distal tubule where reabsorption is transcellular and active in

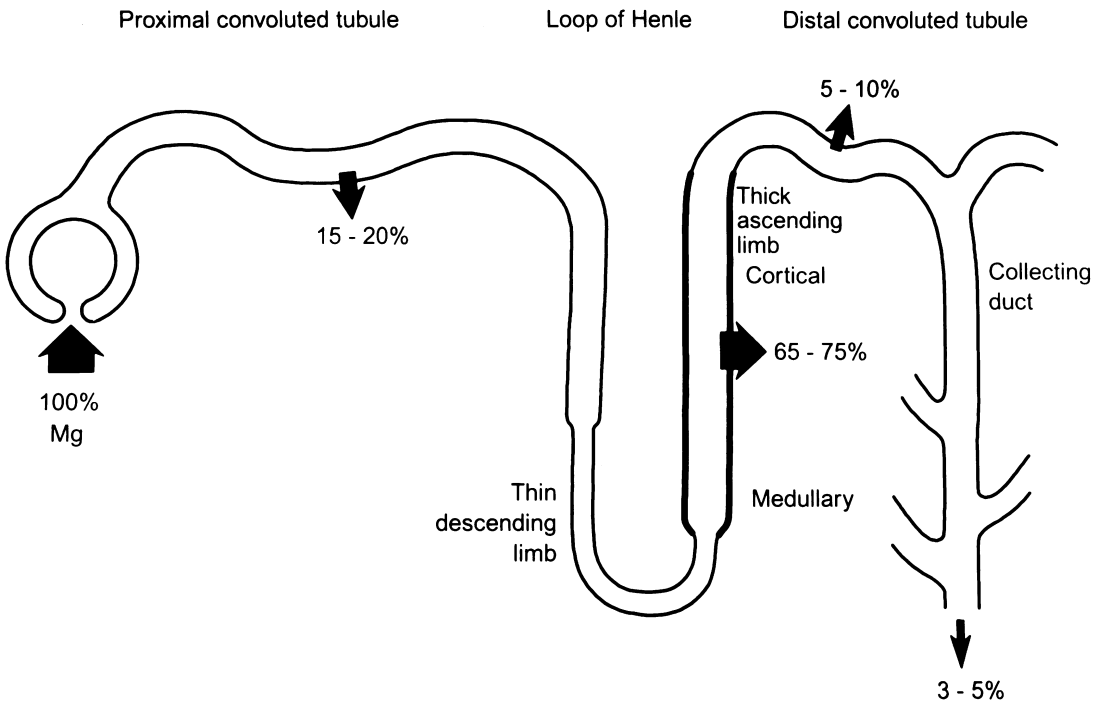

Figure 4 Summary of the tubular handling of magnesium. Schematic illustration of the cellular transport of magnesium within the thick ascending limb of the loop of Henle (Cole and Quamme, 2000).

nature (Quamme and De Rouffignac, 2000; Cole and Quamme, 2000). It is speculated that transport at this site may be regulated hormonally and serve to finely regulate Mg homeostasis.

Micropuncture studies performed during a time in which the concentration of Mg was increased gradually, in either the tubular lumen or in the extracellular fluid, have failed to demonstrate a tubular maximum for Mg (TmMg) in the proximal tubule (De Rouffignac and Quamme, 1994; Quamme, 2000). The rate of Mg reabsorption is dependent on the concentration of Mg in the tubule lumen. Similarly, a TmMg was not reached in the loop of Henle during a graduated increase in the luminal-filtered Mg load. Hypermagnesemia, as discussed earlier, however, results in a marked depression of Mg resorption in this segment. *In vivo* studies in animals and humans, however, have demonstrated a TmMg that probably reflects a composite of tubular reabsorption processes, as shown in Fig. 5 (Rude *et al.,* 1980; Rude and Ryzen, 1986).

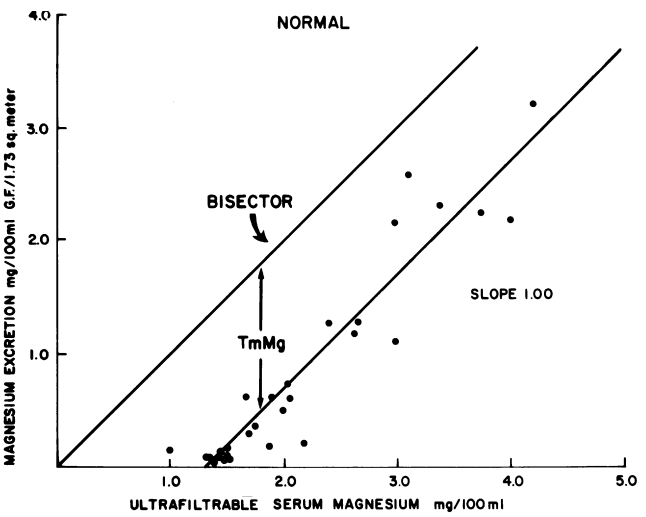

Figure 5 Urinary magnesium excretion is plotted against ultrafiltrable serum Mg in normal subjects before and during magnesium infusion. Data are related to a bisector that corresponds to the theoretical value of magnesium excretion if no magnesium were reabsorbed (Rude *et al.,* 1980).

During Mg deprivation, Mg virtually disappears from the urine (Barnes *et al.,* 1958). Despite the close regulation of Mg by the kidney, there has been no hormone or factor described that is responsible for renal Mg homeostasis. Micropuncture studies have shown that PTH changes the potential difference in the cortical thick ascending limb and increases Mg reabsorption (Quamme and De Rouffignac, 2000; Cole and Quamme, 2000). When given in large doses in humans or other species, PTH decreases urinary Mg excretion (Bethune *et al.,* 1968; Massry *et al.,* 1969). However, patients with either primary hyperparathyroidism or hypoparathyroidism usually have a normal serum Mg concentration and a normal TmMg, suggesting that PTH is not an important physiological regulator of Mg homeostasis (Rude *et al.,* 1980). Glucagon, calcitonin, and ADH also affect Mg transport in the loop of Henle in a manner similar to PTH (Quamme and De Rouffignac, 2000; Cole and Quamme, 2000); the physiological relevance of these actions is unknown. Little is known about the effect of vitamin D on renal Mg handling. An overall view of Mg metabolism is shown in Fig. 6.

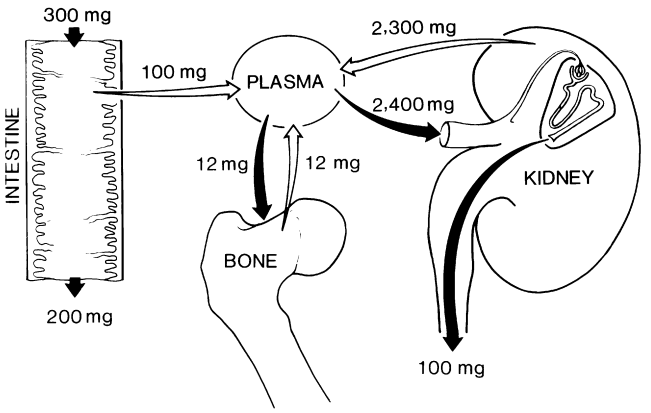

Figure 6 Schematic representation of magnesium metabolism.

Intracellular Mg

Within the cell, Mg is compartmentalized and most of it is bound to proteins and negatively charged molecules such as ATP, ADP, RNA, and DNA; in the cytoplasm, about 80% of Mg is complexed with ATP ((Gupta and Moore, 1980; Frausta da Silva and Wilkens, 1991; Romani *et al.,* 1993a). Significant amounts of Mg are found in the nucleus, mitochondria, and endoplasmic and sarcoplasmic reticulum as well as in the cytoplasm (Gunther, 1986; Romani *et al.,* 1993a). Total cell Mg concentration has been reported to range between 5 and 20 m*M* (Gunther, 1986; Romani *et al.,* 1993a). The concentration of free ionized Mg^{2+}, which has been measured in the cytoplasm of mammalian cells, has ranged from 0.2 to 1.0 m*M,* depending on cell type and means of measurement (Raju *et al.,* 1989; London, 1991; Romani and Scarpa, 1992; Romani *et al.,* 1993b). It constitutes 1–5% of the total cellular Mg. The Mg^{2+} concentration in the cell cytoplasm is maintained relatively constant even when the Mg^{2+} concentration in the extracellular fluid is experimentally varied to either high or low nonphysiological levels (Dai and Quamme, 1991; Quamme *et al.,* 1993; Romani *et al.,* 1993b). The relative constancy of the Mg^{2+} in the intracellular milieu is attributed to the limited permeability of the plasma membrane to Mg and to the operation of specific Mg transport systems, which regulate the rates at which Mg is taken up by cells or extruded from cells (Flatman, 1984; Romani *et al.,* 1993a; Murphy, 2000). Although the concentration differential between the cytoplasm and the extracellular fluid for Mg^{2+} is minimal, Mg^{2+} enters cells down an electrochemical gradient due to the relative electronegativity of the cell interior. Maintenance of the normal intracellular concentrations of Mg^{2+} requires that Mg be actively transported out of the cell (Murphy, 2000).

Studies in mammalian tissues and isolated cells suggest the presence of specific Mg transport systems. Early *in vivo* studies, using the radioactive isotope ^{28}Mg, suggested that tissues vary with respect to the rates at which Mg exchange occurs and the percentage of total Mg that is readily exchangeable (Rogers and Mahan, 1959). The rate of Mg exchange in heart, liver, and kidney exceeded that in skeletal muscle, red blood cells, brain, and testis (Romani *et al.,* 1993a). These studies do show that, albeit slow in some tissues, there is a continuous equilibration of Mg between cells and the extracellular fluid. An increased cellular Mg content has been reported for rapidly proliferating cells, indicating a possible relationship between the metabolic state of a cell and the relative rates of Mg transport into and out of cells (Cameron *et al.,* 1980).

Mg transport out of cells appears to require the presence of carrier-mediated transport systems, possibly regulated by the concentration of Mg^{2+} within the cell (Romani *et al.,* 1993a; Gunther, 1993). The efflux of Mg from the cell is coupled to Na transport and requires energy (Romani *et al.,* 1993a; Gunther, 1993; Murphy, 2000). Muscle tissue, which is incubated in isotonic sucrose, a low sodium buffer, or in the presence of ouabain as a metabolic inhibitor, has been shown to accumulate large amounts of Mg. These studies also suggest that the efflux of Mg from the cell is coupled with the movement of sodium down its electrochemical gradient into the cell. Maintenance of this process would require the subsequent extrusion of sodium by the Na^+,K^+-ATPase. There is also evidence for a Na-independent efflux of Mg, however (Gunther, 1993). Mg influx appears to be linked to Na and HCO_3 transport, but by a different mechanism than efflux (Gunther, 1993; Gunther and Hollriegl, 1993). The molecular characteristics of the Mg transport proteins have not been described. Studies in prokaryotes, however, have identified three separate transport proteins for Mg (Smith and Maguire, 1993).

Mg transport in mammalian cells is influenced by hormonal and pharmacological factors. Mg^{2+} efflux from isolated perfused rat heart and liver (Romani and Scarpa, 1990a,b; Gunther *et al.,* 1991; Gunther, 1993) or thymocytes (Gunther and Vormann, 1990) is stimulated after short-term acute exposure to β-agonists and permeant cAMP. Because intracellular Mg^{2+} does not change, a redistribution from the mitochondria was suggested, as cAMP can induce Mg^{2+} release from this compartment (Romani and Scarpa, 1992) or by altered buffering of Mg within the cell (Murphy, 2000). In contrast, Mg^{2+} influx was stimulated by β-agonists after a more prolonged exposure in hepatocytes, as well as in adipocytes and vascular smooth muscle, presumably mediated by protein kinase A (Zama and Towns, 1986; Ziegler *et al.,* 1992; Gunther, 1993; Romani *et al.,* 1993b). However, the rate of Mg uptake by the mouse lymphoma S49 cell line is inhibited by β-adrenergic agents (Maguire, 1984). Activation of protein kinase C by diacyl-glycerol or by phorbol esters also stimulates Mg^{2+} influx and does not alter efflux (Grubbs and Maguire, 1986; Romani *et al.,* 1993a).

Growth factors may also influence Mg^{2+} uptake by cells. Epidermal growth factor has been shown to increase Mg transport into a vascular smooth muscle cell line (Grubbs, 1991). Insulin and dextrose were found to increase ^{28}Mg uptake by a number of tissues, including skeletal and cardiac muscle, in which total cellular Mg content increased as

well (Lostroh and Krahl, 1973). Increased amounts of total intracellular Mg following treatment with insulin *in vitro* have been reported in uterine smooth muscle and chicken embryo fibroblasts (Aikawa, 1960; Sanui and Rubin, 1978). An insulin-induced transport of Mg into cells could be one factor responsible for the fall in the serum Mg concentration observed during insulin therapy of diabetic ketoacidosis (Kumar *et al.,* 1978). The effect of insulin on total cellular Mg may differ from its effects on intracellular-free Mg^{2+}. Measurements of intracellular-free Mg^{2+} in frog skeletal muscle failed to show an effect of insulin (Gupta and Moore, 1980); however, other studies demonstrated that insulin increases Mg^{2+} in human red blood cells, platelets, lymphocytes, and heart (Hwang *et al.,* 1993; Barbagallo *et al.,* 1993; Hua *et al.,* 1995; Romani *et al.,* 2000).

It is hypothesized that this hormonally regulated Mg uptake system controls intracellular Mg^{2+} concentration in cellular subcytoplasmic compartments. The Mg^{2+} concentration in these compartments would then serve to regulate the activity of Mg-sensitive enzymes.

Role of Magnesium in Bone and Mineral Homeostasis

Because of the prevalence of Mg in both cells and bone, as well as its critical need for numerous biological processes in the body, it is not surprising that this mineral plays a profound role in bone and mineral homeostasis. Our understanding of the role of magnesium has developed principally through observations of the effect of Mg depletion in both humans and animals. Mg influences the formation and/or secretion of hormones that regulate skeletal homeostasis and the effect of these hormones on bone. Mg can also directly affect bone cell function, as well as influence hydroxyapatite crystal formation and growth. These areas are discussed later and are outlined in Table III.

Parathyroid Hormone Secretion

Calcium is the major regulator of PTH secretion. Mg, however, modulates PTH secretion in a manner similar to calcium. A number of *in vitro* and *in vivo* studies have demonstrated that acute elevations of Mg inhibit PTH secretion, whereas an acute reduction stimulates PTH secretion (Sherwood, 1970; Cholst *et al.,* 1984; Ferment *et al.,* 1987; Toffaletti *et al.,* 1991 Rude, 1994). These data suggest that Mg could be a physiologic regulator of PTH secretion. While early investigations indicated that Mg was equipotent to calcium in its effect on parathyroid gland function (Sherwood, 1970), more recent studies demonstrated that Mg has approximately 30–50% the effect of calcium on either stimulating or inhibiting PTH secretion (Wallace and Scarpa, 1982; Ferment *et al.,* 1987; Toffaletti *et al.,* 1991; Rude, 1994). The finding in humans that a 5% (0.03 m*M*) decrease in serum ultrafilterable Mg did not result in any detectable change in intact serum PTH concentration while a 5.5% (.07 m*M*) decrease in ionized calcium resulted in a 400% increase in serum PTH supports this concept (Toffaletti *et al.,* 1991).

The inhibitory effects of Mg on PTH secretion may be dependent on the extracellular calcium concentration (Brown *et al.,* 1984). At physiological calcium and Mg concentrations, these divalent cations were found to be relatively equipotent at inhibiting PTH secretion from dispersed bovine parathyroid cells (Brown *et al.,* 1984). At a low calcium concentration (0.5 m*M*), however, a threefold greater Mg concentration was required for similar PTH inhibition. Altering the Mg concentration did not diminish the ability of calcium to inhibit PTH secretion. Differences have also been noted in the effect of Mg and calcium on the biosynthesis of PTH *in vitro.* Changes in calcium over the range of 0 to 3.0 m*M* resulted in increased PTH synthesis (Hamilton *et al.,* 1971; Lee and Roth, 1975), whereas changes in Mg over the range of 0 to 1.7 m*M* had no effect.

Table III Effect of Mg Depletion on Bone and Mineral Metabolism

Effect	Potential mechanism(s)
1. Decreased PTH secretion	Altered phosphoinositol activity Decreased adenylate cyclase activity
2. Decreased PTH action	Decreased adenylate cyclase activity Altered phosphoinositol activity
3. Decreased serum $1,25(OH)_2D$	Decreased serum PTH Renal PTH resistance (decrease in 1α-hydroxylase activity)
4. Impaired vitamin D metabolism and action	Decreased $1,25(OH)_2D$ formation Decreased intestinal epithelial cell and osteoblast activity Skeletal resistance to vitamin D
5. Impaired bone growth/osteoporosis	Decreased PTH and $1,25(OH)_2D$ formation and action Decreased effect of insulin and IGF-1 Direct effect to decrease bone cell activity Increased cytokine production
6. Altered hydroxyapatite crystal formation	Impaired calcium binding to hydroxyapatite Directly alter crystal growth

The effect of Mg on PTH secretion appears to act through the Ca^{2+}-sensing receptor, which mediates the control of extracellular calcium on PTH secretion (Brown *et al.,* 1993). Mg^{2+} was shown to bind to this receptor, but with much less efficiency than Ca^{2+} (Herbert, 1996). Mg may also regulate calcium transport into the cell through other ion channels (Miki *et al.,* 1997). Acute changes in the serum Mg concentration may therefore modulate PTH secretion and should be considered in the evaluation of the determination of serum PTH concentrations.

Mg Depletion and Parathyroid Gland Function

While acute changes in extracellular Mg concentrations will influence PTH secretion qualitatively similar to calcium, it is clear that Mg deficiency markedly perturbs mineral homeostasis (Rude *et al.,* 1976; Rude, 1994). Hypocalcemia is a prominent manifestation of Mg deficiency in humans (Rude *et al.,* 1976; Rude, 1994), as well as in most other species (Shils, 1980; Anast and Forte, 1983). In humans, Mg deficiency must become moderate to severe before symptomatic hypocalcemia develops. A positive correlation has been found between serum Mg and calcium concentrations in hypocalcemic hypomagnesemic patients (Rude *et al.,* 1976). Mg therapy alone restored serum calcium concentrations to normal in these patients within days (Rude *et al.,* 1976). Calcium and/or vitamin D therapy will not correct the hypocalcemia (Rude *et al.,* 1976; Rude, 1994). Even mild degrees of Mg depletion, however, may result in a significant fall in the serum calcium concentration, as demonstrated in experimental human Mg depletion (Fatemi *et al.,* 1991). One major factor resulting in the fall in serum calcium is impaired parathyroid gland function. Low Mg in the media of parathyroid cell cultures impairs PTH release in response to a low media calcium concentration (Targovnik *et al.,* 1971). Determination of serum PTH concentrations in hypocalcemic hypomagnesemic patients has shown heterogeneous results. The majority of patients have low or normal serum PTH levels (Anast *et al.,* 1972; Suh *et al.,* 1973; Chase and Slatopolsky, 1974; Rude *et al.,* 1976, 1978). Normal serum PTH concentrations are thought to be inappropriately low in the presence of hypocalcemia. Therefore, a state of hypoparathyroidism exists in most hypocalcemic Mg-deficient patients. Some patients, however, have elevated levels of PTH in the serum (Rude *et al.,* 1976, 1978; Algrove *et al.,* 1984). The administration of Mg will result in an immediate rise in the serum PTH concentration regardless of the basal PTH level (Anast *et al.,* 1972; Rude *et al.,* 1976, 1978). As shown in Fig. 7, 10 mEq of Mg administered intravenously over 1 min caused an immediate marked rise in serum PTH in patients with low, normal, or elevated basal serum PTH concentrations. This is distinctly different than the effect of a Mg injection in normal subjects where, as discussed earlier, Mg will cause an inhibition of PTH secretion (Cholst *et al.,* 1984; Fatemi *et al.,* 1991). The serum PTH concentration will gradually fall to normal within several days of therapy with return of the serum calcium concentration to normal (Anast *et al.,*

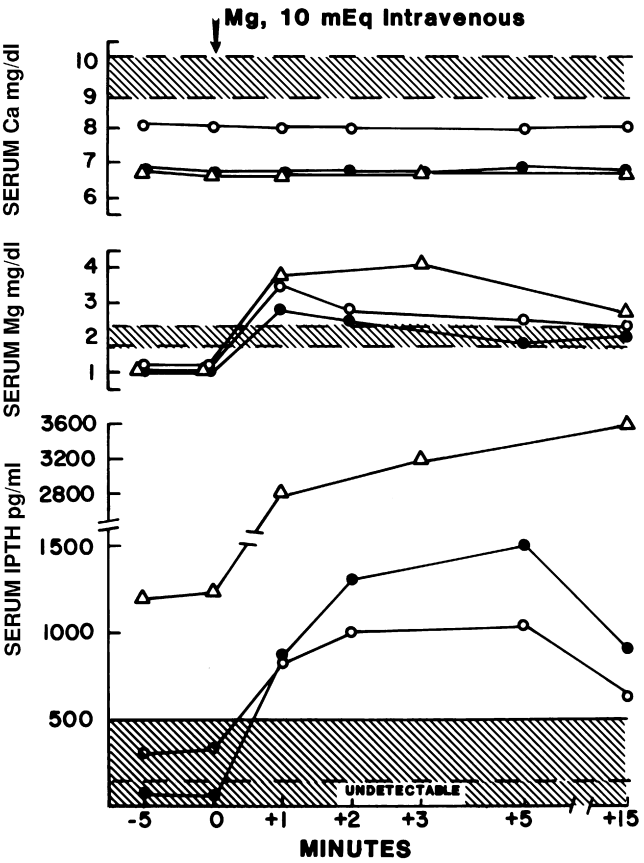

Figure 7 The effect of an IV injection of 10 mEq Mg on serum concentrations of calcium, magnesium, and immunoreactive parathyroid hormone (IPTH) in hypocalcemic magnesium-deficient patients with undetectable (●), normal (○), or elevated (Δ) levels of IPTH. Shaded areas represent the range of normal of each assay. The broken line for the IPTH assay represents the level of detectability. The magnesium injection resulted in a marked rise in PTH secretion within 1 min in all three patients (Rude *et al.,* 1978).

1972; Rude *et al.,* 1976, 1978). The impairment in PTH secretion appears to occur early in Mg depletion. Normal human subjects placed experimentally on a low Mg diet for only 3 weeks showed similar but not as marked changes in the serum PTH concentrations (Fatemi *et al.,* 1991) in which there was a fall in both serum calcium and PTH concentrations in 20 of 26 subjects at the end of the dietary Mg deprivation period. The administration of intravenous Mg at the end of this Mg depletion period resulted in a significant rise in the serum PTH concentration qualitatively similar to that observed in hypocalcemic Mg-depleted patients shown in Fig. 7, whereas a similar Mg injection suppressed PTH secretion prior to the low Mg diet. In this study, as with hypocalcemia hypomagnesemic patients, some subjects had elevations in the serum PTH concentration. The heterogeneous serum PTH values may be explained on the severity of Mg depletion. As the serum Mg concentration falls, the parathyroid gland will react normally with an increase in PTH secretion. As intracellular Mg depletion develops, however, the ability of the parathyroid to secrete PTH is impaired, resulting in a fall in serum PTH levels with a resultant fall in the serum calcium

concentration. This concept is supported by the observation that the change in serum PTH in experimental human Mg depletion is correlated positively with the fall in red blood cell intracellular-free Mg^{2+} (Fatemi *et al.,* 1991). A slight fall in red blood cell Mg^{2+} resulted in a increase in PTH. However, a greater decrease in red blood cell Mg^{2+} correlated with a progressive fall in serum PTH concentrations.

It is conceivable that either PTH synthesis and/or PTH secretion may be affected. However, as the *in vitro* biosynthesis of PTH requires approximately 45 min (Hamilton *et al.,* 1971), the immediate rise in PTH following the administration of intravenous magnesium to Mg-deficient patients strongly suggests that the defect is in PTH secretion.

Mg Depletion and Parathyroid Hormone Action

The above discussion strongly supports the notion that impairment in the secretion of PTH in Mg deficiency is a major contributing factor in the hypocalcemia. However, the presence of normal or elevated serum concentrations of PTH in the face of hypocalcemia (Rude *et al.,* 1976, 1978; Rude, 1994) suggests that there may also be end organ resistance to PTH action. In hypocalcemic Mg-deficient patients treated with Mg, the serum calcium concentration does not rise appreciably within the first 24 hrs, despite elevated serum PTH concentrations (Rude *et al.,* 1976; Rude, 1994), which also suggests skeletal resistance to PTH because exogenous PTH administered to hypoparathyroid patients causes a rise in the serum calcium within 24 hrs (Bethune *et al.,* 1968). Clinical studies have reported resistance to exogenous PTH in hypocalcemic Mg-deficient patients (Estep *et al.,* 1969; Woodard *et al.,* 1972; Rude *et al.,* 1976; Rude, 1994). In one study, the parathyroid extract did not result in elevation in the serum calcium concentration or urinary hydroxyproline excretion in hypocalcemic hypomagnesemic patients as shown in Fig. 8 (Estep *et al.,* 1969). Following Mg repletion, however, a clear response to PTH was observed. PTH has also been shown to have a reduced calcemic effect in Mg-deficient animals (MacManus *et al.,* 1971; Levi *et al.,* 1974; Forbes and Parker, 1980). The ability of PTH to resorb bone *in vitro* is also diminished greatly in the presence of low media Mg (Raisz and Niemann, 1969). In one study of isolated perfused femur in the dog, the ability of PTH to simulate an increase in the venous cyclic AMP was impaired during perfusion with low Mg fluid, suggesting skeletal PTH resistance (Freitag *et al.,* 1979). Not all studies have shown skeletal resistance to PTH, however (Salet *et al.,* 1966; Stromme *et al.,* 1969; Suh *et al.,* 1973, Chase and Slatopolsky, 1974). It appears likely that skeletal PTH resistance may be observed in patients with more severe degrees of Mg depletion. Patients in whom a normal calcemic response to PTH was demonstrated were in subjects who had been on recent Mg therapy (Salet *et al.,* 1966; Stromme *et al.,* 1969; Suh *et al.,* 1973, Chase and Slatopolsky, 1974). Patients who have been found to be resistant to PTH have, in general, not had prior Mg administration (Estep *et al.,* 1969; Woodard *et al.,* 1972; Rude *et al.,* 1976; Rude, 1994). Consistent with this notion is that in the Mg-depleted rat, normal responses to PTH were observed when the serum Mg concentration was 0.95 mg/dl (Hahn *et al.,* 1972); however, in another study, rats with a mean serum Mg of 0.46 mg/dl were refractory to PTH (MacManus *et al.,* 1971). In addition, a longitudinal study of Mg deficiency in dogs demonstrated a progressive decline in responsiveness to PTH with increasing degrees of Mg depletion (Levi *et al.,* 1974).

Calcium release from the skeleton also appears to be dependent on physicochemical processes as well as cellular activity (Pak and Diller, 1970; MacManus and Heaton, 1970). Low Mg will result in a decrease in calcium release from bone (Pak and Diller, 1970; MacManus and Heaton, 1970) and may be another mechanism for hypocalcemia in Mg deficiency.

The renal response to PTH has also been assessed by determining the urinary excretion of cyclic AMP and/or phosphate (Figs. 8 and 9) in response to exogenous PTH. In

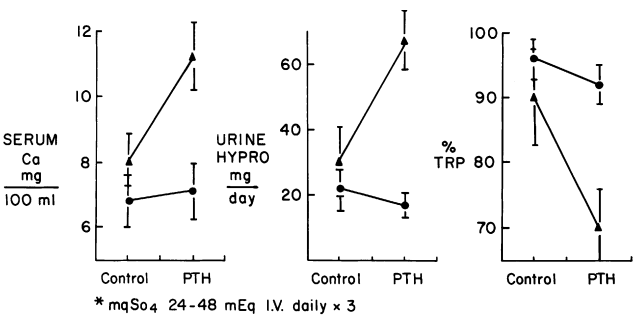

Figure 8 Mean and standard deviations of serum calcium concentration and urinary hydroxyproline and phosphate excretion in hypocalcemic magnesium-deficient patients before (●) and after (▲) 3 days of parenteral magnesium therapy (Estep *et al.,* 1969).

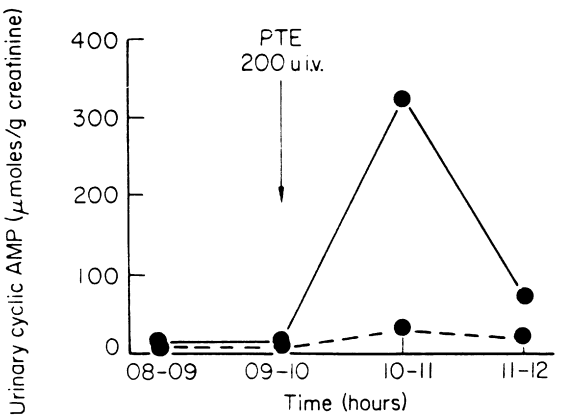

Figure 9 The effect of an IV injection of 200 units of parathyroid extract on the excretion of urinary cyclic AMP in a magnesium-deficient patient before (●---●) and after (●—●) 4 days of magnesium therapy. Urine was collected for four consecutive 1-hr periods, two before and two after the PTE injection. While Mg deficient, the patient had a minimal rise in urinary cyclic AMP in response to PTH, but following Mg therapy the response was normal (Rude *et al.,* 1976).

some patients, a normal effect of PTH on urinary phosphate and cyclic AMP excretion has been noted (Anast *et al.,* 1972; Suh *et al.,* 1973; Chase and Slatopolsky, 1974). In general, these were the same subjects in which a normal calcemic effect was also seen (Anast *et al.,* 1972; Suh *et al.,* 1973; Chase and Slatopolsky, 1974). In other studies, with more severely Mg-depleted patients, an impaired response to PTH has been observed (Estep *et al.,* 1969; Rude *et al.,* 1976; Medalle and Waterhouse, 1973; Rude, 1994). A decrease in urinary cyclic AMP excretion in response to PTH has also been described in the Mg-deficient dog and rat (Levi *et al.,* 1974; Forbes and Parker, 1980).

Mechanism of Impaired Mineral Homeostasis in Mg Depletion

The mechanism for impaired PTH secretion and action in Mg deficiency remains unclear. It has been suggested that there may be a defect in the second messenger systems in Mg depletion. PTH is thought to exert is biologic effects through the intermediary action of cyclic AMP (Bitensky *et al.,* 1973; Neer, 1995). Adenylate cyclase has been universally found to require Mg for cyclic AMP generation, both as a component of the substrate (Mg-ATP) and as an obligatory activator of enzyme activity (Northup *et al.,* 1982). There appears to be two Mg^{2+}-binding sites within the adenylate cyclase complex: one resides on the catalytic subunit and the other on the guanine nucleotide regulatory protein, Ns (Cech *et al.,* 1980; Maguire, 1984). The requisite role that Mg^{2+} plays in adenylate cyclase function suggests that factors that would limit the availability of Mg^{2+} to this enzyme could have significant effects on the cyclic nucleotide metabolism of a cell and hence overall cellular function. It is clear that some patients with severe Mg deficiency have a reduced urinary excretion of cyclic AMP in response to exogenously administered PTH (Rude *et al.,* 1976). In addition, PTH was shown to have a blunted effect in causing a rise in cyclic AMP from isolated perfused tibiae in Mg-deficient dogs (Freitag *et al.,* 1979). These observations correspond well with the impaired calcemic and phosphaturic effects of PTH in Mg-deficient patients and animals as discussed earlier.

While Mg^{2+} is stimulatory for adenylate cyclase, Ca^{2+} may inhibit or activate enzyme activity (Sunahara *et al.,* 1996). Nine isoforms of adenylate cyclase have been identified whose activities are modulated by both Mg^{2+} and Ca^{2+} (Sunahara *et al.,* 1996). In plasma membranes from parathyroid, renal cortex, and bone cells, Ca^{2+} will competitively inhibit Mg^{2+}-activated adenylate cyclase activity (Rude, 1983, 1985; Oldham *et al.,* 1984). In parathyroid plasma membranes, at a Mg^{2+} concentration of 4 m*M,* Ca^{2+} was found to inhibit adenylate cyclase in a bimodal pattern described in terms of two calcium inhibition constants with K_i values of 1–2 and 200–400 μM (Oldham *et al.,* 1984). At a lower Mg^{2+} concentration (0.5 m*M*) the only adenylate cyclase activity expressed was that inhibitable by the high-affinity Ca^{2+}-binding site. With increasing Mg concentrations, the fraction of total adenylate cyclase activity subject to high-affinity calcium inhibition became progressively less. Thus, the ambient Mg^{2+} concentrations can markedly affect the susceptibility of this enzyme to the inhibitory effects of Ca^{2+}. Total intracellular calcium has been observed to rise during Mg depletion (George and Heaton, 1975; Ryan and Ryan, 1979). Mg is not only important for the operation of Mg^{2+}, Ca^{2+}-dependent ATPase, but may also be countertransported during the uptake and release of calcium through calcium channels (Romani and Scarpa, 1992; Romani *et al.,* 1993a). The combination of higher intracellular Ca^{2+} and increased sensitivity to Ca^{2+} inhibition due to Mg depletion could explain the defective PTH secretion in Mg deficiency. An increase in the release of intracellular Ca^{2+} via the phosphoinositol system is also possible, as discussed later. A similar relationship between Mg^{2+} and Ca^{2+} was described for adenylate cyclase obtained from bone (Rude, 1985). Ca^{2+} caused a competitive inhibition of Mg^{2+}-activated skeletal adenylate cyclase with a high-affinity Ca^{2+}-binding site with a K_iCa of 1–2 μM. Lowering the Mg^{2+} concentration increased overall Ca^{2+} inhibition. Thus, a fall in the intracellular Mg^{2+} concentration would render the adenylate cyclase enzyme more susceptible to inhibition by the prevailing intracellular Ca^{2+} concentrations and may be a mechanism by which both PTH secretion and PTH end organ action are compared in Mg deficiency.

Adenylate cyclase is a widely distributed enzyme in the body, and if the hypothesis just given were true, the secretion and action of other hormones mediated by adenylate cyclase might also exhibit impaired activity in Mg deficiency. This has not been found to be true, as the actions of ACTH, TRH, GnRH, and glucagon are normal in Mg depletion (Cohan *et al.,* 1982). Prior investigations have suggested that Mg affinity for adenylate cyclase is higher (lower K_aMg) in liver, adrenal, and pituitary than in parathyroid (for reviews, see Rude and Oldham, 1985; Rude, 1994). In one study, investigation of K_aMg and K_iCa in tissues from one species (guinea pig) demonstrated that under agonist stimulation the K_aMg from liver $<$ thyroid $<$ kidney $=$ bone and the $K_i Ca^{2+}$ for liver $>$ renal $>$ kidney $=$ bone (Rude and Oldham, 1985). These data suggest that adenylate cyclase regulation by divalent cations varies from tissue to tissue and may explain the greater propensity for disturbed mineral homeostasis in Mg deficiency.

While cyclic AMP is an important mediator of PTH action, current studies do not suggest an important role in mediating Ca^{2+}-regulated PTH secretion (Brown, 1991; Dunlay and Hruska, 1990). PTH has been shown to activate the phospholipase C second messenger system (Dunlay and Hruska, 1990). PTH activation of phospholipase C leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 binds to specific receptors on intracellular organelles (endoplasmic reticulum, calciosomes), leading to an acute transient rise in cytosolic Ca^{2+} with a subsequent activation of calmodulin-dependent protein kinases. Diacylglycerol

activates protein kinase C. Mg depletion could perturb this system via several mechanisms. First, a Mg^{2+}- dependent guanine nucleotide-regulating protein is also involved in the activation of phospholipase C (Babich *et al.,* 1989; Litosch, 1991). Mg^{2+} has also been shown to be a noncompetitive inhibitor of IP_3-induced Ca^{2+} release (Volpe *et al.,* 1990). A reduction of Mg^{2+} from 300 to 30 μM increased Ca^{2+} release in response to IP_3 by two-to threefold in mitochondrial membranes obtained from canine cerebellum (Volpe *et al.,* 1990). The Mg concentration required for a half-maximal inhibition of IP_3-induced Ca^{2+} release was 70 μU. In these same studies, Mg^{2+} was also found to inhibit IP_3 binding to its receptor. Mg, at a concentration of 500 μM, decreased maximal IP_3 binding threefold (IC_{50} 200 μM) (Volpe *et al.,* 1990). These Mg^{2+} concentrations are well within the estimated physiologic intracellular range (200–500 μM) and therefore Mg^{2+} may be an important physiological regulator of the phospholipase C second messenger system.

The effect of Mg depletion on cellular function in terms of the second messenger systems is most complex, potentially involving substrate availability, G protein activity, release and sensitivity to intracellular Ca^{2+}, and phospholipid metabolism.

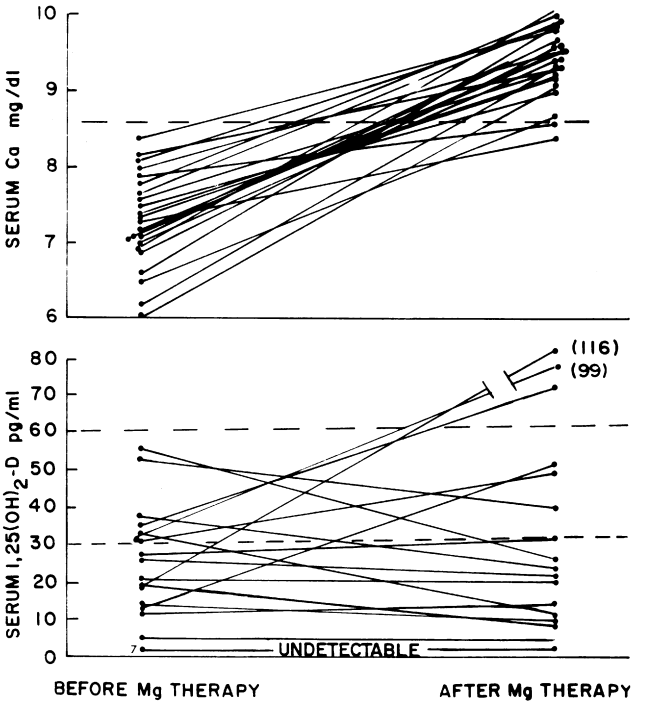

Figure 10 Serum concentrations of calcium and 1,25-diydroxyvitamin D in hypocalcemic magnesium-deficient patients before and after 5–8 days of parenteral magnesium therapy. The broken line represents the upper and lower limits of normal for serum 1,25-dihydroxyvitamin D and the lower limit of normal for the serum calcium (Rude *et al.,* 1985).

Mg Depletion and Vitamin D Metabolism and Action

Mg may also be important in vitamin D metabolism and/or action. Patients with hypoparathyroidism, malabsorption syndromes, and rickets have been reported to be resistant to therapeutic doses of vitamin D until Mg was administered simultaneously (for review, see Rude, 1994). Patients with hypocalcemia and Mg deficiency have also been reported to be resistant to pharmacological doses of vitamin D (Medalle *et al.,* 1976; Leicht *et al.,* 1990), 1α hydroxyvitamin D (Ralston *et al.,* 1983; Selby *et al.,* 1984) and 1,25-dihydroxyvitamin D (Graber and Schulman, 1986). Similarly, an impaired calcemic response to vitamin D has been found in Mg-deficient rats (Lifshitz *et al.,* 1967), lambs (McAlleese and Forbes, 1959), and calves (Smith, 1958).

The exact nature of altered vitamin D metabolism and/or action in Mg deficiency is unclear. Intestinal calcium transport in animal models of Mg deficiency has been found to be reduced in some (Higuchi and Lukert, 1974) but not all (Coburn *et al.,* 1975) studies. Calcium malabsorption was associated with low serum levels of 25-hydroxyvitamin D in one study (Lifshitz *et al.,* 1967), but not in another (Coburn *et al.,* 1975), suggesting that Mg deficiency may impair intestinal calcium absorption by more than one mechanism. Patients with Mg deficiency and hypocalcemia frequently have low serum concentrations of 25-hydroxyvitamin D (Rude *et al.,* 1985; Fuss *et al.,* 1989) and therefore nutritional vitamin D deficiency may be one factor. Therapy with vitamin D, however, results in high serum levels of 25-hydroxyvitamin D without correction of the hypocalcemia (Medalle *et al.,* 1976), suggesting that the vitamin D nutrition is not the major reason. In addition, conversion of radiolabeled vitamin D to 25-hydroxyvitamin D was found to be normal in three Mg-deficient patients (Lukert, 1980). Serum concentrations of 1,25-dihydroxyvitamin D have also been found to be low or low normal in most hypocalcemic Mg-deficient patients (Rude *et al.,* 1985; Fuss *et al.,* 1989; Leicht *et al.,* 1992). Mg-deficient diabetic children, when given a low calcium diet, did not exhibit the expected normal rise in serum 1,25-dihydroxyvitamin D or PTH (Saggese *et al.,* 1988); the response returned to normal following Mg therapy (Saggese *et al.,* 1991). Because PTH is a major trophic for 1,25-dihydroxyvitamin D formation, the low serum PTH concentrations could explain the low 1,25-dihydroxyvitamin D levels. In support of this is the finding that some hypocalcemic Mg-deficient patients treated with Mg have a rise in serum 1,25-dihydroxyvitamin D to high normal or to frankly elevated levels, as shown in Fig. 10 (Rude *et al.,* 1985). Most patients, however, do not have a significant rise within 1 week after institution of Mg therapy, despite a rise in serum PTH and normalization of the serum calcium concentration (Fig. 10) (Rude *et al.,* 1985). These data suggest that Mg deficiency in humans also impairs the ability of the kidney to synthesize 1,25-dihydroxyvitamin D. This is supported by the observation that the ability of exogenous administration of 1-34 human PTH to normal subjects after 3 weeks of experimental Mg depletion resulted in a significantly lower rise in serum 1,25-dihydroxyvitamin D concentrations than before institution of the diet (Fatemi *et al.,* 1991). It appears, therefore, that the renal synthesis of 1,25-dihydroxyvitamin

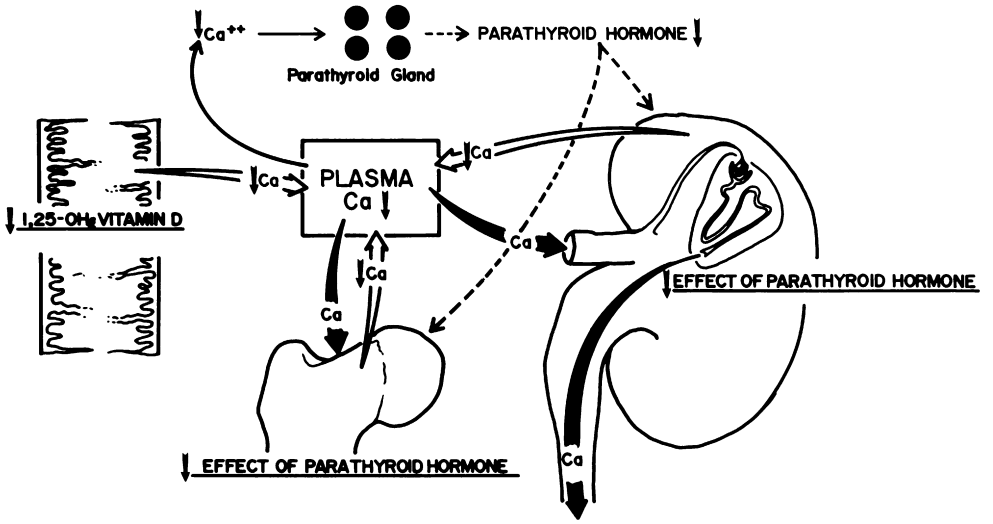

Figure 11 Disturbance of calcium metabolism during magnesium deficiency. Hypocalcemia is caused by a decrease in PTH secretion, as well as renal and skeletal resistance to the action of PTH. Low serum concentrations of 1,25-dihydroxyvitamin D may result in reduced intestinal calcium absorption (Rude and Oldham, 1990).

D is sensitive to Mg depletion. While Mg is known to support 25-hydroxy-1α-hydroxylase *in vitro* (Fisco and Traba, 1992), the exact Mg requirement for this enzymatic process is not known.

The association of Mg deficiency with impaired vitamin D metabolism and action therefore may be due to several factors, including vitamin D deficiency (Rude *et al.,* 1985; Carpener, 1988; Fuss *et al.,* 1989; Leich and Biro, 1992) and a decrease in PTH secretion (Anast *et al.,* 1972; Such *et al.,* 1973; Chase and Slatopolsky, 1974; Rude *et al.,* 1976, 1978), as well as a direct effect of Mg depletion on the ability of the kidney to synthesize 1,25-dihydroxyvitamin D (Rude *et al.,* 1985; Fuss *et al.,* 1989; Fatemi *et al.,* 1991). In addition, Mg deficiency may directly impair intestinal calcium absorption (Higuchi and Lukert, 1974; Rude *et al.,* 1976, 1985). Skeletal resistance to vitamin D and its metabolites may also play an important role (Lifshitz *et al.,* 1967; Ralston *et al.,* 1983; Selby *et al.,* 1984; Graber and Schulman, 1986). It is clear, however, that the restoration of normal serum 1,25-dihydroxyvitamin D concentrations is not required for normalization of the serum calcium level (Fig. 9). Most Mg-deficient patients who receive Mg therapy exhibit an immediate rise in PTH, followed by normalization of the serum calcium prior to any change in serum 1,25-dihydroxyvitamin D concentrations (Rude *et al.,* 1985; Fuss *et al.,* 1989). An overall view of the effect of Mg depletion on calcium metabolism is shown in Fig. 11.

Magnesium Depletion: Skeletal Growth and Osteoporosis

Women with postmenopausal osteoporosis have decreased nutrition markers, suggesting that osteoporosis is associated with nutritional deficiencies (Rico *et al.,* 1993). While low calcium intake is one of these nutritional factors (Rico *et al.,* 1993), a large segment of our population also has low dietary Mg intake (Morgan *et al.,* 1985; Marier, 1986). Mg deficiency, when severe, will disturb calcium homeostasis markedly, resulting in impaired PTH secretion and PTH end organ resistance, leading to hypocalcemia (Rude, 1998). Mg exists in macronutrient quantities in bone, and long-term mild-to-moderate dietary Mg deficiency has been implicated as a risk factor for osteoporosis.

EPIDEMIOLOGICAL STUDIES

Epidemiologic studies have provided a major link associating dietary Mg inadequacy to osteoporosis. One cross-sectional study assessed the effect of dietary nutrients on appendicular (radius, ulna, and heel) bone mineral density (BMD) in a large group of Japanese-Americans living in Hawaii (Yano *et al.,* 1985). In 1208 males (age 61–81), whose mean Mg intake was 238 $\pm$ 111 mg/day, no correlation of Mg intake with BMD was observed at any site. In a subgroup of 259 of these subjects who took Mg supplements, however (mean Mg intake of 381 mg/day), BMD was correlated positively with Mg intake at one or more skeletal sites. In 912 females (age 43–80) whose Mg intake was 191 $\pm$ 36 mg/day, a positive correlation with BMD was also observed. In contrast to males, no correlation with BMD was found in females who took Mg supplements (total Mg intake of 321 mg/day). In a smaller study of women aged 35–65 (17 premenopausal, mean Mg intake 243 $\pm$ 44 mg/day; 67 postmenopausal, mean Mg intake 249 $\pm$ 68 mg/day) in which BMD was measured in the distal forearm, no cross-sectional correlation was observed with Mg intake in either group (Freudenheim *et al.,* 1986). Longitudinal observation over 4 years, however, demonstrated that loss of bone mass was related inversely to Mg intake in premenopausal women ($p < 0.05$) and had a similar trend in the postmenopausal group ($p < 0.085$). In another cross-sectional study, a positive correlation of BMD of the forearm (but not femur or spine) was found in a larger group of 89 premenopausal

women (age 37.8 ± 0.8; Mg intake of 243 ± 9 mg/day), but no similar correlation was found in 71 recently menopausal women age 58.9 ± 0.9 (Mg intake of 253 ± 11 mg/day) (Angus *et al.,* 1988). In contrast, a study of 194 older postmenopausal women (age 69–97; mean Mg intake of 288 mg/day) demonstrated a significant positive correlation with BMD of the forearm (Tranquilli *et al.,* 1994).

Studies have concentrated on BMD of the axial skeleton. Sixty-six premenopausal women (age 28–39) whose Mg intake was 289 ± 73 mg/day had a significant relationship between dietary Mg intake and rate of change of BMD of the lumbar spine and total body calcium over a 1-year period (Houtkooper *et al.,* 1995). A cross-sectional study that combined 175 premenopausal and postmenopausal women aged 28–74 (mean Mg intake was 262 ± 70 mg/day) found no correlation with BMD at the lumbar spine, femoral neck, or total body calcium (Michaelsson *et al.,* 1995). In a study of 994 premenopausal women aged 45–49, whose Mg intake was 311 ± 85 mg/day, New *et al.* (1997) did find a significant correlation of BMD of the lumbar spine with Mg intake. A significant difference was also observed in lumbar spine BMD between the highest and the lowest quartiles of dietary Mg intake. A report by this same group in a study of 65 pre- and postmenopausal women aged 45–55 again found higher bone mass of the forearm (but not femoral neck or hip) in subjects consuming a Mg intake of 326 ± 90 mg/day (New *et al.,* 2000). Women with a high childhood intake of fruits (Mg and potassium) did have higher femoral neck BMD than those on a lower fruit intake, however. Another cross-sectional study assessed Mg intake in older males and females (age 69–97) (345 males and 562 females), as well as a 2-year longitudinal study of a subset of these subjects (229 males and 399 females) (Tucker *et al.,* 1999). In the cross-sectional analysis in males, Mg intake (300 ± 110 mg/day) was correlated with BMD of the radius and hip. In the 4-year longitudinal study of these subjects, a positive inverse relationship between bone loss of the hip and Mg intake was observed. A positive cross-sectional correlation of BMD of the hip was also observed in females (Mg intake of 288 ± 106 mg/day), but not in the longitudinal assessment. Finally, dietary Mg intake in a large population of non-Hispanic white males and females from the NHANES III database was found by multiple regression analysis to predict BMD at several sites in the proximal femur (Carpenter *et al.,* 2000).

In a study of younger individuals, the effect of dietary Mg intake of preadolescence girls (age 9–11) on bone mass/quality in these young women was evaluated at age 18–19 (Wang *et al.,* 1999). Ultrasound determination of bone mass of the calcaneus in 35 black women (Mg intake 237 ± 83 mg/day) and in 26 white women (Mg intake 240 ± 61 mg/day) was performed. Mg intake was related positively to quantitative ultrasound properties of bone, suggesting that this nutrient was important in skeletal growth and development.

In summary, these epidemiological studies link dietary Mg intake to bone mass. Exceptions appear to include women in the early postmenopausal period in which the effect of acute sex steroid deficiency may mask the effect of dietary factors such as Mg. In addition, diets deplete in Mg are usually deficient in other nutrients, which affect bone mass as well. Therefore, further investigations are needed to provide a firm relationship of dietary Mg inadequacy with osteoporosis.

Bone Turnover

In two of the epidemiological studies cited earlier, markers of bone turnover were determined. In one, where no correlation was found between BMD and dietary Mg intake, serum osteocalcin did not correlate with Mg (or any other nutrient) intake (Michaelsson *et al.,* 1995). New *et al.* (2000) also found that serum osteocalcin was not associated with the dietary intake of Mg or other nutrients. Mg intake, however, was significantly negatively correlated with the urinary excretion of pyridinoline and deoxypyridinoline, suggesting that a low Mg diet was associated with increased bone resorption (New *et al.,* 2000).

The affect of short-term administration of Mg on bone turnover in young normal subjects has been conflicting. Magnesium, 360 mg per day, was administered for 30 days in 12 normal males aged 27–36 (mean dietary intake prior to supplementation was 312 mg/day) and markers of bone formation (serum osteocalcin and C terminus of type I procollagen) and bone resorption (type I collagen telopeptide) were compared with 12 age-matched controls (Dimai *et al.,* 1998). Markers of both formation and resorption were suppressed significantly however, only during the first 5–10 days of the study. A similar trial of 26 females aged 20–28 in a double-blind, placebo-controlled, randomized crossover design has been reported (Doyle *et al.,* 1999). Magnesium, 240 mg/day, or placebo was administered for 28 days (mean dietary Mg intake was 271 mg/day prior to and during the study). No effect of Mg supplementation was observed on serum osteocalcin, bone-specific alkaline phosphatase, or urinary pyridinoline and deoxypyridinoline excretion.

Mg Status in Osteoporosis

Few studies have been conducted assessing Mg status in patients with osteoporosis, despite interest in the possible role that dietary Mg insufficiency may play as a risk factor for osteoporosis. A small group of 15 osteoporotic subjects (10 female, 5 male) aged 70–85 (the presence or absence of osteoporosis was determined by radiographic features) was compared to 10 control nonosteoporotic subjects (Cohen and Kitzes, 1983). Both groups had normal serum Mg concentrations, which were not significantly different from each other. The Mg tolerance test, however, revealed a significantly greater retention in the osteoporotic patients (38%) as compared to 10% in the control subjects, suggesting Mg deficiency. In a second study by this group, 12 younger women aged 55–65 with osteoporosis (as determined by X-ray) had significantly lower serum Mg concentrations than 10 control subjects; however, no difference in the Mg tolerance test was observed (Cohen *et al.,* 1983). Red blood cell Mg was found to be significantly lower in 10 postmenopausal women who had at least one vertebral fracture as compared to 10 subjects with degenerative osteoarthritis;

however, no difference in plasma Mg was found (Reginster *et al.,* 1985). In a second report, 10 postmenopausal women aged 68.9 ± 9 with vertebral crush fracture were compared to 10 nonosteoporotic women aged 67.2 ± 6 years (Reginster, 1989). In comparison to the 10 controls, the osteoporotic subjects had a significantly lower serum Mg, but no difference was noted in red blood cell Mg.

The majority of body Mg (50–60%) resides in the skeleton, and skeletal Mg reflects Mg status. In the two studies cited earlier in which a Mg deficit was suggested by either Mg tolerance testing or low serum Mg concentration, the Mg content of iliac crest trabecular bone was reduced significantly in osteoporotic patients (Cohen and Kitzes, 1983; Cohen *et al.,* 1983). Two additional studies also found a lower bone Mg content in elderly osteoporotic patients (Manicourt *et al.,* 1981; Milachowski *et al.,* 1981). However, no difference in bone Mg content between osteoporotic subjects and bone obtained from cadavers was found (Reginster, 1989). Another study found no difference between patients with osteoporosis and control subjects in cortical bone (Basle *et al.,* 1990), while two studies reported higher bone Mg content in osteoporosis (Basle *et al.,* 1990; Burnell *et al.,* 1982).

In summary, Mg status has been assessed in very few osteoporotic patients. Low serum and red blood cell Mg concentrations, as well as a high retention of parenterally administered Mg, suggest a Mg deficit; however, these results are not consistent from one study to another. Similarly, while a low skeletal Mg content has been observed in some studies, others have found normal or even high Mg content. Larger scale studies are needed.

Effect of Mg Therapy in Osteoporosis

The effect of dietary Mg supplementation on bone mass in patients with osteoporosis has not been studied extensively. Administration of 600 mg of Mg per day to 19 patients over 6–12 months (Abraham, 1991) was reported to increase BMD of the calcaneus (11%) compared to a 0.7% rise in 7 control subjects. All subjects were postmenopausal (age 42–75) and on sex steroid replacement therapy. Subjects who received Mg also received 500 mg of calcium per day, as well as many other dietary supplements, however, making if difficult to conclude that Mg alone was the sole reason for the increase in bone mass. In a retrospective study, Mg (200 mg per day) given to 6 postmenopausal women (mean age 59) was observed to have a small nonsignificant 1.6% rise in bone density of the lumbar spine; no change was seen in the femur (Eisinger and Clairet, 1993). Stendig-Linberg *et al.* (1993) conducted a 2-year trial in which 31 postmenopausal osteoporotic women were administered 250 mg Mg per day, increasing to a maximum of 750 mg per day for 6 months depending on tolerance. All subjects were given 250 mg Mg per day from months 6 to 24. Twenty-three age-matched subjects served as controls. At 1 year there was a significant 2.8% increase in bone density of the distal radius. Twenty-two of the 31 subjects had an increase in bone density while 5 did not change. Three subjects that showed a decrease in bone density had primary hyperparathyroidism and one underwent a thyroidectomy. No significant effect of Mg supplementation was shown at 2 years, although only 10 subjects completed the trial. In a small uncontrolled trial, a significant increase in bone density of the proximal femur and lumbar spine in celiac sprue patients who received approximately 575 mg Mg per day for 2 years was reported (Rude and Olerich, 1996). These subjects had demonstrated evidence of reduced free Mg in red blood cells and peripheral lymphocytes.

In summary, the effect of Mg supplements on bone mass has generally led to an increase in bone mineral density, although study design limits useful information. Larger long-term, plabeco-controlled, double-blind investigations are required.

Osteoporosis in Patients at Risk for Mg Deficiency

Osteoporosis may occur with greater than usual frequency in certain populations in which Mg depletion is also common. These include diabetes mellitus (Levin *et al.,* 1976; McNair *et al.,* 1979, 1981; Hui *et al.,* 1985; Saggese *et al.,* 1988; Krakauer *et al.,* 1995), chronic alcoholism (Bikle *et al.,* 1985; Lindholm *et al.,* 1991; Peris *et al.,* 1992), and malabsorption syndromes (Molteni *et al.,* 1990; Mora *et al.,* 1993). Changes in bone and mineral metabolism in patients with diabetes mellitus and alcoholism are surprisingly similar to those in Mg depletion as discussed earlier. Serum PTH and/or $1,25(OH)_2D$ concentrations have been found to be reduced in both human and animal studies (McNair *et al.,* 1979; Hough *et al.,* 1981; Imura *et al.,* 1985; Ishida *et al.,* 1985; Nyomba *et al.,* 1986; Saggese *et al.,* 1988; Verhaeghe *et al.,* 1990). A prospective study of pregnant diabetic women demonstrated a fall in serum $1,25(OH)_2D$ during pregnancy rather than the expected rise observed in normal women (Kuoppala, 1988). These subjects were also found to have reduced serum Mg concentrations. Diabetic children, with reduced serum Mg and calcium concentrations and low bone mineral content, were shown to have an impaired rise in serum PTH and $1,25(OH)_2D$ in response to a low calcium diet; this defect normalized following Mg repletion (Saggese *et al.,* 1988, 1991). Similar observations were found in the diabetic rat (Welsh and Weaver, 1988). Duodenal calcium absorption has also been reported to be low in diabetic rats (Nyomba *et al.,* 1989; Verhaeghe *et al.,* 1990). The calcium malabsorption may be due to low serum $1,25(OH)_2D$, a duodenal calbindin D_{9K} has been found to be reduced (Nyomba *et al.,* 1989; Verhaeghe *et al.,* 1990).

The reduction in bone mass in diabetes mellitus and alcoholism also appears to be related to a decrease in bone formation, similar to what is observed in experimental Mg depletion (see later). A histomorphometric study of bone has shown decreased bone formation, bone turnover, osteoid, and osteoblast number (Tamayo *et al.,* 1981; Goodman and Hori, 1984; Verhaeghe *et al.,* 1990; Hough *et al.,* 1991; Bouillon, 1991). Reduced bone turnover is supported by the finding that serum osteocalcin, a marker of osteoblast activity, is low

in humans (Pietschmann *et al.,* 1988; Rico *et al.,* 1989) and in rats (Ishida *et al.,* 1988; Verhaeghe *et al.,* 1990).

Magnesium Depletion and Osteoporosis: Experimental Animal Models

The effect of dietary Mg depletion on bone and mineral homeostasis in animals has been studied since the 1940s. Most studies have been performed in the rat. Dietary restriction has usually been severe, ranging from 0.2 to 8 mg per 100 chow (normal = 50–70 mg/100 g). A universal observation has been a decrease in growth of the whole body as well as the skeleton (Lai *et al.,* 1975; McCoy *et al.,* 1979; Mirra *et al.,* 1982; Carpenter *et al.,* 1992; Boskey *et al.,* 1992; Kenny *et al.,* 1994; Gruber *et al.,* 1994). The epiphyseal and diaphyseal growth plate is characterized by thinning and a decrease in the number and organization of chrondrocytes (Mirra *et al.,* 1982). Osteoblastic bone formation has been observed by quantitative histomorphometry to be reduced, as shown in Fig. 12 (Carpenter *et al.,* 1992; Gruber *et al.,* 1994, Rude *et al.,* 1999). Serum and bone alkaline phosphatase (Mirra *et al.,* 1982; Loveless and Heaton, 1976; Lai *et al.,* 1975), serum and bone osteocalcin (Boskey *et al.,* 1992; Carpenter *et al.,* 1992; Creedon *et al.,* 1999), and bone osteocalcin mRNA (Carpenter *et al.,* 1992; Creedon *et al.,* 1999) have been reduced, suggesting a decease in osteoblastic function. This is supported by an observed decrease in collagen formation and sulfation of glycosaminoglycans (Trowbridge and Seltzer, 1967). A decrease in tetracycline labeling has also suggested impaired mineralization (Carpenter *et al.,* 1992; Jones *et al.,* 1980). Data on osteoclast function have been conflicting. A decrease in urinary hydroxyproline (MacManus and Heaton, 1969) and dexoypyridinoline (Creedon *et al.,* 1999) has suggested a decrease in bone resorption; however, Rude *et al.* (1999) reported an increase in the number and activity of osteoclasts in the Mg-deficient rat, as shown in Fig. 13. Bone from Mg-deficient rat, has been described as brittle and fragile (Lai *et al.,* 1975; Duckworth *et al.,* 1940). Biomechanical testing has directly demonstrated skeletal fragility in both rat and pig (Boskey *et al.,* 1992; Kenny *et al.,* 1992; Miller *et al.,* 1965; Heroux *et al.,* 1974; Smith and Nisbet, 1968). Osteoporosis has been observed to occur in dietary Mg depletion by 6 weeks or longer (Boskey *et al.,* 1992; Carpenter *et al.,* 1992; Rude *et al.,* 1999; Heroux *et al.,* 1974; Smith and Nisbet, 1968), as shown in Fig. 14. Bone implants into Mg-deficient rats have also shown osteoporosis in the implanted bone (Belanger *et al.,* 1975; Schwartz and Reddi, 1979). The effect of higher than the recommended dietary Mg intake on mineral metabolism in the rat has been reported (Toba *et al.,* 2000). In this study, increasing dietary Mg from 48 to 118 mg/100 g chow

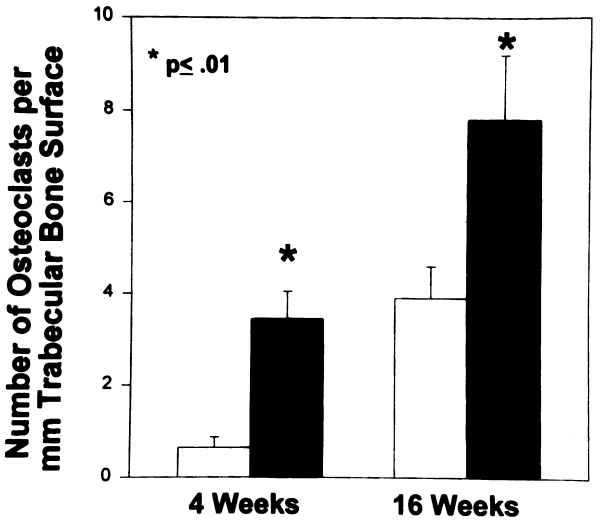

Figure 13 After 4 and 16 weeks of magnesium deficiency in the rat (solid bars), the osteoclast number was elevated significantly compared to controls (open bars) (Rude *et al.,* 1999) $^{*}p < 0.01$.

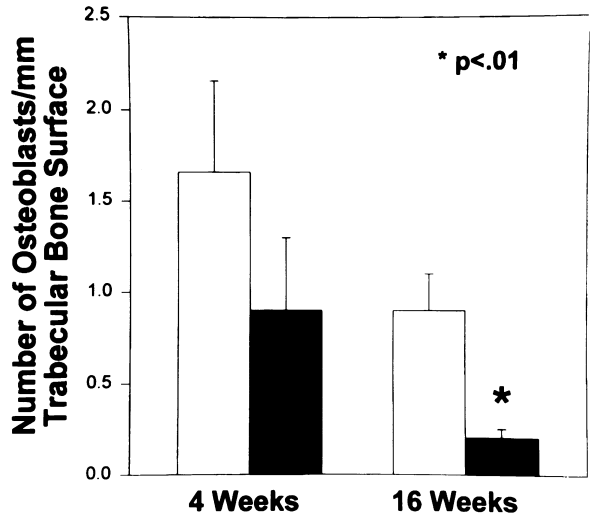

Figure 12 After 16 weeks of magnesium deficiency in the rat (solid bars), the osteoblast number was reduced significantly compared to controls (open bars) (Rude *et al.,* 1999) $^{*}p < 0.01$.

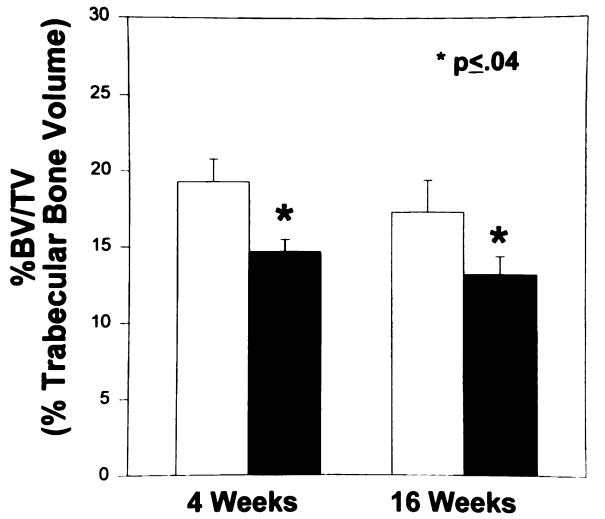

Figure 14 Magnesium deficiency in the rat (solid bars) resulted in a significant reduction in trabecular bone volume compared to control animals (open bars) at both 4 and 16 weeks (Rude *et al.,* 1999) $^{*}p < 0.04$.

resulted in a decrease in bone resorption and an increase in bone strength in ovariectomized rats. No loss of BMD was observed, suggesting a beneficial effect of Mg in acute sex steroid deficiency.

POSSIBLE MECHANISMS FOR MG DEFICIENCY-INDUCED OSTEOPOROSIS

Several potential mechanisms may account for a decrease in bone mass in Mg deficiency. Mg is mitogenic for bone cell growth, which may directly result in a decrease in bone formation (Liu *et al.,* 1988). Mg also affects crystal formation; a lack of Mg results in a larger, more perfect crystal, which may affect bone strength, as discussed later (Cohen *et al.,* 1983).

Mg deficiency can perturb calcium homeostasis and result in a fall in both serum PTH and $1,25(OH)_2D$ as discussed earlier (Rude *et al.,* 1978; Fatemi *et al.,* 1991). Because $1,25(OH)_2D$ stimulates osteoblast activity (Azria, 1989) and the synthesis of osteocalcin and procollagen (Franchesche *et al.,* 1988), decreased formation of $1,25(OH)_2D$ may be a major cause of decreased bone formation, such as that observed in experimental Mg deficiency (Heroux *et al.,* 1975; Jones *et al.,* 1980; Kenney *et al.,* 1994). Similarly, PTH has been demonstrated to be trophic for bone (Marcus, 1994) and therefore impaired PTH secretion or PTH skeletal resistance may result in osteoporosis.

Because insulin promotes amino acid incorporation into bone (Hahn *et al.,* 1971), stimulates collagen production (Wettenhall *et al.,* 1969), and increases nucleotide synthesis by osteoblasts (Peck and Messinger, 1970), insulin deficiency or resistance may alter osteoblast function in diabetes. However, insulin also causes an increase in intracellular Mg, and because Mg has been shown to be trophic for the osteoblast (Liu *et al.,* 1988), insulin deficiency may result in intracellular Mg depletion and impaired osteoblast activity. Serum IGF-1 levels have also been observed to be low in the Mg-deficient rat, which could affect skeletal growth (Dorup *et al.,* 1991).

While the explanation just given may explain low bone formation, it does not explain the observation of an increase in osteoclast bone resorption. Acute Mg depletion in the rat and mouse has demonstrated an immediate rise in substance P followed by a rise in inflammatory cytokines (TNFα, IL-1, IL-6, and IL-11) (Weglicki *et al.,* 1996). These cytokines could contribute to an increase in osteoclastic bone resorption and explain the uncoupling of bone formation and bone resorption observed in the rat (Rude *et al.,* 1999). Whether these possibilities are valid for the suboptimal chronic dietary Mg deficit in human osteoporosis in unknown. Further studies are needed to explore these possibilities.

Magnesium and Mineral Formation

Mg may also independently influence bone mineral formation. In *in vitro* studies, Mg has been shown to bind to the surface of hydroxyapatite crystals and to retard the nucleation and growth of hydroxyapatite and its precrystalline intermediate, amorphorous calcium phosphate (Blumenthal *et al.,* 1977; Bigi *et al.,* 1992; Sojka and Weaver, 1995). Mg has also been demonstrated to compete with calcium for the same absorption site on hydroxyapatite (Aoba *et al.,* 1992). Therefore, surface-limited Mg may play a role in modulating crystal growth in the mineralization process.

In vivo studies have demonstrated that as the Mg content of bone decreases, the hydroxyapatite crystal size increases, whereas high Mg content results in smaller crystals. Rats fed excess Mg have smaller mineral crystal in their bone than control pair-fed animals (Burnell *et al.,* 1986). In contrast, Mg-deficient rats have a significant increase inhydroxyapatite crystal size (Boskey *et al.,* 1992). Clinical studies are also consistent with this effect of Mg on crystal formation. A crystallinity index determined by infrared spectrophotometry has shown larger and more perfect bone mineral crystals along with decreased bone Mg in bone samples obtained from patients with diabetes mellitus, postmenopausal osteoporosis, and alcoholic osteoporosis (Blumenthal *et al.,* 1977; Cohen and Kitzes, 1981; Cohen *et al.,* 1983; Sojka and Weaver, 1995). These conditions are known to have a high incidence of Mg depletion. In contrast, uremic patients, characterized by high serum Mg levels, have smaller, less perfect crystals and high bone Mg (Blumenthal *et al.,* 1977; Cohen and Kitzes, 1981; Cohen *et al.,* 1983; Sojka and Weaver, 1995). An inverse correlation was found to exist between bone Mg and crystallinity index. The effect of these findings on crystallization in terms of bone strength and bone metabolism has yet to be elucidated.

Mg may also have another indirect effect on crystallization by influencing both osteocalcin formation and osteocalcin binding to hydroxyapatite. Osteocalcin has been shown to inhibit the conversion of brushite to hydroxyapatite and the nucleation of mineral formation (Wians *et al.,* 1990). Therefore, the decrease in osteocalcin production, as suggested by decreased serum and bone osteocalcin, in Mg depletion may influence mineralization. However, Mg has also been demonstrated to inhibit the binding of osteocalcin to hydroxyapatite by reducing the number of available hydroxyapatite-binding sites for osteocalcin (Wians *et al.,* 1983). Maximal inhibition occurred at 1.5 m*M* Mg, which is within the physiologically relevant concentration range.

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CHAPTER 22

Metals in Bone

Aluminum, Boron, Cadmium, Chromium, Lead, Silicon, and Strontium

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Introduction

The mineral phase of bone is made up principally of calcium and phosphate. In the course of mineral deposition, a variety of metals are taken up that may be present in the bloodstream as the blood plasma courses over the skeletal tissue. Uptake by the bone mineral is a function of the affinity of a given metal for the bone mineral and extracellular matrix and of the metal's concentration in the plasma. It is also a function of the degree of mineralization of the skeleton. Bone lead content, for example, is higher in persons who have a low calcium intake and presumably have lower bone calcium than in individuals with a high calcium intake (Hernandez-Avila *et al.,* 1996). If, in addition, the metal interacts with bone cells, their metabolism may be affected, which in turn may alter osteoblast and osteoclast function. Fluoride and bisphosphonates are examples of compounds that become part of the bone mineral, but also have an inhibitory effect on osteoclasts.

Once a metal becomes incorporated in the bone mineral, its return to the circulation will depend on when the bone mineral that contains the metal will be resorbed. Metals deposited or exchanged with other metals on the surface of the bone mineral tend to be exchanged rapidly. As additional bone mineral is deposited, the opportunity for isoionic or heteroionic exchange diminishes and osteoclastic resorption becomes the dominant process responsible for the metal's reentry into the circulation. There it is subject to the stochastic processes of loss from the circulation via excretion (urine and stool), redeposition in bone, or soft tissue uptake. The latter usually accounts for only a minute fraction of the loss out of the circulation, but, from a toxicological viewpoint, may constitute the most significant event for the organism. Because the turnover of bone mineral varies in trabecular and cortical bone and in various regions of the skeleton, uptake of a given metal in the skeleton will be in those regions with the highest turnover rate, but retention will be highest in those with the lowest turnover rate. For this reason, one can think of bone not only as a reservoir for a variety of elements, including metals, but as an organ that provides storage for the unexcreted fraction of a body burden. Attempts at predicting the degree of reentry into the circulation of a metal such as lead are at the base of measuring the levels of a metal on bone surfaces (Farias, 1998).

It is also evident that conditions that affect the rate of bone turnover will alter the concentration of a given mineral in the circulation. For example, fetal bone formation and calcification are at a maximum during the third trimester of pregnancy, when deposition of maternal bone mineral in the fetal skeleton becomes important (Franklin *et al.,* 1997), and more so when the newborn is in the low birth weight category (Gonzalez-Cossio *et al.,* 1997). Similarly, end-stage renal failure patients with secondary hyperparathyroidism mobilized more lead from their skeletons, with a dramatic decrease following parathyroidectomy (Kessler *et al.,* 1999).

Aluminum

Interest in the interaction between aluminum (Al) and bone was stimulated as a result of the observation that patients with renal dystrophy accumulated aluminum in their skeleton in quantities that tended to exceed those accumulated by patients with comparable rates of bone turnover, as in hyperparathyroidism (Goodman and Duarte, 1991). Moreover, all accumulation was enhanced markedly as a result of long-term dialysis, leading to bone disease characterized by impaired mineralization and diminished bone cell activity (Goodman and Duarte, 1991). Bone disease of this type has also been described in individuals with chronic renal disease who are not on dialysis (O'Brien *et al.,* 1990) and in persons receiving total parenteral nutrition (Klein *et al.,* 1982), as well as in aluminum welders (Elinder *et al.,* 1991).

Whereas in the majority of healthy subjects, Al plasma concentrations are ~2 μg/liter, many have higher plasma concentrations (Sharp *et al.,* 1993), inasmuch as ingestion of Al-containing substances leads to increased plasma concentrations. Higher plasma levels reflect higher intakes, although differences due to gender (Sharp *et al.,* 1993) or metabolism (absorption, excretion, and bone turnover) have not been explored systematically. What seems reasonably certain is that Al accumulation in bone increases as the plasma concentration increases, whether due to increased intake, as in dialysis with liquids that contain Al, or under conditions of decreased capacity for excretion, as in renal osteodystrophy. Bone accumulation of Al can also increase when turnover is diminished, as in diabetes mellitus (Pei *et al.,* 1993). As aluminum accumulates in the skeleton, it inhibits mineralization and acts on bone cells.

Goodman and Duarte (1991) have reviewed the evidence concerning the effect of aluminum on calcification and the mineralization front, but feel they cannot separate clearly physicochemical from biological effects. Aluminum appears to enter skeletal tissue along with calcium, competing with it, inhibiting hydroxyapatite formation *in vitro* (Blumenthal and Posner, 1984). Data on the *in vivo* effect of Al on bone mineralization are less plentiful, but nevertheless point to the overall toxicity of Al, although Al concentrations that interfere with calcification *in vivo* are likely to be higher. Bouglé *et al.* (1998), for example, reported that both bone mineral density and bone mineral content of the lumbar spine decreased significantly as serum Al levels increased in low birth infants. This was not true for full-term infants. One explanation is that bone development is incomplete in the low birth infants and that Al does indeed interfere with the initiation and progression of bone mineralization. Similarly in adults, Kausz and colleagues (1999) concluded that a patient's plasma Al level does not predict well the presence of aluminum bone disease, a well-described complication encountered in persons undergoing dialysis. As stated earlier, initial bone uptake is proportional to the Al concentration of the plasma, but once taken up by bone, it is the fate of this bone that will determine how much of the initial deposit will remain in that bone site. Therefore, the plasma Al concentration at any time reflects ingested Al, as well as the amount of Al that is released by bone. Moreover, the effect of Al on bone cells is not instantaneous. Inasmuch as the amount of Al found in bone at any instant is a complicated function of plasma levels over time and of bone turnover as a function of time, Al bone and plasma levels at a given moment are not likely to be closely related. The risk of acute Al toxicity can be assessed by plasma analysis. High bone levels, to be sure, indicate high prior exposure, but single plasma or bone analyses are unlikely to provide information on Al toxicity. This statement is illustrated by the report of Suzuki *et al.* (1995), who found Al accumulation in the bones of their patients on chronic hemodialysis, even though the water and dialysis fluid contained less than 10 μg Al/liter over the preceding decade. Goodman and Duarte (1991) have pointed out that an effect of aluminum on bone may require concentrations of 30–40 mg Al/kg dry bone, a concentration equivalent to 1 to 2% of the calcium content (Widdowson and Dickerson, 1964), whereas the Al concentration in bone of normal subjects is only 5–7 mg/kg dry bone (Hodsman *et al.,* 1982).

In addition to inhibiting the formation of calcium hydroxyapatite, detectable only at high or with prolonged rates of Al entry into the skeleton, Al also interferes with the formation of calcified and uncalcified nodules in primary cultures of neonatal mouse calvarial cells (Sprague *et al.,* 1993). Those nodules are specific for isolated calvarial cells, which are osteoblastic in nature. Bellows and colleagues (1995) have shown that Al inhibits *in vitro* mineralization of osteoid nodules, both in its initiation and in its progression phases. More recently, these investigators, working with long-term rat calvaria cell cultures, showed that Al initially accelerated the rate of osteoprogenitor cell differentiation. Al also initially accelerated the formation of osteoid nodules, while at the same time inhibiting mineralization. Ultimately, however, Al exerted toxic effects, with nodules and matrix disintegrating by days 17 to 19 of the cultures (Bellows *et al.,* 1999). Kidder *et al.* (1993) have reported that Al suppressed the proliferation of marrow fibroblast-like stromal cells, as well as of calvarial osteoblasts (cf. Sprague *et al.,* 1993). Interestingly enough, in more mature, confluent cultures, the addition of Al stimulated DNA synthesis and collagen production independently of the presence of 1,25-dihydroxycholecalciferol, the active vitamin D metabolite (Kidder *et al.,* 1993). The fact that fluoride appears to inhibit Al accumulation in rat bone (Ittel *et al.,* 1993) may be taken as supporting a cellular effect of Al, although a physicochemical effect cannot be excluded.

Goodman and Duarte (1991) stated that the amount of surface stainable aluminum is the best available indicator of aluminum toxicity and estimated that when surface levels of Al exceed 30%, bone formation and/or mineralization is affected adversely. Al toxicity that results from Al accumulation is by now a well-known complication of patients in chronic renal failure. Chelators are the treatment of choice

for ridding the body of metals. In the case of Al toxicity, desferrioxamine treatment and elimination of all exogenous Al sources are indicated with careful attention to avoid or at least minimize side affects, including infections (D'Haese *et al.,* 1996).

Specific mechanisms by which Al acts on bone and other cells have not been elucidated. Jeffrey *et al.* (1997) discussed possible effects on cell signaling, mechanisms by which Al inhibits hemoglobin synthesis, and effects of Al on PTH secretion. These reviewers also list a series of recommendations for further study of the multiple actions of Al in the mammalian organism.

Boron

It is uncertain whether boron (B) an essential element for many plant species, is essential for mammals. However, as reported by Nielsen and Hunt (cited by King *et al.,* 1991), a low B diet appears to exacerbate the effects of vitamin D deficiency in chicks, with B supplementation reducing the effects of vitamin D deficiency. It was therefore suggested that B may play a role in bone metabolism. Moreover, Nielsen *et al.* (1987) had reported that increasing the dietary intake of B from 0.25 to 3.25 mg/day in postmenopausal women increased plasma estradiol and testosterone concentrations and decreased urinary calcium output. For this reason, B may play a role in postmenopausal osteoporosis. This possibility was investigated by Beattie and Peace (1993), who studied six postmenopausal women volunteers on a metabolic ward on two levels of B intake, 0.33 and 3.33 mg/day, each subject on each of the two B intakes for 3 weeks. There was no effect on minerals, steroids, or urinary pyridinium cross-link excretion, a measure of collagen turnover. All subjects in this study, as they shifted from an acclimation period diet that they consumed for 2 days to the low B diet, supplemented with additional B 3 weeks later, experienced an increase in Ca absorption and urinary Ca excretion. This increase in absorption and excretion was, however, unaffected by the later increase in B intake and cannot, therefore, be attributed to B.

Hegsted and colleagues (1991) studied the effect of B addition on vitamin D deficiency in rats. They placed weanling rats, 21 days of age, on a vitamin D-deficient diet and 12 weeks later, when both the B-supplemented and B-deficient rats were hypocalcemic, the supplemented groups had higher net calcium absorption and were in somewhat more positive balance. However, there were no effects on soft tissue calcium levels and none on a variety of bone parameters (bone mineral density and length of femur, bone and ash weight, bone Ca, Mg, and P). Hypocalcemia, which on a low calcium diet can be brought about in 2–3 weeks, takes much longer to develop and is less severe when calcium intake has been high (Bronner and Freund, 1975). It is also uncertain whether these animals developed genuine vitamin D deficiency, as their intestinal calbindin D_{9k} content, the molecular measure of vitamin D deficiency (Bronner and Freund, 1975), was not determined.

In a careful study of the effect of B on chick nutrition, Hunt and colleagues (1994) found that B addition modified the effects of vitamin D deficiency and proposed that the plasma B level is regulated homeostatically. The effects on vitamin D_3 deficiency were minor, and the inference concerning B homeostasis was not based on rigorous experimentation because urinary B output was not measured. Conceivably, a zero intake of B may aggravate metabolic defects due to vitamin D deficiency, but B is so widely distributed in nature that a genuine B deficiency can probably be achieved only under strict laboratory conditions.

Utilizing young adult male rats, Chapin *et al.* (1997) studied the effect of increasing B intake, in the form of boric acid, from 0 to 9000 ppm boric acid for 9 weeks. They found that bone B increased in all treated animals and that even though within 1 week of the cessation of feeding B in the diet, serum and urine B values had dropped to normal, bone retained its B level for as long as 32 weeks after cessation of the B diet. The only change in bone these investigators found was a 5–10% increase in vertebral resistance to crush force. The authors point out that these increases occurred at exposure levels that were "substantially below those that were previously reported to be toxic." Future studies are needed to evaluate possible benefits of B intake on bone metabolism and strength.

Cadmium

Cadmium (Cd) intoxication, whether acute or chronic, is principally the result of heavy metal mining, i.e., for lead, zinc, or copper, with Cd often not the object of the mining process, but constituting a contaminant. Cd mining, as in certain areas of Belgium, and Cd smelting also constitute major sources of Cd and lead to Cd toxicity in exposed workers. The most dramatic and attention-drawing incident of Cd poisoning occurred in Japan during the latter part of World War II, although the nature of the disease, which became known as the itai-itai disease, and its relationship to Cd poisoning were not fully understood until the 1960s (Nogawa, 1981). "Itai" is Japanese for the exclamation "ouch," associated with tenderness and pain to the touch. The main symptoms of this disease were osteomalacia in postmenopausal women, traced to a high Cd content of rice grown in certain areas whose irrigation water came from a river that had become severely contaminated with Cd because of upstream mining (Nogawa, 1981; Tsuchiya, 1981).

The three organ systems that are principally affected by Cd poisoning are the respiratory system, implicated particularly in acute poisoning due to Cd contamination of dust, the kidney, and the skeleton. Principal renal symptoms are proteinuria, glycosuria, and microglobulinuria. Osteomalacia and osteoporosis are the skeletal symptoms that have been identified in patients with itai-itai disease and in others that have

had a chronic low-dose exposure for a long time (Tsuchiya, 1981). Uriu and colleagues (2000) have shown that "chronic Cd exposure exacerbated the uncoupling between bone formation and resorption in ovariectomized rats. . . . " Their findings thus add weight to the reported bone effects of chronic cadmium exposure, including decreased mechanical strength. In a similar earlier study, Hiratsuka *et al.* (1997) also showed that chronic Cd intoxication caused osteomalacic lesions in ovariectomized rats. In individuals with skeletal symptoms, calcium deficiency aggravated the disease, and high doses of vitamin D, leading to increased calcium absorption, have been reported to overcome or minimize the symptoms (Nogawa, 1981).

The effect of Cd is very much a function of the dose taken in. Cd appears to have an effect on epithelial cells in the intestine and to react with bone cells. It causes diminished calcium absorption and increased calcium loss from bone (Wilson and Bhattacharyya, 1997). It appears to bind to cells as well as to proteins, causing cell desquamation in the intestine and changes in cell-to-cell binding in the kidney. The latter lead to direct or indirect interference with the hydroxylation of 25-hydroxyvitamin D_3 (Kjellstrom, 1992) so that biosynthesis of the intestinal and renal calbindins is diminished (Kimura, 1981; Sagawara, 1974). This in turn leads to a diminution of the active, transcellular transport of calcium in the duodenum (Bronner *et al.,* 1986) and to diminished active reabsorption of calcium in the distal convoluted tubule (Bronner, 1989, 1991). Moreover, Cd binds to calbindin D, displacing Ca^{2+} (Fullmer and Wasserman, 1977), so that active Ca transport is interfered with. A direct linear relationship exists between Cd intake and Ca excretion in the urine (Nogawa, 1981), doubtless due to Cd-induced damage of the tight junctions of the renal tubule. As a result, less calcium is reabsorbed in the renal distal tubule and calciuria results. Thus, Cd input induces Ca loss.

The effect of Cd on bone is a dual one, direct interaction with bone cells, diminishing their ability to mineralize (Miyahara *et al.,* 1988), inhibiting procollagen C-proteinases (Hojima *et al.,* 1994), thereby preventing collagen self-assembly in the extracellular matrix and effectively decreasing collagen production (Miyahara *et al.,* 1988).

Blumenthal and colleagues (1995) reported that Cd has an inhibitory effect on hydroxyapatite formation *in vitro* and suggested that "the interference of Cd with mineralization can be partially explained by its inhibitory effect on hydroxyapatite nucleation and growth. . . . " This would be in addition to any direct effect of Cd on bone cell function. Thus, Long (1997) has reported that $200-500\ \mu M$ Cd causes changes in cell morphology and causes a decrease in osteoblast and osteoclast number and in alkaline phosphatase activity, all of which are likely to contribute to diminished collagen production and impaired mineralization. A second indirect effect of Cd is to accelerate bone turnover, particularly bone resorption (Chang *et al.,* 1981), a result of the induced calcium deficiency. It is uncertain whether the increase is due to the stimulation of parathyroid hormone release resulting from the tendency toward hypocalcemia.

Shank and Vetta (1981) have calculated that if Cd were administered five times, with intervals of 48 hr between each administration, then 48 hr after the last dose the liver content would account for about two-thirds of the dose and the kidney for 7 to 8%. It is not surprising, therefore, that bone effects are not manifested until later in chronic exposure and that these result from direct effects of Cd on bone cells and hydroxyapatite formation, as well as from the consequences of changes in calcium metabolism. It is because these changes are similar to those of calcium and vitamin D deficiency that bone effects resulting from Cd accumulation are aggravated by conditions that intensify or aggravate calcium needs.

It is not surprising, therefore, that Cd may be a risk factor for osteoporosis (Jarup *et al.,* 1998).

Chromium

The element chromium (Cr) belongs to the first series of the transition elements and occurs in several oxidation states, with the trivalent the most stable (Mertz, 1969). It is thought to be an essential micronutrient, appears essential for optimal glucose utilization, and its deficiency "can be a cause of or an aggravating factor in the glucose intolerance of infants" that suffer from protein calorie insufficiency or have noninsulin-dependent diabetes (Hambidge, 1974). Its role in skeletal metabolism is largely unexplored, with interest stimulated by the increasing use, especially in elderly adults, of metal-containing prostheses, where Cr constitutes part of the alloy. For example, Berry *et al.* (1993) have reported that extensive osteolysis occurred around an aseptic, well-fixed, stable, uncemented total knee prosthesis and concluded that debris resulting from wear, in the form of polyethylene, metal, or both, may be responsible for the breakdown of bone.

Kinetic analyses of the distribution Cr in the body have shown (Onkelinx, 1977) in rats that about 40% of the ^{51}Cr that is lost out of the central compartments flows to a "sink," consisting of various soft tissues and bone. Total bone content is some 2.5 times higher than that of all other tissues after 262 hr, i.e., when uptake approaches a plateau. DoCauto *et al.* (1995) did a kinetic study in humans and found that the compartment with slowest turnover, presumably similar to the "sink" in the rat study (Onkelinx, 1977), reached a near-plateau of about 35% of the injected dose Cr(III) between days 7 and 58 after dose administration; thereafter this compartment began to empty out so that by day 248 it contained only 17% of the dose. In the rat study (Onkelinx, 1977), the bone gained Cr with time, whereas the other tissues either lost or held onto their Cr. Thomann *et al.* (1994) have identified a "major storage compartment" in rats that received Cr in their drinking water for 6 weeks and were studied 140 days later, a period during which they no longer received Cr. The half-life of Cr in that storage compartment, made up of bone, skin, hair, and muscle, was in excess of 100 days. Their study thus confirms the

essential findings reported by Onkelinx (1977). Thomann *et al.* (1994) suggested, as can be inferred from the Onkelinx (1977) study as well, that the storage compartment may function to maintain " . . . elevated body burdens and tissue concentrations of Cr. . . . " If the findings in rats apply to humans, bone could constitute a Cr reservoir.

Cr deficiency or Cr excess may lead to bone changes. Deficiency is difficult to produce in the laboratory and unlikely to be encountered in humans, even though low intakes in the elderly have been associated with glucose intolerance (Hambidge, 1977). The association of high local metal concentrations, including Cr with failed joint prostheses, has been reported (James *et al.* 1993), but the specifics of the effects of Cr excess on bone cells and tissues have not.

Lead

Lead (Pb) contamination of the environment, largely due to the widespread use of Pb compounds in paints and in gasoline, has become a major public health problem. The use of Pb-containing gasoline has been severely restricted in the United States and paints, both exterior and interior, are now formulated without Pb in many countries. Nevertheless, Pb contamination continues to remain an important problem throughout the world. As is true for many trace elements, the body Pb burden is located largely in the skeleton, which serves as the major site of Pb deposit (Aufderheide and Wittmers, 1992). Even when there is little or no further Pb ingestion or inhalation, Pb is liberated from bone as bone turns over (Durbin, 1992). Consequently, the skeleton is not only the major site of Pb deposit, but can become the major source of endogenous Pb (Berglund *et al.,* 2000). As a result, as pointed out by Rust *et al.* (1999), even if intervention such as removal of Pb paint could reduce a child's Pb exposure by 50%, the actual decline in blood Pb may be only 25% because of bone turnover and the consequent release of Pb from bone into the circulation. It is Pb that is released into the circulation and enters soft tissues that constitutes the major health hazard.

It may be obvious that greater bone turnover leads to greater Pb release. A telling illustration is the report by Hac and Krechniak (1996), who showed that after cessation of Pb exposure, the accumulated Pb content of rat hair declined very rapidly to the preexposure level, whereas in that same period only one-third of the accumulated bone Pb was lost, hair obviously turning over much faster than bone.

The importance of bone as a reservoir for Pb and the effects of bone turnover have been illustrated in several reports. For example, Gulson *et al.* (1998) have shown that in the postpregnancy period of women who breast fed their infants the change in blood Pb concentration was significantly greater than during the second and third trimesters. A portion of the calcium in breast milk is of skeletal origin (Bronner, 1960), and bone turnover must therefore be greater than in women who do not breast feed.

Another interesting example of the importance of bone turnover are the observations that Pb is a risk factor for hypertension (Houston and Johnson, 1999; Hu *et al.,* 1996). A possible explanation may be that higher calcium intakes favor lower blood pressure (McCarron *et al.,* 1989) and that Pb in bone, in replacing calcium, lowers, if only slightly, the blood calcium level. This in turn may affect blood pressure by lowering angiotension release or by an indirect effect on the vasculature. This inference derives support from deCastro and Medley (1997), who, on the basis of blood pressure and blood Pb measurements in high school students, suggested a possible association exists between chronic bone Pb accumulation and later adolescent hypertension.

When Pb is ingested, it largely follows the routes of calcium. It binds to calbindin in the duodenum, therefore vitamin D enhances Pb absorption (Fullmer, 1992). In situations of calcium deficiency, when intestinal calbindin levels are high, Pb absorption is enhanced (Fullmer, 1992). On high calcium intakes, most of the calcium and therefore, presumably, most of the Pb are absorbed by the paracellular route, largely in the ileum (Marcus and Lengemann, 1962; Pansu *et al.,* 1993; Duflos *et al.,* 1995). Pb that has entered the body fluids leaves these via the urine, via the intestine, and by entry into bone. Figure 1 is a model of the rates of Pb entry to and return from the two major bone compartments, cortical and trabecular, and of the loss rate via urine. The model, derived from findings in chronically exposed Pb workers, does not take into account losses of endogenous Pb in the stool; O'Flaherty (1993) stated that the ratio for urinary Pb clearance to that of endogenous Pb in the feces varies from 1:1 to 3:1. O'Flaherty also stated (1993) that Pb behaves like calcium in movements into, within, and out of bone. If one assumes that Pb in general behaves like calcium, one can calculate from Fig. 2 that when Pb leaves the body pool, it has a 70% chance of entering bone, whereas the corresponding figure from Fig. 1 would be 55%, neglecting endogenous fecal output. Also, according to Fig. 1, nearly two-thirds of the Pb flow would be to cortical bone and a little over one-third to trabecular bone. However, trabecular bone turns over faster than cortical bone so that in Pb that enters the circulation from bone, a larger proportion than one-third would be of trabecular origin.

Rabinowitz *et al.* (1976) did a kinetic analysis of Pb, given as a stable isotope tracer, in five healthy male volunteers

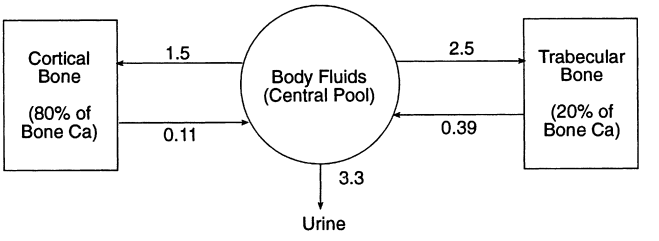

Figure 1 Model of how the adult body handles lead. Arrows refer to turnover rates per year. Based on data of chronically exposed lead workers (Christofferson *et al.,* 1987).

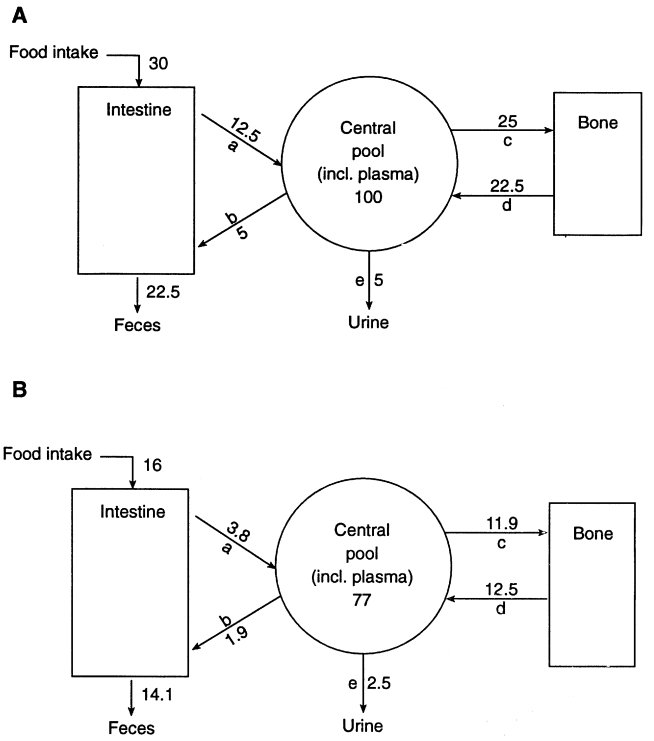

Figure 2 Models of calcium metabolism in a 14-year-old girl (A) and a 62-year-old postmenopausal woman (B). Arrows refer to flows, mmol $Ca \cdot day^{-1}$; units for the central pools are mmol Ca (from Bronner, 1994).

and found that 54–78% of the Pb leaving the blood per day was excreted in the urine and that the body pool of Pb consisted of three compartments, with the third and largest assigned to bone. Pb in the first compartment had a mean life of 36 ± 5 days, of 30 to 55 days in compartment 2, and a much longer life (10^4 days) in compartment 3. Pb absorption was calculated to vary from 6.5 to 13.7%, averaging ~10%.

O'Flaherty (1991) has listed the various routes by which Pb, like calcium, enters bone, i.e., exchange with calcium in the bone mineral, and accretion, i.e., the net transfer into a single microscopic volume of bone, by an increase in either volume (apposition) or mineralization (increase in density). The end result is that some 90% of the body burden of Pb accumulates in bone (Aufderheide and Wittmers, 1992). Because the surface uptake or binding of calcium and therefore presumably of Pb to the bone mineral is the dominant process of entry into bone (Bronner and Stein, 1992), the bone surface tends to have the highest Pb content. Ultimately, some of the surface Pb is lost by removal or by being "buried" by newly deposited bone mineral. For this reason, the microdistribution of Pb in bone is of importance and that is why the bone surface content of Pb has been considered more important for evaluating the body Pb burden than total bone content. How this can and should be evaluated is controversial (Jones *et al.,* 1992).

Some studies have reported on the effect of Pb addition on cultured bone cells. Schanne *et al.* (1989) found that Pb concentrations in the culture medium of 5 and 25 μM increased the intracellular calcium ion concentration in ROS 17/2.8 cells and speculated that Pb toxicity may be mediated by disturbances of intracellular $[Ca^{2+}]$. However, it seems unlikely that raising the $[Ca^{2+}]$ from 0.13 to 0.25 μM would significantly impair the cell's ability to function. In a later paper these authors (Schanne *et al.,* 1992) reported that the addition of Pb to a culture of ROS 17/2.8 cells, which are classified as osteoblastic osteosarcoma cells, interfered with a $1,25(OH)_2D_3$- induced increase in intracellular Ca^{2+}. Moreover, $1,25(OH)_2D_3$ raised $[Ca^{2+}]$ to 0.24 μM, i.e., the value that in the earlier paper was considered potentially harmful. Somewhat similar observations have been reported by Long and Rosen (1994). Klein and Wiren (1993), studying the same cell line, found that Pb concentrations between 2 and 200 μM had no effect on cell number, DNA, or protein synthesis. However, Pb addition caused a decrease in mRNA concentrations of alkaline phosphatase, type 1 procollagen, and osteocalcin.

Hicks *et al.* (1996) have reported that sublethal doses of Pb caused suppression of alkaline phosphatase in isolated chick chondrocytes, as well as of type II and type X collagen expression, and a decrease in thymidine incorporation. The authors suggested that Pb may inhibit endochondral bone formation. Gonzalez-Risla and colleagues (1997) found that Pb exposure inhibited development of the cartilage growth plate of rats and suggested that this may be one cause of the adverse effects of Pb exposure on skeletal development. Another cause may involve disruption of mineralization during growth (Hamilton and O'Flaherty, 1995).

Dowd *et al.* (1994) have reported that submicromolar concentrations of free Pb compete with Ca^{2+} binding to osteocalcin and, because Pb^{2+} inhibits osteocalcin binding to hydroxyapatite, may lead to significant osteocalcin inactivation and ultimately might affect bone mineral dynamics. A related observation was made by Sauk *et al.* (1992), who found that Pb addition inhibited the release of osteonectin/SPARC by ROS 17/2.8 cells and lowered the cellular content of osteonectin/SPARC mRNA. How these *in vitro* effects relate to the *in vivo* effects of Pb poisoning is not yet known.

Miyahara *et al.* (1994) reported that Pb may induce the formation of osteoclast-like cells by increasing the intracellular concentrations of Ca^{2+} and cAMP. Conceivably, therefore, Pb alters Ca channels in a way that allows increased inflow of extracellular Ca^{2+}. Those Pb-sensitive channels, which also permit increased Pb inflow (Schanne *et al.,* 1989), appear therefore to be located in both osteoclasts and osteoblasts. What role these events play in altering bone metabolism is uncertain.

The overall effect of Pb poisoning on bone is unclear. Aufderheide and Wittmers (1992) state that toxic Pb levels may distort the normal macrodistribution of Pb in bone, but this is not established. Koo *et al.* (1991) failed to find significant changes in vitamin D metabolism, bone mineral content, and Ca and P_i plasma concentrations in children of adequate nutritional status who had been chronically exposed to low to moderate Pb levels.

However, Needleman and colleagues (1996) concluded from their study of the relationship between body Pb burden and social adjustment in public school children that high bone Pb levels were associated with attention deficit, aggression, and delinquency and that these effects followed a developmental course. Indeed, it is the overall effect of raised blood levels of Pb on development and behavior of children that led to the advocacy of Pb removal from the environment and the policy of minimizing Pb contamination.

Thus, regardless of the direct effect of Pb on bone, the skeleton clearly constitutes the major Pb store in the body. Consequently, skeletal Pb content will reflect and determine overall exposure and risk.

Silicon

Silicon (Si) atomic number 14, atomic weight 28.03, belongs to the periodic group IVb and is classified as part of the carbon family, which includes germanium, tin, and Pb (Moeller, 1952). Carlisle (1986) has listed the Si concentration in various soft tissues of rats and monkeys as varying typically between 1 and 2 μg/g wet weight; in humans the Si concentration appears to be about one order of magnitude higher. Connective tissues tend to have a high Si content, mainly because Si is an integral component of glycosaminoglycans and their protein complexes, which contribute to the tissue structures (Carlisle, 1986)

In 1972, Schwarz and Milne reported that the addition of 50 mg Si/100g diet increased the growth rate of rats by some 30%, and Carlisle, in the same year, found a comparable increase in the daily weight gain of chicks. Carlisle (1986) has reported that the addition of Si hastened mineralization in weanling rats and that Si deficiency in chicks (Carlisle, 1972) led to abnormally shaped bones. Over the past 20-odd years, there have been many reports that have or have not verified the initial findings (see Carlisle, 1986, for a review of reports until the mid-1980s). Thus, Seaborn and Nielsen (1994) have reported that when Si, as sodium metasilicate, was fed to weanling rats at the rate of 25 μg/g fresh diet, the decreased Ca and Mg content of the femur found in the control animals was reversed. In these experiments, it proved possible to substitute germanium for Si, but germanium did not replace Si in other effects that result from Si deficiency. However, Eliot and Edwards (1991) concluded on the basis of 16-day experiments with broiler chicks that "dietary silicon supplementation has no effect on growth and skeletal development. . . . " Eisinger and Clairet (1993) analyzed bone mineral density in 53 women with osteoporosis and found that in 8 subjects Si supplementation induced a statistically significant increase in femoral bone mineral density.

Rico and colleagues (2000) studied the effect of 30-day Si supplementation in ovariectomized rats and showed that supplementation overcame the losses of bone mass in the fifth lumbar vertebra and in the femur found in the ovariectomized controls. The authors concluded that Si "may have a potential therapeutic application in the treatment of involutive osteoporosis."

One reason for interest in possible Si effects on bone is that granules of special glasses have been used for the repair of bone defects in the dental field (Gatti and Zaffi, 1991a). These vitreous materials contain Si, as do materials used to complete suturectomy for the treatment of craniosynostosis (Antikainen, 1993). In the case of the granules, analyses of the embedded jaws showed (Gatti and Zaffi, 1991b) that Si had diffused into the surrounding tissue. There also was no osteoinduction, but it is not clear whether this was caused by Si or the procedure. As such procedures or prostheses become more prevalent, it may be desirable to study potential effects of Si on bone tissue and cells. The effect of Si on bone structure and bone strength needs further study.

Strontium

Strontium (Sr) like calcium, is a periodic group IIa element and, while not very abundant, constituting only 0.03% of the igneous rocks of the earth, is usually classified as a "familiar element" because of the existence of readily available natural sources (Moeller, 1952). It is not an essential element and interest in Sr metabolism stems from the fact that ^{90}Sr "is an abundant and potentially hazardous by-product of nuclear fission" (Underwood, 1977). Although tests of nuclear explosions have largely ceased, the fact that Sr behaves metabolically like calcium to a large extent has helped maintain interest in this element (Blumsohn *et al.,* 1994; Kollenkirchen, 1995).

In a detailed formal study (Bronner *et al.,* 1963) it was found that although Sr and Ca followed the same metabolic pathway qualitatively, there were significant quantitative differences in how the body handled these two elements. The major difference was in the urine, with the fraction of Sr excreted in the urine three times that of Ca on average. In the stool, the fecal loss of endogenous Sr was greater than that of Ca, but only moderately so (10%). In terms of the fraction of injected isotope that was calculated to reach bone, there was no difference on the average, but the ratio "varied from patient to patient and was not consistent in a given patient." Blumsohn *et al.* (1994) have reexplored the relationship between Sr and Ca absorption in patients with osteoporosis and with chronic renal failure. Sr absorption was approximately half that of Ca absorption, but the time course of the two was similar when evaluated by deconvolution. Stable Sr is less expensive than stable Ca, but differences in absorption between the two elements are sufficiently great that measuring calcium absorption with a calcium isotope seems more meaningful. Treatment with $1,25(OH)_2D_3$ stimulated Sr absorption more than Ca absorption. A possible explanation is that Sr binds more tightly than Ca^{2+} to the newly induced calbindin (Fullmer and Wasserman, 1977)

Fed in large amounts, Sr has long been known to cause rickets in experimental animals (Lehnerdt, 1910, quoted by Neufeld and Boskey, 1994). In Turkish children growing up in regions where the soil Sr content was >350 ppm, the incidence of rickets was nearly twice that in children from regions where the Sr content was lower (Ozgur *et al.,* 1996). Strontium interferes with intestinal calcium absorption and synthesis of $1,25(OH)_2D_3$ (Omdahl and DeLuca, 1972) and interferes with mineralization (Sobel and Hanok, 1952), apparently via direct action on bone cells (Neufeld and Boskey, 1994), although the nature of this action is as yet unclear. One way in which bone formation may be interfered with by Sr is to delay the natural progression of osteoid to bone, i.e., at the stage of conversion of cartilage to bone. This interference is consistent with the greater accumulation of complexed acidic phospholipids in Sr-fed rats or in mesenchymal cell micromass cultures (Neufeld and Boskey, 1994). Davis *et al.* (2000) reported that Sr becomes associated with the collagen matrix produced in cell culture. It would be interesting to know to what extent the inhibitory effect of Sr can be attributed to displacing Ca^{2+} in other calcium-mediated processes.

There have been reports that nontoxic amounts of Sr may be beneficial in osteoporosis (Storey, 1961 and McCaslin and James, 1981, quoted by Morohashi *et al.* 1994, Brandi, 1993) and in rats, where 0.19% $SrCl_2$ in the diet stimulated bone formation (as evaluated histomorphometrically) and raised the trabecular calcified bone volume by 10% (Marie *et al.,* 1985). More recently, Marie *et al.* (1993) have reported that an organic distrontium salt, S12911, inhibited the increase in bone resorption in ovariectomized rats, without reducing bone formation. It is unclear, however, how much of this effect is due to Sr. In mice, 0.27% $SrCl_2$ in the diet increased the osteoid surface, but had no effect on trabecular calcified bone volume (Marie and Hott, 1985). Grynpas *et al.* (1996) fed 0.2% Sr to 28-day old rats consuming a 0.5% Ca and 0.5% P diet and found that the number of bone-forming sites and the vertebral bone volume had increased by 17% compared with controls. No detectable adverse effects on mineralization, mineral profile, or mineral chemistry were observed in the Sr-fed animals. Similarly, Morohashi *et al.* (1994) found no harmful effects when rats were fed 0.05 or 0.10% Sr in a semisynthetic vitamin D-deficient diet, whereas 0.5% Sr depressed bone calcium content and the bone calcium deposition rate. In these studies, the limiting Sr concentration was 175 μM; beyond that level, calcium metabolism was depressed. However, at the lower Sr intakes, there also was no beneficial effect of Sr intake. It would thus seem that as Sr replaces Ca, the metabolism of calcium is depressed, with high Sr intakes leading to rickets and poor bone formation and mineralization.

An interesting use of Sr has been in the radiotherapy of painful bone metastases with ^{89}Sr (Robinson *et al.,* 1995, Pons *et al.,* 1997; Papatheophanis, 1997). Such treatment has been reported more widely for pain relief in patients with prostatic cancer, but has also found application in patients with breast cancer (Pons *et al.,* 1997). Immediately following the injection of $^{89}SrCl_2$, patients experienced a flare reaction of pain. Thereafter pain relief lasted on the average 6 months, with treatment effectively repeatable for another 6 months (Pons *et al.,* 1997). As yet there is no indication that ^{89}Sr is tumoricidal.

Concluding Remarks

All of the metals discussed in detail in this chapter appear to have an effect on the skeleton, but in nearly all there is no clear distinction between cellular effects and effects due to accumulation in the bone mineral and/or the extracellular matrix. Significant accumulation of a given metal in a mineral can be expected to alter the characteristics of the mineral, but it is important to know these changes in detail. Furthermore, by studying the detailed mechanisms of metal accumulation—heteroionic exchange and/or deposition—one might learn more about mineralization. Similarly, by studying the qualitative and quantitative effects on bone cells in culture, mechanisms of cellular action might be laid bare. From a practical viewpoint, moreover, the role of metals used in prostheses needs and is now getting intensive exploration. Dental implants and knee and hip prostheses have raised questions concerning the possible toxicity of the materials used in these procedures. Thus, Wang *et al.* (1996) investigated the cause of osteolysis, a major cause of aseptic loosening in total joint arthroplasty. They studied the effect of titanium, cobalt, and chromium, metals used commonly in joint prostheses, and found a metal-induced increase in the release of bone cytokines. The authors then raised the question whether this can contribute to osteolysis, an event that in turn can severely compromise the outcome of total joint arthroplasty. However, Pohl and colleagues (2000) found no impairment of periodontal healing following the insertion of titanium posts. Piatelli *et al.* (1998) had come to a similar conclusion regarding titanium implants. Aluminum oxide is used with ceramics, and studies on the effect of this metal on biocompatability of the implants have generally found no major problems (Chang *et al.,* 1996; Piatelli *et al.,* 1996, 1998; Shinzato *et al.,* 1999; Okada *et al.,* 2000), but truly long-term results are still outstanding. It seems clear that the study of metals in bone continues to provide wide opportunities for research to all with an interest in either a given metal or the biology of bone.

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CHAPTER 23

Biology of the Extracellular Ca^{2+}-Sensing Receptor

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Introduction

Complex, free-living terrestrial organisms, such as humans, maintain their level of extracellular ionized calcium (Ca_o^{2+}) within a narrow range of about 1.1–1.3 m*M* (Bringhurst *et al.,* 1998; Brown, 1991). This near constancy of Ca_o^{2+} ensures that Ca^{2+} ions are available for their extracellular roles, including serving as a cofactor for clotting factors, adhesion molecules, and other proteins and controlling neuronal excitability (Brown, 1991). Moreover, calcium and phosphate salts form the mineral phase of bone, which affords a rigid framework that protects vital bodily structures and permits locomotion and other movements. The skeleton also provides a nearly inexhaustible reservoir of these ions when their availability in the diet is insufficient for the body's needs (Bringhurst *et al.,* 1998).

In contrast to Ca_o^{2+}, the cytosolic-free calcium concentration (Ca_i^{2+}) has a basal level—about 100 n*M*—that is nearly 10,000-fold lower (Pozzan *et al.,* 1994). Ca_i^{2+}, however, can increase 10-fold or more when cells are stimulated by extracellular signals acting on their respective cell surface receptors as a result of influx of Ca^{2+} and/or its release from intracellular stores (Pozzan *et al.,* 1994). Ca_i^{2+} plays central roles in regulating cellular processes as varied as muscular contraction, cellular motility, differentiation and proliferation, hormonal secretion, and apoptosis (Pietrobon *et al.,* 1990). Because all intracellular Ca^{2+} ultimately originates from that present in the extracellular fluids (ECF), maintaining near constancy of Ca_o^{2+} also ensures that this ion is available for its myriad intracellular roles.

The level of Ca_o^{2+} is maintained by a homeostatic mechanism in mammals that comprises the parathyroid glands, calcitonin (CT)-secreting C cells, kidney, bone, and intestine (Bringhurst *et al.,* 1998; Brown, 1991). A key element of this system are cells that are capable of sensing small deviations in Ca_o^{2+} from its usual level and responding in ways that normalize it (Brown, 1991). Calcium ions have long been known to traverse the plasma membrane through various types of ion channels and other transport mechanisms (Pietrobon *et al.,* 1990); however, the actual mechanism by which Ca_o^{2+} was "sensed" remained an enigma for many years. This chapter provides an update on our present understanding of the process of Ca_o^{2+} sensing, which has increased greatly over the past decade, especially as it relates to the mechanism that maintains Ca_o^{2+} homeostasis.

It is becoming increasingly clear, however, that Ca_o^{2+} serves as a versatile extracellular first messenger—in many instances acting via the Ca^{2+}-sensing receptor (CaR)—that controls numerous physiological processes beyond those governing Ca_o^{2+} homeostasis (for review, see Brown *et al.,* (1999). While it is beyond the scope of this chapter to review the "nonhomeostatic" roles of the CaR in detail, an emerging body of evidence supports the concept that the receptor participates in important interactions between the system regulating Ca_o^{2+} metabolism and other homeostatic systems (i.e., that controlling water metabolism). These interactions may be crucial for the successful adaptation of complex life forms to the terrestrial environment. This chapter will likewise address these recently emerging homeostatic relationships.

Cloning of the CaR

Just a decade ago, the concept that there was a specific Ca_o^{2+}-sensing "receptor" was only supported by indirect evidence derived from studies of a very limited number of

Ca_o^{2+}-sensing cells, particularly parathyroid cells (Brown, 1991; Juhlin *et al.,* 1987; Nemeth and Scarpa, 1987; Shoback *et al.,* 1988). It was necessary, therefore, in devising a strategy for cloning the putative receptor to employ an approach that detected its Ca_o^{2+}-sensing activity using a bioassay—namely, expression cloning in *Xenopus laevis* oocytes. Racke *et al.* (1993) and Shoback and co-workers (Chen *et al.,* 1994) both demonstrated that *X. laevis* oocytes became responsive to Ca_o^{2+}-sensing receptor agonists after they were injected with messenger RNA (mRNA) extracted from bovine parathyroid glands but not with water as a negative control. Brown *et al.* (1993) were then able to implement this strategy to screen a bovine parathyroid cDNA library, permitting the isolation of a full-length, functional clone of the Ca_o^{2+}-sensing receptor. The use of conventional, hybridization-based approaches subsequently permitted the cloning of cDNAs coding for CaRs from human parathyroid (Garrett *et al.,* 1995b) and kidney (Aida *et al.,* 1995b), rat kidney (Riccardi *et al.,* 1995), brain (namely striatum) (Ruat *et al.,* 1995), and C cell (Garrett *et al.,* 1995c); rabbit kidney (Butters *et al.,* 1997); and chicken parathyroid (Diaz *et al.,* 1997) (reviewed in Brown *et al.,* 1999). All exhibit very similar predicted structures and represent tissue and species homologues of the same ancestral gene.

Predicted Structure of the CaR and Its Relationships to Other G Protein-Coupled Receptors

Topology of the CaR protein predicted from its nucleotide sequence is illustrated in Fig. 1. It has three principal structural domains, which include (a) a large, 600 amino acid extracellular amino-terminal domain (ECD), (b) a "serpentine" seven membrane-spanning motif that is characteristic of the superfamily of G protein-coupled receptors (GPCRs), and (c) a sizable carboxyl-terminal (C-) tail of about 200 amino acids. Several different subfamilies of GPCRs have been

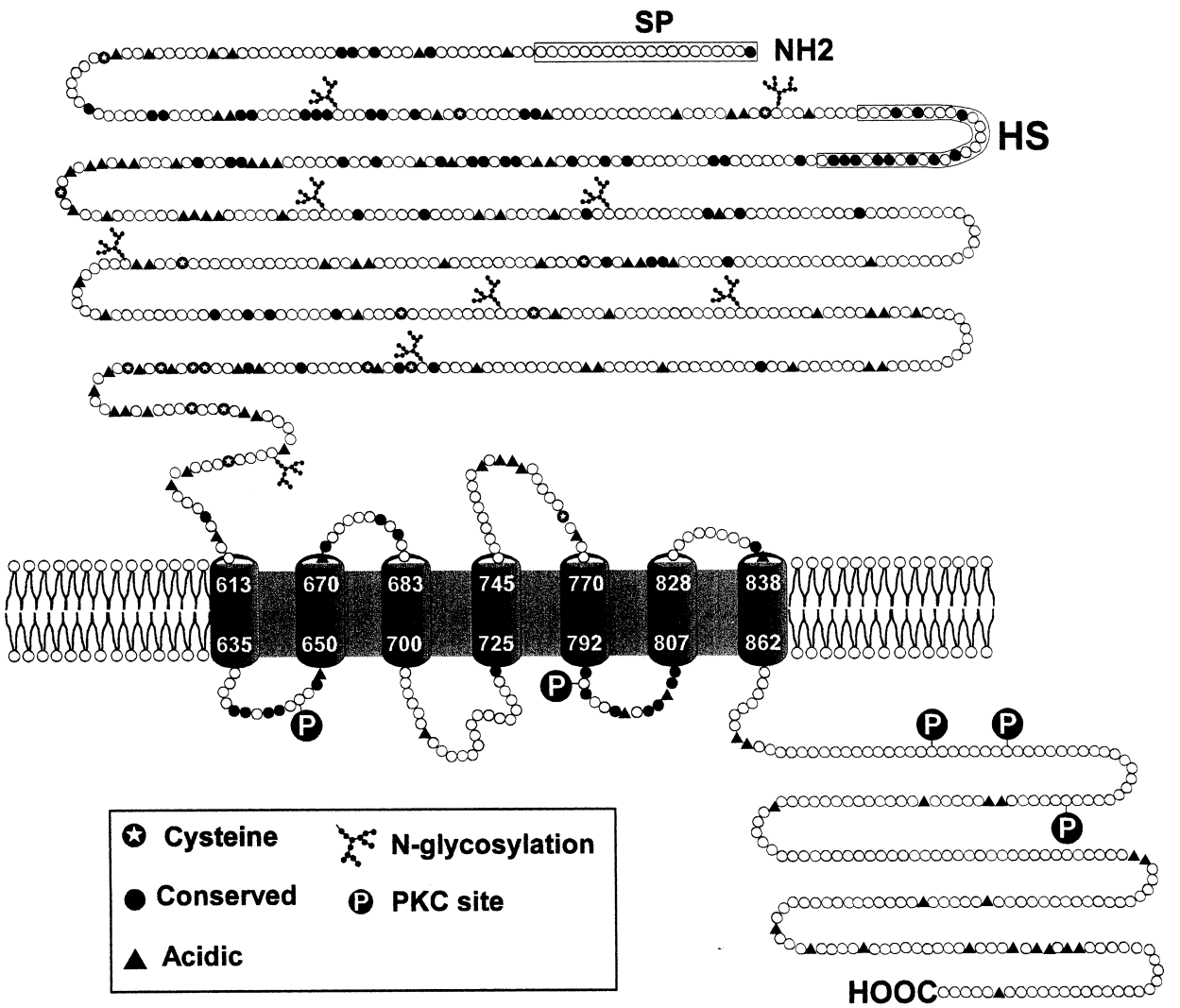

Figure 1 Predicted structure of the CaR (see text for additional details). SP, signal peptide; HS, hydrophobic segment. Reproduced with permission from Brown *et al.* (1993).

identified that share striking topological similarities with the CaR, particularly in their respective, large ECDs. They also share a modest (20–30%) overall degree of amino acid identity with the CaR. These structurally related GPCRs are designated family C receptors (Kolakowski, 1994). They comprise three separate groups: metabotropic glutamate receptors (mGluRs) (group I), the CaR and a family of putative pheromone receptors (group II), and the $GABA_B$ receptors (group III). mGluRs are activated by glutamate, the major excitatory neurotransmitter in the central nervous system (CNS) (Nakanishi, 1994). The $GABA_B$ receptors are GPCRs that recognize γ-aminobutyric acid (GABA), the principal inhibitory neurotransmitter of the CNS (Kaupmann *et al.,* 1997; Ng *et al.,* 1999). The putative pheromone receptors within group II reside solely in neurons of the rat vomeronasal organ (VNO) expressing the guanine nucleotide regulatory (G) protein, $G\alpha_o$ (Matsunami and Buck, 1997). The VNO is a sensory organ that controls instinctual behavior, particularly in rodents, via input from environmental pheromones (Matsunami and Buck, 1997). Additional GPCRs that are closely related to the CaR and pheromone receptors have been characterized in mammals (Hoon *et al.,* 1999) and fish (Cao *et al.,* 1998). These are, respectively, taste and putative odorant receptors. The latter may be evolutionary precursors of the pheromone receptors identified in rats. Both exhibit the topology characteristic of the family C GPCRs.

Therefore, all of the family C GPCRs share the property of having small molecules as ligands that provide environmental cues (i.e., pheromones) or serve as extracellular messengers within the CNS (e.g., glutamate or GABA) or in bodily fluids more generally (namely Ca_o^{2+}). As detailed later ligands of the CaR and the other family C GPCRs are thought to bind to their respective ECDs. In contrast, most other GPCRs binding small ligands, such as epinephrine or dopamine, have binding sites within their TMDs and/or extracellular loops (ECLs). The ligand-binding capacity of the family C ECDs probably has its origin in a family of extracellular binding proteins in bacteria (O'Hara *et al.,* 1993), the so-called periplasmic binding proteins (PBPs). These serve as receptors for a wide variety of small ligands present in the environment, including ions (including Mg_o^{2+}, but apparently not Ca_o^{2+}), amino acids, and other nutrients (Tam and Saier, 1993). PBPs promote bacterial chemotaxis toward these environmental nutrients and other substances and facilitate their cellular uptake by activating specific transport systems in the cell membrane (Tam and Saier, 1993).

The family C GPCRs, therefore, can be thought of as representing fusion proteins, which comprise an extracellular ligand-binding motif (the ECD) linked to a signal-transducing motif (the seven transmembrane domains) that couples the sensing of extracellular signals to intracellular signaling systems (i.e., G proteins and their associated second messenger pathways). It is of interest that some of the biological functions regulated by the CaR are the same as those controlled by the PBPs, namely chemotaxis [e.g., of monocytes toward elevated levels of Ca_o^{2+} (Sugimoto *et al.,* 1993)] and cellular transport [i.e., of Ca_o^{2+} by CaR-regulated, Ca^{2+}-permeable channels (Chang *et al.,* 1995)]. Moreover, as detailed later, the CaR binds not only Ca_o^{2+} but also additional ligands, including amino acids (Conigrave *et al.,* 2000), which further supports its evolutionary and functional relationships to the other family C GPCRs and, ultimately, to PBPs.

Biochemical Properties of the CaR

Studies using chimeric receptors comprising the ECD of the CaR coupled to the TMDs and C tail of the mGluRs (and vice versa) have demonstrated that Ca_o^{2+} binds to the ECD of the CaR (Hammerland *et al.,* 1999). Studies have indicated that specific residues within the ECD (e.g., Ser147 and Ser170) may be involved, directly or indirectly, in the binding of Ca_o^{2+}. These residues are equivalent to those that are thought to participate in binding glutamate to the mGluRs and GABA to the $GABA_B$ receptor (Brauner-Osborne *et al.,* 1999). Given the apparent "positive cooperativity" of the CaR and the resultant steep slope of the curve describing its activation by various polycationic agonists (e.g., Ca_o^{2+} and Mg_o^{2+}) (Brown, 1991), however, it is likely that the CaR binds several calcium ions. Further work is needed, therefore, in defining more precisely the identity of this putative Ca_o^{2+}-binding site(s). Interestingly, the CaR resides on the cell surface primarily in the form of a dimer (Bai *et al.,* 1998a; Ward *et al.,* 1998). CaR monomers within the dimeric receptor are linked by disulfide bonds within their ECDs that involve the cysteines at amino acid positions 129 and 131 (Ray *et al.,* 1999). Moreover, functional interactions can occur between the monomeric subunits of the dimeric CaR because two individually inactive CaRs that harbor inactivating mutations in different functional domains (e.g., the ECD and C tail) can reconstitute substantial biological activity when they heterodimerize after cotransfection in human embryonic kidney (HEK293) cells (Bai *et al.,* 1999). Therefore, even though the individual CaRs lack biological activity, they "complement" one another's defects through a mechanism that must involve intermolecular interactions to form a partially active heterodimer.

As noted previously, the CaR exhibits apparent positive cooperativity, which is essential to ensure that it responds over the narrow range of Ca_o^{2+} regulating, for instance, PTH secretion. This cooperativity could result, at least in part, from the presence of Ca_o^{2+}-binding sites on both of the individual ECDs of the dimeric CaR and/or at the site(s) where the two ECDs in the dimer interact with one another. Eventually, solution of the three-dimensional structure of the receptor's ECD by X-ray crystallography will no doubt illuminate how the CaR binds Ca_o^{2+} and its other agonists and modulators.

The ECD of the CaR on the cell surface is N-glycosylated extensively with complex carbohydrates (Bai *et al.,*

1996). Eight of the predicted N-glycosylation sites in the ECD of the human CaR are glycosylated (Ray *et al.,* 1998). Disrupting four or five of these sites decreases the cell surface expression of the receptor by 50–90%. Therefore, glycosylation of at least three sites is required for robust cell surface expression, although glycosylation *per* se does not appear to be critical for the capacity of the CaR to bind Ca_o^{2+} and activate its intracellular signaling pathways (Ray *et al.,* 1998).

Within its intracellular loops and C tail, the human CaR harbors five predicted protein kinase C (PKC) and two predicted protein kinase A (PKA) phosphorylation sites (Bai *et al.,* 1998b; *Garrett et al.,* 1995b). The functional significance of the PKA sites is not known. Activation of PKC diminishes CaR-mediated stimulation of phospholipase C (PLC), and studies utilizing site-directed mutagenesis have demonstrated that phosphorylation of a single, key PKC site in the C tail of the CaR at thr888 can account for most of the inhibitory effect of PKC on the function of the receptor (Bai *et al.,* 1998b). Therefore, PKC-induced phosphorylation of the C tail may afford a means of conferring negative feedback regulation on the coupling of the receptor to PLC. That is, PLC-elicited activation of protein kinase C — through the ensuing phosphorylation of the CaR at thr888 — limits further activation of this pathway.

Intracellular Signaling by the CaR

Activation of the CaR by its agonists stimulates the activities of phospholipases C, A_2 (PLA_2), and D (PLD) in bovine parathyroid cells and in HEK293 cells stably transfected with the human CaR (Kifor *et al.,* 1997). In most cells, CaR-evoked activation of PLC involves the participation of the pertussis toxin-insensitive G protein(s), $G_{q/11}$ (Hawkins *et al.,* 1989), although in some it can take place through a pertussis toxin-sensitive pathway, most likely via one or more isoforms of the G_i subfamily of G proteins (Emanuel *et al.,* 1996). In bovine parathyroid cells and CaR-transfected HEK293 cells, CaR-mediated stimulation of PLA_2 and PLD occurs through PKC-dependent mechanisms, presumably via receptor-dependent activation of PLC (Kifor *et al.,* 1997).

The high Ca_o^{2+}-induced, transient increase in Ca_i^{2+} in bovine parathyroid cells and CaR-transfected HEK293 cells likely results from activation of PLC (Kifor *et al.,* 1997) and resultant IP_3-mediated release of intracellular Ca^{2+} stores (Bai *et al.,* 1996). High Ca_o^{2+} also elicits sustained elevations in Ca_i^{2+} in both CaR-transfected HEK293 cells (Bai *et al.,* 1996) and bovine parathyroid cells (Brown, 1991), acting through an incompletely defined influx pathway(s) for Ca^{2+}. Via the patch-clamp technique, we have shown that the CaR activates a Ca_o^{2+}-permeable, nonselective cation channel (NCC) in CaR-transfected HEK cells (Ye *et al.,* 1996). An NCC with similar properties is present in bovine parathyroid cells and is likewise activated by high Ca_o^{2+} — an effect presumably mediated by the CaR (Chang *et al.,* 1995). This latter NCC may participate in the high Ca_o^{2+}-induced, sustained elevation in Ca_i^{2+} in parathyroid cells (Brown *et al.,* 1990).

High Ca_o^{2+} reduces agonist-stimulated cAMP accumulation in bovine parathyroid cells markedly (Chen *et al.,* 1989), an action that is thought to involve inhibition of adenylate cylcase by one or more isoforms of G_i, as it is pertussis toxin sensitive (Chen *et al.,* 1989). Other cells, however, can exhibit high Ca_o^{2+}-elicited diminution of cAMP accumulation that involves indirect pathways, including inhibition of a Ca_i^{2+}-inhibited isoform of adenylate cyclase due to the associated rise in Ca_i^{2+} (de Jesus Ferreira *et al.,* 1998). The CaR also activates mitogen-activated protein kinase (MAPK) in several types of cells, including rat-1 fibroblasts (McNeil *et al.,* 1998b), ovarian surface cells (McNeil *et al.,* 1998a), and CaR-transfected but not nontransfected HEK293 cells (Kifor *et al.,* 2001). As is the case with other GPCRs, the CaR stimulates the activity of MAPK through both PKC- and tyrosine kinase-dependent pathways. The latter involves, in part, c-Src-like cytoplasmic tyrosine kinases. The PKC-dependent activation of MAPK is presumably downstream of G_q-mediated activation of PLC, whereas that involving tyrosine kinases may utilize the G_i-dependent pathway involving $\beta\gamma$ subunits released as a consequence of the activation of this G protein (Kifor *et al.,* 2001; McNeil *et al.,* 1998b).

The CaR Gene and Its Regulation

Relatively little is presently known regarding the structure of the CaR gene, especially its upstream regulatory regions and the factors controlling its expression. The human CaR gene is on the long arm of chromosome 3, as demonstrated by linkage analysis (Chou *et al.,* 1992), and in band 3q13.3–21, as documented by fluorescent *in situ* hybridization (Janicic *et al.,* 1995). Rat and mouse CaR genes reside on chromosomes 11 and 16, respectively (Janicic *et al.,* 1995). The CaR gene has seven exons: the first includes the upstream untranslated region, the next five encode various regions of the ECD, and the last encodes the rest of the CaR from its first TMD to the C terminus (Pearce *et al.,* 1995). Characterization of the upstream regulatory regions of the gene will be of great interest because expression of the CaR can change in a various physiologically relevant circumstances — some of which are delineated next.

Several factors increase CaR expression. Both high Ca_o^{2+} and $1,25(OH)_2D_3$ upregulate expression of the gene in certain cell types. High Ca_o^{2+} increases expression of the CaR in ACTH-secreting, pituitary-derived AtT-20 cells (Emanuel *et al.,* 1996), whereas administration of $1,25(OH)_2D_3$ elevates expression of the CaR *in vivo* in kidney and parathyroid of the rat in some (Brown *et al.,* 1996) but not all studies (Rogers *et al.,* 1995). Interleukin-1β increases the level of CaR mRNA modestly in bovine parathyroid gland fragments, which may contribute to the associated reduction in PTH secretion that was observed

in this study (Nielsen *et al.,* 1997). In rat kidney, substantial upregulation of the CaR takes place during the peri- and immediate postnatal period; the resultant higher level of expression of the receptor then persists throughout adulthood (Chattopadhyay *et al.,* 1996). There is likewise a developmental increase in expression of the CaR in rat brain. In contrast to that occurring in the kidney, however, the rise in the expression of the CaR in the brain occurs about a week postnatally (Chattopadhyay *et al.,* 1997). Moreover, the increase in CaR expression in the brain is only transient—it decreases severalfold approximately 2 weeks later and reaches a lower level that remains stable thereafter (Chattopadhyay *et al.,* 1997). The biological importance of these developmental changes in the expression of the receptor is currently unknown.

In contrast, several instances have been defined in which CaR expression decreases. Calf parathyroid cells exhibit a rapid, marked (80–85%) reduction in expression of the receptor after they are placed in culture (Brown *et al.,* 1995; *Mithal et al.,* 1995), which is likely to be a major factor that contributes to the associated reduction in high Ca_o^{2+}-evoked inhibition of PTH secretion. The expression of the CaR in the kidney decreases in a model of chronic renal insufficiency in the rat induced by subtotal nephrectomy (Mathias *et al.,* 1998). This latter reduction in CaR expression might contribute to the hypocalciuria that occurs in human renal insufficiency, as reduced renal CaR expression and/or activity increases tubular reabsorption of Ca^{2+} in humans with inactivating mutations of the receptor (Brown, 1999). Because $1,25(OH)_2D_3$ upregulates renal CaR expression (Brown *et al.,* 1996), the reduction in CaR expression in the setting of impaired renal function could be the result, in part, of the concomitant decrease in circulating levels of $1,25(OH)_2D_3$ (Bringhurst *et al.,* 1998). The mechanisms that underlie these alterations in expression of the CaR gene, however, including the relative importance of changes in gene transcription vs posttranscriptional mechanisms, require further investigation.

Roles of the CaR in Tissues Maintaining Ca_o^{2+} Homeostasis

Parathyroid

The parathyroid glands of several species express high levels of CaR mRNA and protein, including those of humans (Kifor *et al.,* 1996), rats (Autry *et al.,* 1997), mice (Ho *et al.,* 1995), rabbits (Butters *et al.,* 1997), and chickens (Diaz *et al.,* 1997). Indeed, the level of CaR expression in the parathyroid chief cells is one of the highest, if not the highest, in the cells and tissues examined to date. Abundant evidence, reviewed later, supports the importance of the CaR as the key mediator of the inhibitory action of elevated Ca_o^{2+} on PTH secretion. Despite several decades of study, however, the principal intracellular signaling mechanism(s) through which it exerts this action remains unclear (for review, see Diaz *et al.,* 1998). Evidence supporting the role of the CaR in Ca_o^{2+}-regulated PTH release is as follows: As noted earlier, the reduced CaR expression in bovine parathyroid cells maintained in culture is associated with a progressive loss of high Ca_o^{2+}-induced inhibition of PTH secretion (Brown *et al.,* 1995; Mithal *et al.,* 1995). In addition, individuals with familial hypocalciuric hypercalcemia (FHH), who are heterozygous for inactivating mutations of the CaR gene (Brown, 1999), or mice that are heterozygous for targeted disruption of the CaR gene (Ho *et al.,* 1995) exhibit modest right shifts in their set points for Ca_o^{2+}-regulated PTH secretion (the level of high Ca_o^{2+} half-maximally inhibiting PTH release), indicative of "Ca_o^{2+} resistance." Moreover, humans and mice homozygous for loss of the normal CaR (Brown, 1999; Ho *et al.,* 1995) show much greater impairment of high Ca_o^{2+}-elicited suppression of PTH release, showing that that the "Ca_o^{2+} resistance" of the parathyroid is related inversely to the number of normally functioning CaR alleles. Therefore, the biochemical findings in mice in which the CaR has been "knocked-out," as well as in humans with naturally occurring inactivating mutations of the CaR, prove the central role of the CaR in Ca_o^{2+}-regulated PTH release.

Another aspect of parathyroid function that is likely to be CaR regulated is PTH gene expression. Garrett *et al.* (1995a) showed in preliminary studies that the "calcimimetic" CaR activator NPS R-568, which activates the receptor allosterically through a mechanism involving an increase in the apparent affinity of the CaR for Ca_o^{2+} (Nemeth *et al.,* 1998b), decreases the level of PTH mRNA in bovine parathyroid cells. Finally, the CaR, directly or indirectly, tonically inhibits the proliferation of parathyroid cells, as persons homozygous for inactivating mutations of the CaR (Brown, 1999) or mice homozygous for "knockout" of the CaR gene (Ho *et al.,* 1995) exhibit marked parathyroid cellular hyperplasia. Treatment of rats with experimentally induced renal impairment with the calcimimetic NPS R-568 prevented the parathyroid hyperplasia that would otherwise have been anticipated to occur in this setting (Wada *et al.,* 1998), providing further evidence that activation of the CaR suppresses parathyroid cellular proliferation.

C Cells

Unlike the effect of Ca_o^{2+} on PTH release, elevating Ca_o^{2+} stimulates CT secretion—a response that conforms to the classical, positive relationship between Ca^{2+} and activation of exocytosis in most other hormone-secreting cells (Bringhurst *et al.,* 1998; Brown, 1991). This observation was one of several pieces of indirect evidence that the mechanism underlying Ca_o^{2+} sensing in C cells might differ in a fundamental way from that in parathyroid cells. More recent data, however, have demonstrated that rat, human, and rabbit C cells express the CaR (Butters *et al.,* 1997; Freichel *et al.,* 1996; Garrett *et al.,* 1995c). Moreover,

cloning of the CaR from a rat C cell tumor cell line showed it to be identical to that expressed in rat kidney (Garrett *et al.,* 1995c). Available evidence indicates that the CaR mediates the stimulatory effect of high Ca_o^{2+} on CT secretion; however, studies definitively proving the involvement of the CaR in high Ca_o^{2+}-evoked CT secretion, e.g., by "knocking out" the CaR in C cells *in vitro* using a dominant-negative construct or in mice *in vivo* utilizing targeted inactivation of the CaR gene, have not yet been reported. Tamir and co-workers have suggested that the following sequence of steps mediates CaR-stimulated CT secretion (McGehee *et al.,* 1997). High Ca_o^{2+} first stimulates phosphatidylcholine-specific PLC, which generates diacylglycerol, thereby activating an NCC through a PKC-dependent mechanism. The activated NCC then enhances cellular uptake of Na^+ and Ca^{2+}, producing cellular depolarization and consequent stimulation of voltage-dependent, principally L-type Ca^{2+} channels that elevate Ca_i^{2+} and activate exocytosis of 5-hydroxytryptamine- and CT-containing secretory granules.

Kidney

In the kidney of the adult rat, the CaR is expressed along almost the entire nephron, with the highest levels of protein expression at the basolateral aspect of the epithelial cells of the cortical thick ascending limb (CTAL) (Riccardi *et al.,* 1998). The latter plays a key role in the hormone (e.g., PTH)-regulated reabsorption of divalent minerals (De Rouffignac and Quamme, 1994; Friedman and Gesek, 1995). The CaR is also present on the basolateral membranes of the cells of the distal convoluted tubule (DCT), where Ca^{2+} reabsorption—similar to that in the CTAL— is stimulated by PTH. Further sites of renal CaR expression include the base of the microvilli of the proximal tubular brush border (Riccardi *et al.,* 1998), the basolateral surface of the tubular cells of the medullary thick ascending limb (MTAL) (Riccardi *et al.,* 1998), and the luminal side of the epithelial cells of the inner medullary collecting duct (IMCD) (Sands *et al.,* 1997). None of these latter nephron segments play major roles in renal Ca_o^{2+} handling, but the CaR expressed in them could conceivably modulate the handling of other solutes and/or water. As will be discussed in more detail later, the CaR in the CTAL, in addition to modulating reabsorption of Ca^{2+} and Mg^{2+}, also participates in controlling the renal handling of Na^+, K^+, and Cl^- (Hebert *et al.,* 1997). Finally, the CaR expressed in the IMCD likely mediates the well-recognized inhibitory action of high Ca_o^{2+} on vasopressin-evoked water reabsorption (Sands *et al.,* 1997, 1998), which is one cause of the defective urinary-concentrating ability in some patients with hypercalcemia (Brown, 1999; Brown *et al.,* 1999).

The localization of the CaR on the basolateral membrane in the CTAL indicates that it might represent the mediator of the previously demonstrated inhibitory effect of high peritubular but not luminal levels of Ca_o^{2+} on Ca^{2+} and Mg^{2+} reabsorption in perfused tubular segments from this portion of the nephron (De Rouffignac and Quamme, 1994). Figure 2 shows a schematic illustration of how the CaR may inhibit PTH-enhanced divalent cation reabsorption in the CTAL. As indicated in detail in Fig. 2, the CaR acts in a "lasix-like" fashion to diminish overall activity of the Na^+-K^+-$2Cl^-$ cotransporter, which generates the lumen-positive, transepithelial potential gradient that normally drives passive paracellular reabsorption of about 50% of NaCl and most of the Ca^{2+} and Mg^{2+} in this part of the nephron (Hebert *et al.,* 1997). It is of interest that individuals with FHH manifest a markedly reduced ability to upregulate urinary excretion of Ca^{2+} despite their hypercalcemia—even when they have been rendered aparathyroid by total parathyroidectomy (Attie *et al.,* 1983). Thus, the PTH-independent, excessive reabsorption of Ca^{2+} in FHH likely results, in part, from a decreased complement of normally functioning CaRs in the CTAL. This defect renders the tubule "resistant" to Ca_o^{2+} and reduces the normal, high Ca_o^{2+}-evoked hypercalciuria that occurs in this nephron segment (Brown, 1999). Thus, in normal persons, hypercalcemia-evoked hypercalciuria likely has two distinct CaR-mediated components: (1) suppression of PTH secretion and (2) direct inhibition of tubular reabsorption of Ca^{2+} in the CTAL. It is not presently known whether the CaR also modulates PTH-enhanced Ca^{2+} reabsorption in the DCT. The Ca^{2+}-permeable channels, ECaC1 (Hoenderop *et al.,* 1999) and CaT2 (Peng *et al.,* 2000) (which were cloned from rabbit and rat kidney, respectively, and are orthologs of the same gene), may participate in the apical entry of Ca^{2+} in this part of the nephron. They represent potential targets for the mutually antagonistic regulation of Ca^{2+} reabsorption in the DCT and connecting segment by PTH and the CaR.

Intestine

The intestine is a key participant in maintaining Ca_o^{2+} homeostasis by virtue of its capacity for the regulated absorption of dietary Ca_o^{2+} through the action of $1,25(OH)_2D_3$, the most active naturally occurring metabolite of vitamin D (Bringhurst *et al.,* 1998; Brown, 1991). The duodenum is the principal site for l,25-dihydroxyvitamin D_3 [$l,25(OH)_2D_3$]-stimulated intestinal Ca^{2+} absorption through a transcellular pathway of active transport. The latter is thought to involve initial Ca^{2+} entry through the newly cloned, Ca^{2+}-permeable channel known as CaT1 (Peng *et al.,* 1999) or ECaC2 (Hoenderop *et al.,* 1999) (both are products of the same gene and are closely related to but distinct from CaT2 and ECaC1). Calcium ions subsequently diffuse across the cell—a process facilitated by the vitamin D-dependent Ca^{2+}-binding protein, calbindin—and are eventually extruded at the basolateral cell surface by the Ca^{2+}-ATPase and, perhaps, the Na^+-Ca^{2+} exchanger. Jejunum and ileum absorb less Ca^{2+} than the duodenum (particularly when expressed as absorption per unit surface area). They also secrete Ca^{2+}, which may chelate fatty acids and bile acids, thereby producing insoluble "calcium soaps" and mitigating possible damaging effects of free fatty

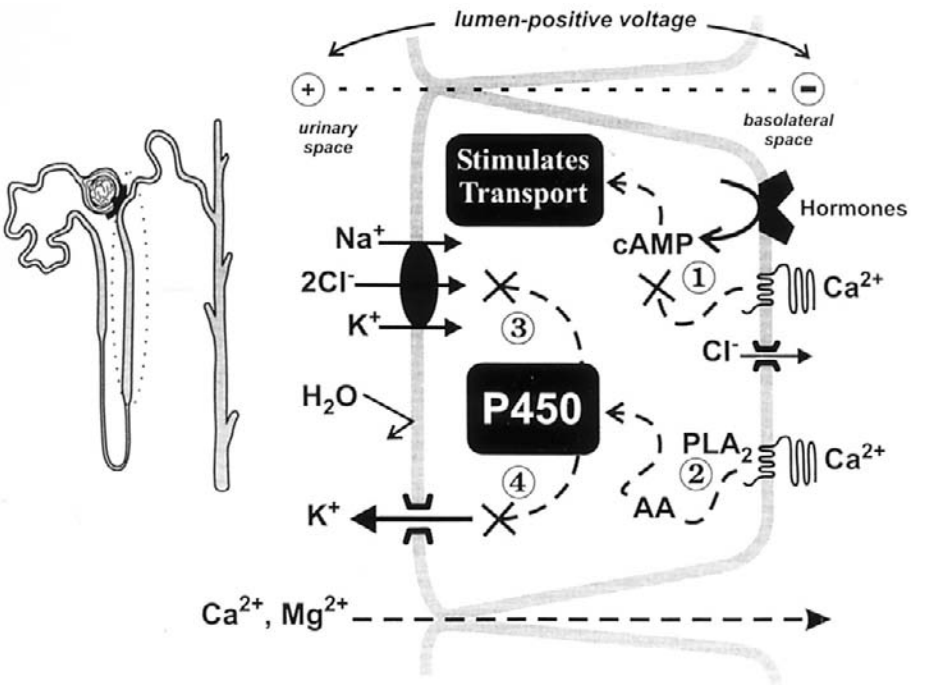

Figure 2 Possible mechanisms by which the CaR modulates intracellular second messenger pathways and ionic transport in the CTAL. Hormones that elevate cAMP (i.e., PTH) stimulate paracellular Ca^{2+} and Mg^{2+} reabsorption by increasing the activities of the Na^+-K^+-$2Cl^-$ cotransporter and an apical K^+ channel and, therefore, increasing the magnitude of V_t. The CaR, which like the PTH receptor is on the basolateral membrane, inhibits PTH-stimulated adenylate cyclase and activates PLA_2 (2). This latter action increases free arachidonic acid, which is metabolized by the P450 pathway to an inhibitor of the apical K^+ channel (4) and, perhaps, the cotransporter (3). Actions of the CaR on both adenylate cyclase and PLA_2 will reduce V_t and, therefore, diminish paracellular divalent cation transport. Reproduced with permission from Brown and Hebert (1997a).

acids and bile salts on colonic epithelial cells. The major function of the colon in fluid and electrolyte metabolism is the absorption of water and Na^+. Nevertheless, it absorbs substantial amounts of Ca^{2+} in humans by both vitamin D-dependent and -independent routes, especially in its proximal segments (Favus, 1992), where it expresses levels of CaT1 similar to those in the duodenum (Peng *et al.,* 1999).

The CaR is expressed in all segments of the intestine in the rat. The highest levels of expression of the receptor are on the basal surface of the small intestinal absorptive cells, the epithelial cells of the crypts of both the small intestine and colon, and in the enteric nervous system (Chattopadhyay *et al.,* 1998). Does the CaR have any role in systemic Ca_o^{2+} homeostasis in these locations? The CaR expressed in the enteric nervous system, which regulates the secretomotor functions of the gastrointestinal tract, could potentially mediate known effects of high and low Ca_o^{2+} to reduce and increase GI motility, respectively, in hyper- and hypocalcemic individuals (Bringhurst *et al.,* 1998). Such an effect on gastrointestinal motility, however, even if it were CaR mediated, would not have any obvious relevance to systemic Ca_o^{2+} homeostasis, other than perhaps indirectly, by modulating the time available for absorption of Ca^{2+} and other nutrients. Currently available evidence, however, does suggest a possible role for the CaR in directly modulating intestinal Ca^{2+} absorption. Hypercalcemia is known to inhibit dietary Ca^{2+} absorption (Krishnamra *et al.,* 1994). Moreover, direct measurements of Ca_o^{2+} within the interstitial fluid underneath small intestinal absorptive epithelial cells has shown that Ca_o^{2+} increases by nearly two-fold if luminal levels of Ca_o^{2+} are elevated to 5–10 m*M*—similar to those achieved after the intake of Ca^{2+}-containing foods (Mupanomunda *et al.,* 1999). This level of Ca_o^{2+} would be more than sufficient to stimulate the CaR expressed on the basolateral surface of small intestinal absorptive cells. Thus it is conceivable that there is a homeostatically relevant, negative feedback regulation of intestinal Ca^{2+} absorption occurring via the local increases in Ca_o^{2+} that take place during the absorptive process. It is not presently known if the CaR modulates small interstitial or colonic Ca^{2+} secretion, although hypercalcemia has been shown to stimulate intestinal Ca^{2+} secretion in some studies (Krishnamra *et al.,* 1994).

Bone and Cartilage

The levels of Ca_o^{2+} achieved within the bony microenvironment probably vary substantially during the regulated

turnover of the skeleton via osteoclastic resorption of bone followed by its restoration by bone-forming osteoblasts—a process that totally replaces the human skeleton approximately every 10 years (Bringhurst *et al.,* 1998). In fact, Ca_o^{2+} directly beneath resorbing osteoclasts can be as high as 8–40 m*M* (Silver *et al.,* 1988). Moreover, Ca_o^{2+} has a variety of actions on bone cell functions *in vitro* that could serve physiologically useful purposes, although it has not yet known if these same actions occur *in viv*o. For example, high Ca_o^{2+} stimulates parameters of osteoblastic functions that could enhance their recruitment to sites of future bone formation, including chemotaxis and proliferation, and promote their differentiation to osteoblasts with a more mature phenotype (Quarles, 1997; Yamaguchi *et al.,* 1999). Furthermore, elevated levels of Ca_o^{2+} suppress both the formation (Kanatani *et al.,* 1999) and the activity (Zaidi *et al.,* 1999) of osteoclasts *in vitro.* Therefore, Ca_o^{2+} has effects on cells of both osteoblastic and osteoclastic lineages and/or their precursors that are homeostatically appropriate. Raising Ca_o^{2+} would, for example, produce net movement of Ca^{2+} into bone by stimulating bone formation and inhibiting its resorption. In addition, locally elevated levels of Ca_o^{2+} produced by osteoclasts at sites of active bone resorption could potentially contribute to "coupling" bone resorption to the ensuing replacement of the missing bone by osteoblasts, by promoting proliferation and recruitment of preosteoblasts within the vicinity to these sites and enhancing their differentiation (Quarles, 1997; Yamaguchi *et al.,* 1999). As detailed later, the molecular identity of the Ca_o^{2+}-sensing mechanism(s) in bone cells remain(s) controversial, although the CaR has been found by several groups of investigators to be present in at least some cells of both osteoblast and osteoclast lineages and could, therefore, potentially participate in this process.

Substantial indirect evidence amassed prior to and around the time that the CaR was cloned suggested that the Ca_o^{2+}-sensing mechanism in osteoblasts and osteoclasts differed in certain pharmacological and other properties from those exhibited the CaR (Quarles, 1997; Zaidi *et al.,* 1999). Moreover, some investigators have been unable to detect expression of the CaR in osteoblast-like (Pi *et al.,* 1999) and osteoclast-like cells (Seuwen *et al.,* 1999). More recent studies, however, have provided strong support for the presence of the CaR in a variety of cells originating from the bone and bone marrow, although its physiological and functional implications in these cells remain uncertain. These CaR-expressing cells include hematopoietic precursors of some (i.e., erythroid and platelet progenitors) but not all lineages (e.g., myeloid precursors) (House *et al.,* 1997), some osteoblast-like and osteoclast-like cell lines, and cells of both lineages when studied *in situ* in sections of bone (Yamaguchi *et al.,* 1999). ST-2 stromal cells (Yamaguchi *et al.,* 1998a), a stromal cell line derived from the same mesenchymal stem cells giving rise to osteoblasts, express CaR mRNA and protein, as do osteoblast-like cell lines (e.g., the Saos-2, MC-3T3-E1, UMR-106, and MG-63 cell lines) (Chang *et al.,* 1999b; Yamaguchi *et al.,* 1998b,c). Furthermore, Chang *et al.* (1999b) have shown that both CaR mRNA and protein are expressed in most osteoblasts in sections of murine, rat, and bovine bone. Regarding cells of the osteoclast lineage, more than 80% of human peripheral blood monocytes, which arise from the same hematopoietic lineage giving rise to osteoclast precursors, express abundant levels of CaR mRNA and protein (Yamaguchi *et al.,* 1998d). Preosteoclast-like cells generated *in vitro* also show expression of the CaR (Kanatani *et al.,* 1999), and osteoclasts isolated from rabbit bone likewise express the receptor (Kameda *et al.,* 1998). In murine, rat, and bovine bone sections, in contrast, only a minority of the multinucleated osteoclasts expressed CaR mRNA and protein (Chang *et al.,* 1999b). Additional studies are required to clarify whether primarily osteoclast precursors, rather than mature osteoclasts, express the CaR. Furthermore, more work is necessary in which the activity of the CaR in bone cells and their precursors is "knocked out," utilizing genetic and/or pharmacological methodologies to determine if the CaR actually mediates some or even all of the known actions of Ca_o^{2+} on these cells. One study failed to detect expression of the CaR in transformed osteoblast-like cells derived from either wild-type mice or those with knock-out of the CaR (Pi *et al.,* 2000). However, these cells still showed some responses to Ca_o^{2+} and Al^{3+} (e.g., mitogenesis), suggesting the presence of another Ca_o^{2+} sensor, as this group has suggested in earlier studies (Quarles *et al.,* 1997).

While chondrocytes—the cells that form cartilage—do not participate directly in systemic Ca_o^{2+} homeostasis, they play a key role in skeletal development and growth by providing a cartilaginous model of the future skeleton that is gradually replaced by actual bone. Furthermore, the growth plate represents a site where chondrocytes play a crucial role in the longitudinal growth that persists until the skeleton is fully mature at the end of puberty. The availability of Ca^{2+} is important for ensuring proper growth and differentiation of chondrocytes and resultant skeletal growth *in vivo* (Jacenko and Tuan, 1995; Reginato *et al.,* 1993). Moreover, changing the level of Ca_o^{2+} *in vivo* modulates the differentiation and/or other properties of cells of the cartilage lineage (Bonen and Schmid, 1991; Wong and Tuan, 1995). Chondrocytes arise from the same mesenchymal stem cell lineage that gives rise to osteoblasts, smooth muscle cells, adipocytes, and fibroblasts (Boyan *et al.,* 1999; Dennis *et al.,* 1999). It is interesting, therefore, that the rat cartilage cell line RCJ3.1C5.18 showed readily detectable levels of CaR mRNA and protein (Chang *et al.,* 1999a). In addition, some cartilage cells in sections of intact bone express CaR mRNA and protein, including the hypertrophic chondrocytes in the growth plate, which are key participants in the growth of long bones (Chang *et al.,* 1999b). Thus the CaR is a candidate for mediating, at least in part, the previously described direct actions of Ca_o^{2+} on chondrocytes and cartilage growth.

In fact, elevating Ca_o^{2+} exerts several direct effects on RCJ3.1C5.18 cells dose dependently, reducing the levels of the mRNAs that encode a major proteoglycan in

cartilage, aggrecan, the α_1 chains of type II and X collagens, and alkaline phosphatase (Chang *et al.,* 1999a). In addition, treatment of this cell line with a CaR antisense oligonucleotide for 48–72 hrs reduced the level of CaR protein expression significantly, in association with enhanced expression of aggrecan mRNA (Chang *et al.,* 1999a), suggesting a mediatory role of the CaR in regulating this gene. These results demonstrate, therefore, that (1) Ca_o^{2+} modulates the expression of several important genes in this chondrocytic cell line, (2) this cartilage-like cell line expresses CaR mRNA and protein, and (3) the receptor mediates at least some of these actions of Ca_o^{2+} in this chondrocytic model. Thus the CaR could potentially not only modulate bone turnover and/or the coupling of bone resorption to its later replacement by osteoblasts through its actions on bone cells and/or their precursors, but might also participate in the control of skeletal growth through its effects on chondrocytes.

The CaR and Integration of Calcium and Water Metabolism

In addition to its roles in tissues involved directly in Ca_o^{2+} homeostasis, increasing evidence indicates that the CaR is located in other cells and tissues where it contributes to integrating the functions of distinct homeostatic systems, as discussed in this section. An illustrative example is the capacity of the CaR to integrate certain aspects of mineral and water metabolism. Some hypercalcemic patients exhibit reduced urinary-concentrating capacity and, occasionally, frank nephrogenic diabetes insipidus (Gill and Bartter, 1961; Suki *et al.,* 1969). The presence of CaR in several segments of the nephron that participate in regulating urinary concentration (Riccardi *et al.,* 1998; Sands *et al.,* 1997) has suggested a novel mechanism(s) for the long-recognized but poorly understood inhibitory actions of high Ca_o^{2+} on renal-concentrating capacity. Studies have shown that high Ca_o^{2+}, probably by activating CaRs residing on the apical membrane of cells of the IMCD, reversibly inhibits vasopressin-elicited water flow by about 35–40% in perfused rat IMCD tubules (Sands *et al.,* 1997). Indeed, the CaR is present in the same apical endosomes that contain the vasopressin-regulated water channel aquaporin-2 (Sands *et al.,* 1997). This observation indicates that the receptor potentially reduces vasopressin-enhanced water flow in the IMCD by either promoting the endocytosis or blocking the exocytosis of these endosomes (Sands *et al.,* 1997). Furthermore, induction of chronic hypercalcemia in rats through treatment with vitamin D reduces the level of expression of the aquaporin-2 protein but not its mRNA (Sands *et al.,* 1998), which would further decrease vasopressin-activated water flow in the terminal-collecting duct. In addition to the mechanisms just described, high Ca_o^{2+}-elicited, CaR-mediated reduction in the reabsorption of NaCl in the MTAL (Wang *et al.,* 1996, 1997), by decreasing the medullary countercurrent gradient, would diminish even further the maximal urinary-concentrating power of hypercalcemic patients (Fig. 3).

What is the evidence that these effects of high Ca_o^{2+} on the renal concentrating mechanism are mediated by the CaR? Of interest in this regard, persons with inactivating mutations of the CaR are capable of concentrating their urine normally despite their hypercalcemia (Marx *et al.,* 1981), presumably because they are "resistant" to the usual suppressive effects of high Ca_o^{2+} on the urinary-concentrating mechanism. Conversely, individuals with activating CaR mutations can develop symptoms of diminished urinary-concentrating capacity (e.g., polyuria and polydipsia), even at normal levels of Ca_o^{2+}, when treated with vitamin D and calcium supplementation, probably because their renal CaRs are overly sensitive to Ca_o^{2+} (Pearce *et al.,* 1996). These experiments in nature, therefore, support the postulated, CaR-mediated link between Ca_o^{2+} and water homeostasis.

Is the defective renal handling of water in hypercalcemic patients of any physiological relevance? We have suggested previously that it provides a mechanism that integrates the renal handling of divalent cations, especially Ca^{2+}, and water, thereby allowing appropriate "trade-offs" in how the kidney coordinates calcium and water metabolism under specific physiological conditions (Hebert *et al.,* 1997) (Fig. 3). For example, in a situation where a systemic load of Ca^{2+} must be disposed of, the resultant CaR-mediated inhibition of PTH secretion and direct inhibition of tubular reabsorption of Ca^{2+} promote calciuria. The consequent elevation in luminal Ca_o^{2+} in the IMCD, particularly in a dehydrated individual, could predispose to the formation of Ca^{2+}-containing renal stones, were not for the concomitant, CaR-mediated inhibition of maximal urinary concentration. Furthermore, there are abundant CaRs in the subfornical organ (SFO) (Rogers *et al.,* 1997)—an important hypothalamic thirst center (Simpson and Routenberg, 1975)—that may ensure a physiologically appropriate increase in drinking. This increased intake of free water could prevent dehydration that might otherwise be a consequence of renal loss of free water due to the concomitant, CaR-induced inhibition of the urinary-concentrating mechanism (Fig. 3).

Finally, available data support the existence of a "calcium appetite" in rats (Tordoff, 1994) that may furnish a mechanism for modulating the intake of calcium-containing foods in a physiologically relevant manner during hypo- and hypercalcemia. Some reduction in the intake of Ca_o^{2+}-containing foods might also occur as a result of the activation of CaRs in the area postrema of the brain—a "nausea center" (Rogers *et al.,* 1997)—due to the resultant anorexia/nausea. We have hypothesized, therefore, that the CaR mediates multiple layers of integration and coordination of the homeostatic systems governing water and calcium metabolism. In doing so, it may contribute to the ability of terrestrial organisms to adjust to the only intermittent availability of environmental calcium and water (Hebert *et al.,* 1997).

CaR-mediated modulation of vasopressin-induced water flow in the IMCD represents an example of "local" Ca_o^{2+}

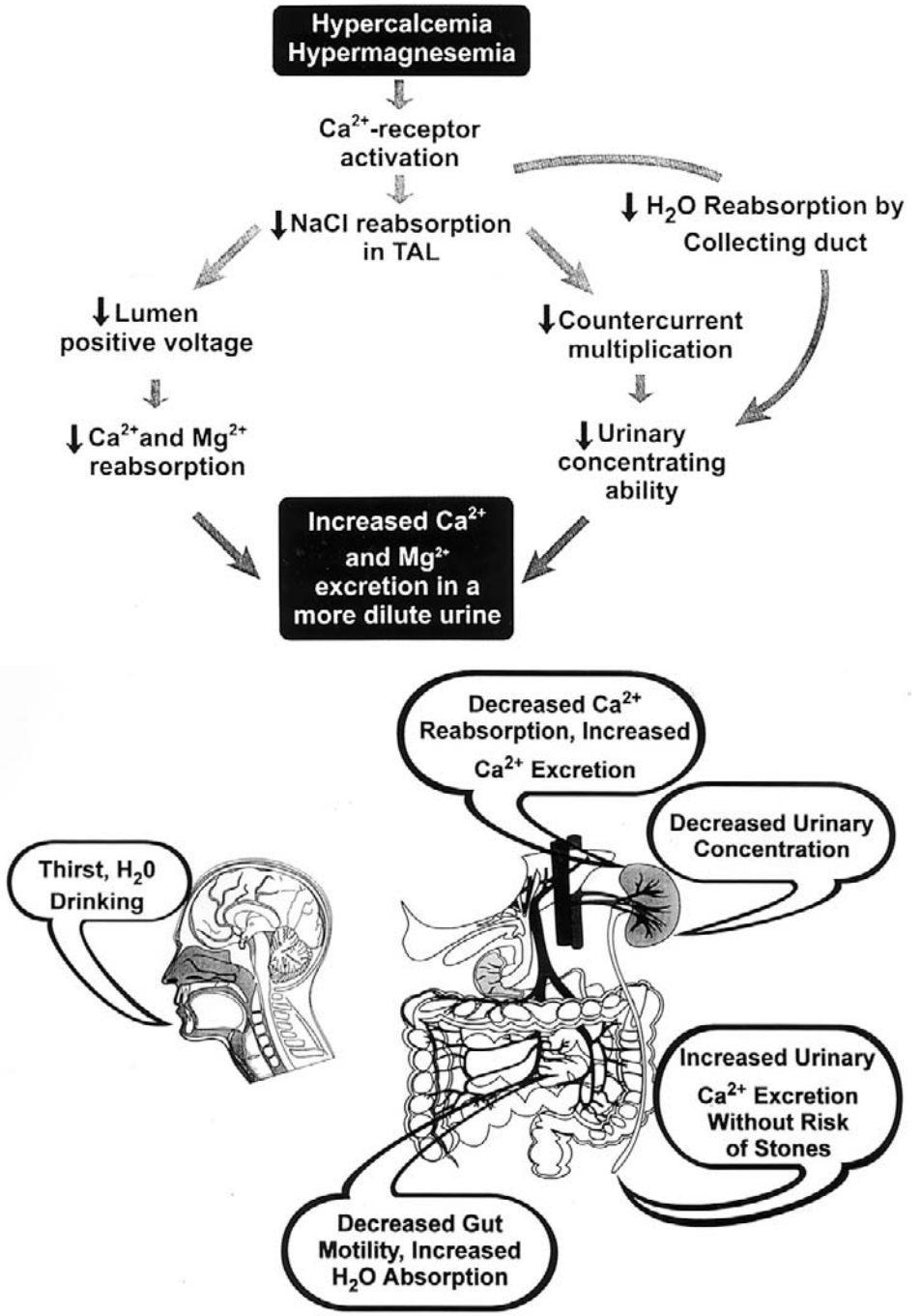

Figure 3 Mechanisms that may interrelate systemic Ca_o^{2+} and water homeostasis (see text for further details). (Top) Mechanisms through which the CaR reduces maximal urinary-concentrating ability. Reproduced with permission from Brown and Hebert (1997b). (Bottom) Additional extrarenal mechanisms integrating Ca_o^{2+} and water homeostasis, such as Ca_o^{2+}-evoked activation of the CaR in the SFO, which would enhance water intake and mitigate loss of free water that would otherwise result from a diminished maximal urinary-concentrating capacity. Reproduced with permission from Brown *et al.* (1996).

homeostasis. That is, Ca_o^{2+} within a specific microenvironment, which is outside of the blood and the various compartments of the ECF in direct contact with the circulation, is only allowed to rise to a certain level (Brown *et al.,* 1999). Interestingly, changes in the level of Ca_o^{2+} resulting from the mechanism governing systemic Ca_o^{2+} homeostasis are traditionally thought to occur through fine adjustments of the movements of Ca^{2+} into or out of the ECF (i.e., by intestine, bone, or kidney) (Brown, 1991). In contrast, CaR-mediated regulation of Ca_o^{2+} in the IMCD primarily results

from alterations in the movement of water. Perhaps even this formulation is oversimplified. On the one hand, vasopressin is known to increase distal tubular reabsorption of calcium (Hoenderop *et al.,* 2000), which would also reduce the level of Ca_o^{2+} in the collecting duct. On the other, the increased thirst in hypercalcemic patients, in addition to providing more free water so as to mitigate any associated rise in Ca_o^{2+} in the IMCD, would also dilute Ca_o^{2+} in the ECF. Further studies will no doubt illuminate additional subtleties related to how the body integrates divalent mineral and water metabolism.

Other CaR Agonists and Modulators: The CaR as an Integrator of Physiological Signals and as a "Nutrient Sensor"

A variety of divalent cations (Sr_o^{2+}), trivalent cations (e.g., Gd_o^{2+}), and even organic polycations [i.e., spermine (Quinn *et al.,* 1997)] are effective CaR agonists. It is likely that they all interact with one or more binding sites within the ECD of the receptor (Brown *et al.,* 1999). Only a few of these polycationic agonists, however, are thought to be present within biological fluids at levels that would activate the CaR (Quinn *et al.,* 1997). In addition to Ca_o^{2+}, Mg_o^{2+} and spermine are two such putative, physiological CaR agonists. It is probable that in specific microenvironments [e.g., within the gastrointestinal (GI) tract and central nervous system] the concentrations of spermine are high enough to activate the CaR even at levels of Ca_o^{2+} that are insufficient to do so by themselves (Brown *et al.,* 1999; Quinn *et al.,* 1997). In fact, all of the polycationic CaR agonists potentiate one anothers' stimulatory effects on the receptor. In other words, only small increments in the level of any given agonist (i.e., spermine) may be sufficient to activate the CaR when a threshold level of another agonist is present in the local microenvironment (e.g., Ca_o^{2+}) (Brown *et al.,* 1999).

Is the CaR also a Mg_o^{2+} -sensing receptor? Some evidence supporting the role of the CaR in sensing and, therefore, "setting" Mg_o^{2+} comes from the experiments in nature that firmly established the role of the CaR as a central element in Ca_o^{2+} homeostasis. Namely, persons with hypercalcemia due to heterozygous-inactivating mutations of the CaR (e.g., FHH) exhibit serum Mg^{2+} levels that are in the upper part of the normal range or mildly elevated. Moreover, some patients with neonatal severe hyperparathyroidism due to homozygous-inactivating CaR mutations can have more pronounced hypermagnesemia (Aida *et al.,* 1995a). Conversely, persons harboring activating mutations of the CaR can manifest mild reductions in Mg_o^{2+} (Brown, 1999). Mg_o^{2+} is about 2-fold less potent than Ca_o^{2+} on a molar basis in activating the CaR (Brown *et al.,* 1993; Butters *et al.,* 1997). One might justifiably ask, therefore, how Mg_o^{2+} could regulate its own homeostasis by modulating PTH secretion — an important component of CaR-mediated control of Ca_o^{2+} — when circulating levels of Mg_o^{2+} are, if anything, lower than those of Ca_o^{2+} (Bringhurst *et al.,* 1998)? It is possible that even small changes in Mg_o^{2+} can modulate the activity of the CaR in parathyroid cells because the receptor has been sensitized by ambient levels of Ca_o^{2+} that are close to its "set point" (i.e., on the steepest portion of the curve relating PTH to Ca_o^{2+}). A more likely scenario, however, is that Mg_o^{2+} acts on the CaR in the CTAL to regulate its own level in the ECF, as follows: the fraction of Mg^{2+} reabsorbed in the proximal tubule is less than for other solutes (e.g., Ca^{2+}, Na^+, Cl^-, and water). As a result, there is a 1.6- to 1.8-fold rise in the level of Mg_o^{2+} in the tubular fluid of the CTAL (De Rouffignac and Quamme, 1994), which should, therefore, reach a sufficiently high level to activate the CaR in this nephron segment and, therefore, reduce the reabsorption of Mg_o^{2+}. Recall that not only Ca_o^{2+} but also Mg_o^{2+} inhibits the reabsorption of both divalent cations in perfused CTAL (De Rouffignac and Quamme, 1994).

Another factor modulating the actions of Ca_o^{2+} and other polycations on the CaR is ionic strength per se (e.g., alterations in the concentration of NaCl) (Quinn *et al.,* 1998). Elevating ionic strength decreases and reducing ionic strength enhances the sensitivity of the CaR to activation by Ca_o^{2+} and Mg_o^{2+}. The impact of changing ionic strength on the responsiveness of the CaR to divalent cations may be especially relevant in particular microenvironments, such as the GI or urinary tracts, where ionic strength can vary greatly, easily encompassing the range over which this parameter modulates the function of the receptor (Quinn *et al.,* 1998).

In addition to the polycationic CaR agonists just noted, novel "calcimimetic," allosteric activators of the receptor have been developed. These are small hydrophobic molecules, which are derivatives of phenylalkylamines and activate the CaR by increasing its apparent affinity for Ca_o^{2+}. They do so by interacting with the transmembrane domains of the receptor (Nemeth *et al.,* 1998b), in contrast to Ca_o^{2+}, which binds to the ECD (Hammerland *et al.,* 1999). Calcimimetics are called "modulators" rather than "agonists" because they only activate the CaR in the presence of Ca_o^{2+}. In contrast, the polycationic agonists of the CaR (e.g., Gd_o^{2+}) activate it even in the nominal absence of Ca_o^{2+} (Nemeth *et al.,* 1998b). Calcimimetics are currently in phase II/III clinical trials for the treatment of primary and uremic hyperparathyroidism, and results to date strongly suggest that these agents will provide the first effective medical therapy for controlling the hypersecretion of PTH in these two conditions. CaR antagonists, so-called "calcilytics," are also entering clinical trials. The principal therapeutic application envisioned for these agents at the moment is in the treatment of osteoporosis (Nemeth *et al.,* 1998a). Because intermittent exogenous administration of PTH can produce sizable increases in bone mineral density, once daily administration of a short-acting calcilytic would presumably accomplish the same goal by producing a "pulse" of endogenous PTH secretion (Gowen *et al.,* 2000; Nemeth *et al.,* 1998a).

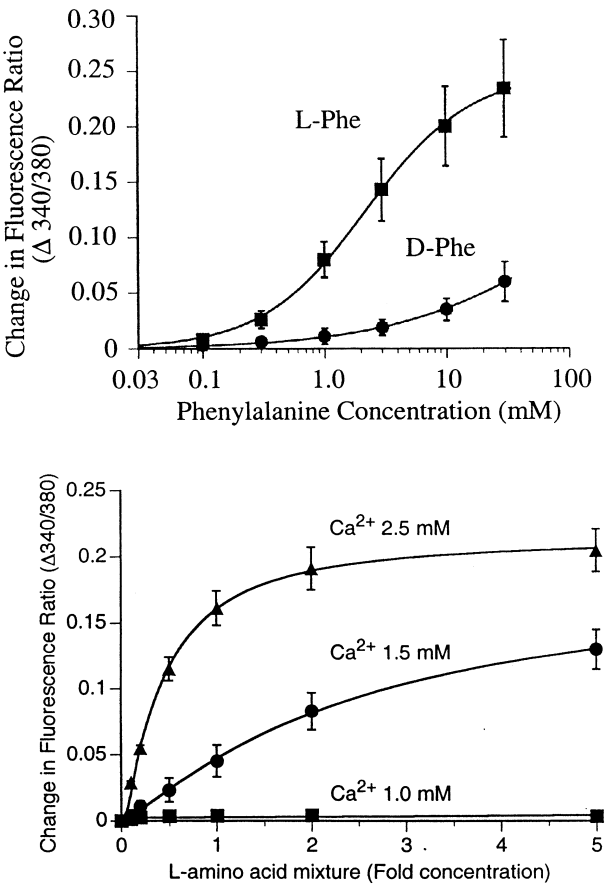

Figure 4 Amino acid sensing by the CaR. (Top) Activation of the CaR by phenylalanine (L-phenylalanine > D-phenylalanine) at 2.5 m*M* Ca_o^{2+} in HEK293 cells transfected stably with the CaR as reflected by amino acid-induced increases in the cytosolic Ca^{2+} concentration in cells loaded with the Ca^{2+}-sensitive intracellular dye fura-2. Increases in the fluorescence ratio (340/380 nm) indicate CaR-mediated increases in Ca_i^{2+}. (Bottom) Marked impact on the level of Ca_o^{2+} on the capacity of the CaR to sense the mixture of L amino acids that emulates that present in the blood under fasting conditions (see text for details). Reproduced with permission from Conigrave *et al.* (2000).

We have identified another class of endogenous CaR modulators, namely certain amino acids (Conigrave *et al.,* 2000) (Fig. 4). Activation of the CaR by specific amino acids, particularly the aromatic amino acids, phenylalanine, tyrosine, histidine, and tryptophan, only occurs when Ca_o^{2+} is 1 m*M* or higher. Although individual amino acids are of relatively low potency (e.g., they act at concentrations of 0.1–1 m*M* or higher), a mixture of amino acids with a composition similar to that present in human serum under fasting conditions substantially enhances the sensitivity of the CaR to Ca_o^{2+}. That is, optimal concentrations of the mixture reduce the EC_{50} for Ca_o^{2+} (the level of Ca_o^{2+} half-maximally activating the receptor) by nearly 2 m*M* (e.g., by 40–50%) in HEK293 cells stably transfected with the CaR (Conigrave *et al.,* 2000).

While the implications of these direct actions of amino acids on the CaR are not yet clear, they could potentially explain several long-standing but poorly understood observations appearing to link protein and Ca_o^{2+} metabolism. Additionally, they suggest future lines of research directed at elucidating the possible role of the receptor in "nutrient sensing" more generally, rather than just as a sensor of divalent cations. For example, ingestion of a high protein diet can nearly double the rate of urinary calcium excretion relative to that observed on a low protein intake (Insogna and Broadus, 1987). This effect of dietary protein has traditionally been ascribed to the buffering of acidic products of protein metabolism by bone as well as a direct calciuric action of the acid load (Lemann *et al.,* 1966). However, direct activation of CaRs in the CTAL as a result of increases in serum levels of amino acids might contribute as well. Moreover, reducing dietary protein intake has been shown to have marked effects on circulating calciotropic hormones, nearly doubling serum PTH and $1,25(OH)_2D_3$ levels in normal women (Kerstetter *et al.,* 1997). Could these latter changes result from the concomitant decrease in the circulating levels of amino acids, despite little, if any, change in Ca_o^{2+}? Is it possible that the reduced intake of dietary protein commonly recommended for patients with chronic renal insufficiency (Bringhurst *et al.,* 1998) actually exacerbates their secondary hyperparathyroidism?

These observations suggest that the CaR is not solely a Ca_o^{2+} (and probably a Mg_o^{2+}) receptor but may also serve as a more general "nutrient" and environmental sensor, which detects changes in Ca_o^{2+} and Mg_o^{2+}, not in isolation, but in the context of the ambient levels of certain amino acids. Further testing of this hypothesis may enhance our understanding of the mechanisms by which complex organisms coordinate homeostatic systems that have traditionally been thought of as functioning largely independently, such as those controlling protein and mineral metabolism. This homeostatic integration may be particularly important within specific parts of the life cycle, such as during somatic growth. Skeletal growth in childhood requires the precisely coordinated deposition of both bone matrix and mineral. Moreover, mineral ions and amino acids must also be assimilated during the growth of soft tissues—all of which contain varying mixtures of mineral ions and protein. For instance, smooth muscle cells contain half as much calcium as bone when expressed on the basis of wet weight (Brown and MacLeod, 2001).

Coordinating mineral ion and protein metabolism might be particularly relevant in the GI tract. Indeed, the presence of an "amino acid receptor" regulating the secretion of gastrin, gastric acid, and cholecystokinin has been postulated (Conigrave *et al.,* 2000). Furthermore, the pharmacological profile of the actions of different amino acids on these parameters is strikingly similar to that for the effects of the same amino acids on the CaR (Mangel *et al.,* 1995; McArthur *et al.,* 1983; Taylor *et al.,* 1982; Conigrave *et al.,* 2000). CaRs in the GI tract system could serve as a particularly suitable target for sensing the availability of dietary protein and mineral ions, which are generally ingested together (i.e., in milk). Further studies are needed, therefore, to investigate whether the CaR represents, in

fact, this putative amino acid receptor. Such investigations may reveal whether the sensing of amino acids by the CaR, taken in the context of ambient levels of Ca_o^{2+} and Mg_o^{2+} within the GI tract and elsewhere, provides the molecular basis for a physiologically important link between the systems governing protein and mineral metabolism.

Are There Additional Ca_0^{2+} Sensors?

As noted earlier and described in detail in other reviews (Quarles, 1997; Zaidi *et al.,* 1999), Ca_o^{2+} sensors in addition to the CaR may exist on osteoblasts and osteoclasts. Moreover, studies have revealed that some of the mGluRs can sense Ca_o^{2+} in addition to recognizing glutamate as their principal physiological agonist, although the physiological importance of this Ca_o^{2+} sensing is not clear at present. Kubo *et al.* (1998) showed that mGluRs 1, 3, and 5 sense levels of Ca_o^{2+} between 0.1 and 10 m*M,* whereas mGluR2 is substantially less responsive to Ca_o^{2+}. All three of the mGluRs that are capable of sensing Ca_o^{2+} have identical serines and threonines, respectively, at amino acid positions that are equivalent to amino acid residues 165 and 188 in mGluR1a (Brauner-Osborne *et al.,* 1999). These two residues have been shown to play key roles in the binding of glutamate to the respective ECDs of the MGluRs (O'Hara *et al.,* 1993). In contrast, while mGluRs 1a, 3, and 5 have serines at positions equivalent to amino acid residue 166 in mGluR1a, mGluR2 has an aspartate rather than a serine at this position (Kubo *et al.,* 1998). Changing this serine to an aspartate in mGluRs 1a, 3, and 5 substantially reduces their capacities to respond to Ca_o^{2+}, whereas substituting the aspartate in mGluR2 with a serine enhances its apparent affinity for Ca_o^{2+} to a level comparable to those of mGluRs 1, 3, and 5 (Kubo *et al.,* 1998). Thus the serines in mGluRs 3 and 5 at amino acid positions homologous to residue 166 in mGluR1a appear to play important roles in the capacities of these three receptors to sense Ca_o^{2+}.

Interestingly, another study has shown that changes in Ca_o^{2+} also modulate the function of the activated $GABA_B$ receptors, whereas Ca_o^{2+} has no effect on these receptors in the absence of added GABA (Wise *et al.,* 1999). Ca_o^{2+} potentiates the stimulatory effect of GABA on the binding of GTP to the receptor and increases the coupling of the $GABA_B$ receptor to stimulation of a K^+ channel and inhibition of forskolin-stimulated adenylate cyclase activity. The effects of Ca_o^{2+} on the $GABA_B$ receptor, unlike those on the CaR, were not reproduced by other polyvalent cations. Thus, similar to the CaR, which senses Ca_o^{2+} but is modulated by various amino acids (although not by glutamate) (Conigrave *et al.,* 2000), mGluRs and $GABA_B$ receptors sense their primary physiological ligands, glutamate and GABA, respectively, as well as Ca_o^{2+}. These observations further emphasize the structural, functional, and evolutionary relationships among the three types of receptors.

Finally, Ca_o^{2+} could also, of course, modulate the functions of proteins other than GPCRs. For instance, the recently cloned Ca_o^{2+} channels CaT1, CaT2, ECaC1, and ECaC2 can be viewed as operating on a macroscopic level as facilitated transporters. That is, they exhibit Michaelis–Menten-like kinetics, their activities (measured as $^{45}Ca^{2+}$ uptake in *X. laevis* oocytes) increase with the level of Ca_o^{2+} until Ca^{2+} uptake saturates above about 1 m*M* Ca_o^{2+}. They could potentially function, therefore, as Ca_o^{2+} sensors. That is, they would tend to "set" the level of Ca_o^{2+} within the local ECF by increasing Ca^{2+} uptake when Ca_o^{2+} is high and reducing it when it is low.

Summary

The discovery of the CaR has provided a molecular mechanism that mediates many of the known actions of Ca_o^{2+} on the cells and tissues that participate directly in systemic Ca_o^{2+} homeostasis, such as parathyroid and certain renal cells. There is still much to be learned, however, about the various functions of the receptor in these tissues, particularly in intestinal and bone cells, as well as in the numerous other CaR-expressing cells that are not directly involved in systemic Ca_o^{2+} homeostasis. In these latter, "nonhomeostatic" cells, the CaR probably serves a variety of roles that enable it to serve as a versatile first messenger, capable of regulating numerous cellular functions. Moreover, the ability of the CaR to integrate and coordinate several different ionic and nutritional signals may permit it to act as a central homeostatic element not only for mineral ion homeostasis, but also for processes relevant to Mg_o^{2+}, water, and protein metabolism.

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CHAPTER 24

Receptors for Parathyroid Hormone (PTH) and PTH-Related Peptide

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Introduction

The biological actions of parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP) have attracted ever wider interest in recent years because of the rapid advances in the study of the developmental biology of bone in which PTHrP and its receptor play a major role, as well as demonstration of the therapeutic potential of PTH in fracture prevention in osteoporosis.

One principal receptor, the type-1 PTH/PTHrP receptor (PTH1R), is the chief mediator of both the homeostatic actions of PTH and the paracrine actions of PTHrP on endochondral bone development. This receptor interacts equivalently with the amino-terminal domains of PTH and PTHrP. As discussed later, however, additional receptors clearly interact differentially with PTH versus PTHrP and/or with regions of the two ligands other than their amino-terminal domains. The tools of molecular biology have been central in the efforts to clone and express the PTH1R and the closely related PTH-2 receptor, as well as to characterize both ligand-binding requirements and signaling properties of these receptors. Work with the receptors for PTH and PTHrP has proven pivotal in studies aimed at understanding the physiological role of PTH in calcium and phosphate homeostasis in greater depth and the critical paracrine role played by PTHrP in the complex network of different signaling factors that directs endochondral bone development.

At the same time that the reductionist approaches based on analyses of cloned receptors expressed in cell lines have helped clarify initial steps in PTH action and provided a detailed analysis of ligand/receptor/signaling events *in vitro,* the tools of molecular biology have also made possible a new level of integrative physiological analyses of bone biology *in vivo* through the use of mice modified genetically through selective gene knockout and/or transgenic overexpression of the PTH1 receptor and/or its ligands. Much of this latter work, as well as the overall biological actions and physiological role of PTH, is outlined in subsequent chapters on PTH. This chapter focuses on the receptors per se, particularly those that are cloned and well characterized, such as the PTH1R and PTH2R, and those that are still uncloned but of potential biological significance in overall PTH or PTHrP action, especially the receptor for the carboxyl-terminal portion of PTH for which much recent biochemical data have accumulated.

Receptors for PTH, PTHrP, and TIP39: The PTH1R and PTH2R

Cloning, Gene Structure, Evolution, and Expression

Because of the pleiotropic actions of PTH, which involve both direct and indirect effects, as well as multiple signal

transduction mechanisms, it was initially thought that several different receptors mediated the biological responses of this peptide hormone. Furthermore, the realization that some of these actions were PTHrP rather than PTH dependent seemed to increase the probability that more than one receptor would be involved. It was somewhat surprising therefore that initial cloning approaches led to the isolation of cDNAs encoding only a single G protein-coupled receptor, the PTH/PTHrP receptor or PTH-1 receptor (PTH1R). The recombinant PTH1R interacts equivalently with PTH and PTHrP and activates at least two distinct second messenger pathways: adenylate cyclase/protein kinase A (AC/PKA) and phospholipase C/protein kinase C (PLC/PKC) (Abou-Samra *et al.,* 1992; Jüppner *et al.,* 1991; Schipani *et al.,* 1993). These findings with the recombinant receptor confirmed earlier studies using different clonal cell lines or renal membrane preparations that had shown that PTH and PTHrP bind to and activate the same G protein-coupled receptor with similar efficiency and efficacy (Jüppner *et al.,* 1988; Nissenson *et al.,* 1988; Orloff *et al.,* 1989; Shigeno *et al.,* 1988). Based on these and subsequent findings, such as the similar phenotypes observed in mice that are null for either PTHrP or the PTH-1 receptor (Karaplis *et al.,* 1994; Lanske *et al.,* 1996; Vortkamp *et al.,* 1996), it now seems very likely that most of the endocrine actions of PTH and paracrine/autocrine actions of PTHrP on bone development are mediated through the PTH-1 receptor. Studies have identified two other G protein-coupled receptors that are closely related to the PTH1R. One of these, the PTH-2 receptor (PTH2R), responds selectively to TIP39, a recently discovered hypothalamic peptide (Usdin, 1999; Usdin *et al.,* 1995), and the other, the PTH-3 receptor (PTH3R), was identified in zebrafish and responds to human PTHrP more efficiently than to human PTH (Rubin and Jüppner, 1999), although it responds to rat PTH more efficiently than either hPTH(1-34) or hPTHrP(1-34) (Hoare *et al.,* 2000b). A review of the published sequence of the human genome indicates that there is no gene sequence detected that might be expected to yield the PTH-3 receptor (Venter *et al.,* 2001).

The PTH-1 receptor belongs to a distinct family of G protein-coupled receptors (GPCR), called class II (or family B) receptors (see the G protein-coupled receptor data base at www.gpcr.org/7tm/). The first cDNAs encoding mammalian PTH-1 receptors were isolated through expression cloning techniques from cell lines that had been widely used in classical PTH/PTH receptor studies: the opossum kidney cell line OK and the rat osteosarcoma cell line ROS 17/2.8 (Abou-Samra *et al.,* 1992; Jüppner *et al.,* 1991). Subsequently, cDNAs encoding human (Eggenberger *et al.,* 1997; Schipani *et al.,* 1993; Schneider *et al.,* 1993), mouse (Karperien *et al.,* 1994), rat (Pausova *et al.,* 1994), chicken (Vortkamp *et al.,* 1996), porcine (Smith *et al.,* 1996), dog (Smock *et al.,* 1999), frog (Bergwitz *et al.,* 1998), and fish (Rubin and Jüppner, 1999) PTH-1 receptors were isolated through hybridization techniques from various tissue and cell sources, i.e., kidney, brain, whole embryos, osteoblast-like cells, and embryonic stem cells. Northern blot and *in situ* studies (Tian *et al.,* 1993; Urena *et al.,* 1993; van de Stolpe *et al.,* 1993), as well as data provided through available public (expressed sequence tag) (EST) databases, confirmed that the PTH-1 receptor is expressed in a wide variety of fetal and adult tissues. With the exception of the tetraploid African clawed frog *Xenopus laevis,* which expresses two non-allelic isoforms of the PTH-1 receptor (Bergwitz *et al.,* 1998), all investigated species have only one copy of the PTH-1 receptor per haploid genome.

The possible existence of other receptors for PTH or PTHrP with unique, organ-specific pharmacological characteristics had been suggested by the distinct ligand binding (Chorev *et al.,* 1990a,b; McKee *et al.,* 1988) and second messenger signaling profiles observed in different clonal cell lines (Cole *et al.,* 1987; Yamaguchi *et al.,* 1987a,b). However, the molecular cloning of identical full-length PTH-1 receptor cDNAs from human kidney, brain, and bone-derived cells (Eggenberger *et al.,* 1997; Schipani *et al.,* 1993; Schneider *et al.,* 1993) suggested that the previously observed pharmacological differences arose from species-specific variations in the receptor primary sequence rather than the tissue-specific expression of distinct receptors.

The gene encoding the human PTH-1 receptor is located on chromosome 3 (locus 3p22-p21.1). The intron/exon structure of the gene has been analyzed in detail (Bettoun *et al.,* 1997; Manen *et al.,* 1998; Schipani *et al.,* 1995) and was shown to have an organization similar to that of genes encoding the rat and mouse homologues (Kong *et al.,* 1994; McCuaig *et al.,* 1994) (Fig. 1). In each of these mammals, the PTH1R gene spans at least 20 kbp of DNA and consists of 14 coding exons and at least three noncoding exons. The size of the coding exons in the human PTH-1 receptor gene ranges from 42 bp (exon M7) to more than 400 bp (exon T); the size of the introns varies from 81 bp (between exons M6 and M6/7) to more than 10 kbp (between exons S and E1).

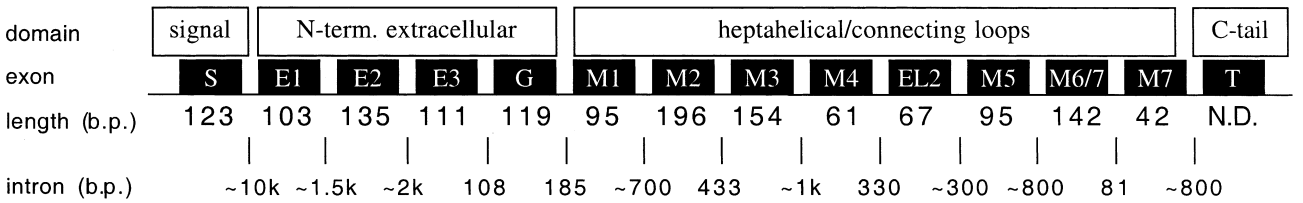

Figure 1 Intron/exon structure of the human PTH-1 receptor gene. The 14 coding exons of the human PTH1R gene are indicated as black boxes, and the corresponding receptor domains are indicated above in open boxes. The lengths in nucleotide basepairs (k = 1/1000) of the exons and the intervening introns are also indicated.

Two promoters for the PTH-1 receptor have been described in rodents (Joun *et al.,* 1997; Kong *et al.,* 1994; McCuaig *et al.,* 1994, 1995). The P1 promoter (also referred as U3) is active mainly in the adult kidney, whereas the P2 promoter (also referred to as U1) is active in several fetal and adult tissues, including cartilage and bone. In humans, a third promoter, P3 (also referred to as S), also appears to be active in some tissues, including kidney and bone (Bettoun *et al.,* 1998; Giannoukos *et al.,* 1999; Manen *et al.,* 1998). Several frequent polymorphisms were identified within the human PTH-1 receptor gene; these include an intronic *Bsm*I polymorphism located between the 5′ noncoding exon U1 and the coding exon S (Hustmyer *et al.,* 1993) and a silent *Bsr*DI polymorphism in exon M7 (nucleotide 1417 of human PTH-1 receptor cDNA) (Schipani *et al.,* 1994).

At the protein level, all mammalian PTH-1 receptors have a relatively long amino-terminal extracellular domain (~170 amino acids in the human PTH1R after removal of the signal sequence by signal peptidase cleavage). This domain is encoded by five exons: S (encoding the signal sequence), E1, E2, E3, and G [encoding the four N-linked glycosylation sites (Zhou *et al.,* 2000)]. Genes encoding other class II G protein-coupled receptors for which the genomic structure has been explored have a similar organization, except that the equivalent of exon E2 is lacking (Jüppner, 1994; Jüppner and Schipani, 1996). The protein segment encoded by exon E2 is also missing in the PTH-2 receptors, as well as in the PTH-1 receptors from *X. laevis* and zebrafish (Bergwitz *et al.,* 1998; Rubin *et al.,* 1999). Earlier *in vitro* mutational studies showed that the E2 segment of the PTH1R can be modified or deleted without a measurable impact on receptor surface expression or function (Jüppner *et al.,* 1994; Lee *et al.,* 1994). Taken together, these findings led to the conclusion that the addition of this nonessential exon in the mammalian PTH-1 receptors was a relatively recent evolutionary modification to the PTH-1 receptor gene (Rubin and Jüppner, 1999); the biological role of this receptor region, if any, is unknown.

Class II Receptors

The molecular cloning of the PTH-1 receptor (Abou-Samra *et al.,* 1992; Jüppner *et al.,* 1991), along with the receptors for secretin (Ishihara *et al.,* 1991) and calcitonin (Lin *et al.,* 1991) that same year, made it clear that these peptide hormone receptors formed a distinct GPCR family. Except for the structural similarity provided by the seven membrane-spanning helices, members of the class II (family B) peptide hormone GPCR family share virtually no amino acid sequence homology with most other GPCRs, such as the β-adrenergic receptor, a class I GPCR. All members of the secretin/calcitonin/PTH receptor family, including an insect and several other invertebrate peptide hormone receptors (Reagan, 1994, 1996; Sulston *et al.,* 1992), share about 45 strictly conserved amino acid residues. Furthermore, all receptors of this family have a relatively long amino-terminal, extracellular domain, and most use at least two different signal transduction pathways, adenylate cyclase and phospholipase C (Jüppner, 1994; Jüppner and Schipani, 1996). Each of these related receptors contains up to four sites for potential asparagine-linked glycosylation, eight conserved extracellular cysteine residues that appear to be important for ligand/receptor interaction and/or proper receptor processing or folding (Gaudin *et al.,* 1995; Knudsen *et al.,* 1997; Lee *et al.,* 1994; Qi *et al.,* 1997), and several other "signature" residues. It is predicted that within the membrane-embedded region there is an overall topological similarity between these class II heptahelical receptors and G protein-coupled receptors of other families, such as the β-adrenergic receptor (Sheikh *et al.,* 1999), or those represented by the metabotropic glutamate receptor and the calcium-sensing receptor (class III receptors; reviewed in Chapter 23), although the receptors from each class share no primary sequence homology.

A distinctive subgroup of class II receptors has been identified. These receptors have, in addition to the usual hallmarks of the peptide hormone-binding class II receptors, extremely large (>600 amino acid) extensions of the amino-terminal extracellular domain, in which are found arrays of protein sequence motifs that are typically seen in single membrane-spanning proteins involved in cell adhesion (e.g., cadherin, laminin, thrombospondin, lectin, and mucin) (Abe *et al.,* 1999; Baud *et al.,* 1995; Hamann *et al.,* 1995; Usui *et al.,* 1999). The biological roles of these distinctive heptahelical proteins, the identity of their cognate ligands, and their evolutionary relationship to the other class II receptors remain to be established.

Mechanisms of Ligand Recognition and Activation by PTH Receptors

Current data indicate that the PTH receptor interacts with multiple regions of PTH peptide ligands; these contacts establish binding affinity and/or promote receptor activation. Much information on these interactions has been gained from studies that used intact native PTH receptors expressed in various cell systems and synthetic PTH and PTHrP analogs. PTH(1-34) and PTHrP(1-34) bind to and activate the PTH-1 receptor with affinities and potencies in the low nanomolar range. The first 13 amino acids of PTH and PTHrP have been highly conserved in evolution with eight identities; the (15-34) regions share only moderate homology with three amino acid identities. The N-terminal portions of the two peptides play key roles in receptor activation, whereas the (15-34) portions are required for high-affinity receptor binding (Abou-Samra *et al.,* 1989; Caulfield *et al.,* 1990; Nussbaum *et al.,* 1980).

Ligand Determinants of PTH Receptor Activation

cAMP Signaling Response

Amino-terminally truncated PTH or PTHrP analogs, such as PTH(3-34), PTH(7-34), and PTHrP(7-34), bind to the PTH-1 receptor with high affinity and elicit little or no

increase in cAMP accumulation. Such fragments yield the most potent PTH-1 receptor competitive antagonists (Nutt *et al.,* 1990). Bulky amino acid modifications within the amino-terminal portion of (1-34)-length peptides (e.g., at positions 2, 3, and 6) also confer antagonistic properties to the peptides (Behar *et al.,* 1999; Carter *et al.,* 1999a; Cohen *et al.,* 1991; Gardella *et al.,* 1991). Peptides consisting only of the amino-terminal residues of PTH exhibit severely diminished receptor-binding affinity and hence cAMP-signaling potency. The shortest amino-terminal peptide of the native sequence that retains full PTH-1 receptor-binding affinity and cAMP-signaling potency is PTH(1-31) (Whitfield and Morley, 1995). PTH(1-14) has been shown to be the shortest native amino-terminal PTH peptide for which at least some cAMP agonist activity can be detected, albeit the EC_{50} of the cAMP response induced by PTH(1-14) in LLC-PK1 porcine kidney cells transfected stably with high levels of the human PTH1R (~100 μM) is markedly higher than that observed for PTH(1-34) (~3 n*M*) (Luck *et al.,* 1999). A series of structure–activity relationship studies on the PTH(1-14) scaffold peptide was undertaken as part of an effort to better understand how residues in the amino-terminal portion of PTH mediate receptor activation (Carter and Gardella, 2001; Luck *et al.,* 1999; Shimizu *et al.,* 2001; Shimizu *et al.,* 2000b). An alanine scan analysis of PTH(1-14) demonstrated the functional importance of residues (1-9) and suggested that this sequence represents a minimum-length receptor-activation domain (Luck *et al.,* 1999). Further substitution analyses revealed that the PTH(1-14) sequence could accommodate amino acid changes at a number of positions (Carter and Gardella, 2001; Shimizu *et al.,* 2000b, 2001) many of which improved signaling potency and binding affinity. The most active analog was [$Ala^{3,12}$, Gln^{10}, homoArg^{11}, Trp^{14}]PTH(1-14), which is ~2000-fold more potent as a cAMP agonist in stably transfected LLC-PK1 cells than was native PTH(1-14) and is only ~60-fold weaker than PTH(1-34) (Shimizu *et al.,* 2001). The relevant substitutions also conferred activity to the otherwise inactive PTH(1-11) fragment; these modified PTH(1-11) analogs are currently the shortest free peptide sequences that can activate the PTH-1 receptor (Shimizu *et al.,* 2000b, 2001).

NON-cAMP SIGNALING RESPONSES

While it is well established that the amino-terminal residues of PTH mediate AC/PKA signaling, there is still some uncertainty regarding the ligand determinants of PLC/PKC/calcium signaling. Several studies have indicated that residues in the C-terminal portion of PTH(1-34) mediate PKC activation; perhaps most notably, the tetrapeptide PTH(29-32) was shown to be sufficient for activating PKC in ROS 17/2 rat osteosarcoma cells (Jouishomme *et al.,* 1994), as well as in Chinese hamster ovary cells transfected with the rat PTH-1 receptor (Azarani *et al.,* 1996). Stimulation of PKC is generally thought to be mediated through PLC signaling; however, other data indicate that determinants of PLC activation reside at the amino terminus of PTH. Thus, even minor N-terminal truncations, as in [desNH_2-Gly^1]PTH(1-34), PTH(2-34), or PTH(3-34), severely diminish the capacity of the peptide to stimulate inositol polyphosphate (IP) production via PLC in porcine kidney LLC-PK1 cells transfected with the human PTH-1 receptor (Takasu *et al.,* 1999a). In addition, the activity-enhanced PTH(1-14) analogs mentioned above stimulate IP production in transfected COS-7 cells, indicating that residues in this N-terminal portion of the ligand are sufficient for PLC signaling (Shimizu *et al.,* 2000b). One possible explanation for the apparent discrepancy in the mapping of PKC and PLC activation determinants is that residues (29-32) of PTH mediate PKC activation via a phospholipase other than PLC. In support of this possibility, Friedman and co-workers (1999) have shown that in the distal tubule cells of the kidney, the PTH-1 receptor couples to phospholipase D, whereas in the proximal tubule cells it couples to phospholipase C; moreover, distinct structural components of the ligand were required for the altered signaling responses in the two different cell types. The PTH-1 receptor may therefore be capable of recognizing different portions of the ligand as activation determinants for various phospholipases, a capacity that may be modulated by the cellular milieu (Whitfield *et al.,* 2001). While these possibilities need further investigation, current data suggest that it should be possible to develop signaling-selective PTH analogs that could be used to dissect the metabolic pathways by which PTH exerts its biological effects. As examples, PTH(1-31) (Whitfield and Morley, 1995) and a conformationally constrained PTH(1-28) analog (Whitfield *et al.,* 2000) have been shown to be osteogenic in a rat model of osteoporosis, thus demonstrating that the bone anabolic effect of PTH is not dependent on the PKC response induced by PTH residues 29-32. The amino-terminally substituted analog [Gly^1]PTH(1-28), in which PLC signaling, but not AC/cAMP signaling, is severely impaired (Takasu *et al.,* 1999a) could similarly be used to examine the role of PLC in the PTH-induced bone formation response. There is interest in whether signaling-selective PTH analogs could be developed as more effective PTH-based therapies for osteoporosis (Neer *et al.,* 2000; Whitfield *et al.,* 2001; Whitfield *et al.,* 2000).

Ligand Determinants of PTH Receptor Binding

For both PTH and PTHrP, the (15-34) fragment can inhibit the binding of either radiolabeled PTH(1-34) or PTHrP(1-34) to the PTH-1 receptor with an IC_{50} in the micromolar range, thus demonstrating that the (15-34) domain contains the principal determinants of receptor-binding affinity (Abou-Samra *et al.,* 1989; Caulfield *et al.,* 1990). The (15-34) domains of both ligands are predicted to form amphiphilic α helices with the hydrophobic face of PTH being formed principally by Trp-23, Leu-24, and Leu-28 (Epand *et al.,* 1985; Neugebauer *et al.,* 1992). Substitution of Leu-24 or Leu-28 in PTH(1-34) by glutamate results in 100-fold reductions in binding affinity, consistent with the view that the hydrophobic face plays a key role in

A

```
                  1                   10                  20                  30
PTH(1-37)         S V S E I Q L M H N L G K H L N S M E R V E W L R K K L Q D V H N F V A L
PTHrP(1-37)       A V S E H Q L L H D K G K S I Q D L R R R F F L H H L I A E I H T A E I R
TIP39       S L A L A D D A A F R E R A R L L A A L E R R H W L N S Y M H K L L V L D A F
```

B

	hPTH1R		hPTH2R		zPTH3R	
	Binding	cAMP	Binding	cAMP	Binding	cAMP
PTH(1-34)	+	+	+	+	+	+
PTHrP(1-34)	+	+	(+)	-	+	+
TIP39	(+)	-	+	+	(+)	-

Figure 2 Ligand sequences and specifity profiles for the PTH receptor subtypes. (A) An alignment of human PTH(1-37), human PTHrP(1-37), and human TIP39. Homologous residues are in boldface type. (B) Ligand selectivities of the human PTH1R, human PTH2R, and zebrafish PTH3R. A minus sign indicates negligable activity; a plus sign indicates high affinity (low nanomolar K_d) or high potency for cAMP generation ($EC_{50} < 10$ n*M*); a plus sign in parentheses indicates low-binding affinity (K_d ~100 to ~1 μM). Note that the rat PTH2R (not shown) responds poorly to PTH(1-34) (EC_{50} 140 n*M*, $E_{max} = 43\%$) as well as PTHrP (Hoare *et al.,* 1999a).

receptor binding (Gardella *et al.,* 1993). It has been suggested for PTH (Epand *et al.,* 1985; Neugebauer *et al.,* 1992; Rölz *et al.,* 1999) that this role involves nonspecific interaction of the hydrophobic surface of the peptide with the phospholipid bilayer of the cell membrane, which then facilitate a two-dimensional diffusion of the hormone to the receptor. Such a model has been suggested for other peptide hormones (Sargent and Schwyzer, 1986). However, it seems likely that the hydrophobic surface of the (15-34) domain directly contacts the receptor. The cross-linking of a PTHrP(1-36) analog having tryptophan-23 replaced by the photolabile benzophenone-containing amino acid analog benzoylphenylalanine (Bpa) to a 16 amino acid segment at the extreme amino terminus of the PTH-1 receptor suggests that at least some residues in the (15-34) domain of the ligand interact with the amino-terminal domain of the receptor (Mannstadt *et al.,* 1998).

TIP39 and the PTH-2 Receptor

The PTH-2 receptor subtype was initially identified through hybridization cloning methods in a human brain cDNA library. At the amino acid level, this receptor is 51% identical to the human PTH-1 receptor (Usdin *et al.,* 1995). The cloning of the PTH-2 receptor prompted a search for a peptide ligand that is its naturally occurring agonist and would selectively activate the PTH-2 receptor. The effort resulted in the discovery of a peptide of 39 amino acids that potently activates both rat and human PTH-2 receptor subtypes without activating the PTH-1 receptor (Usdin, 1999). The human PTH-2 receptor responds to PTH but not to PTHrP, whereas the rat PTH-2 receptor responds to neither PTH nor PTHrP (Hoare *et al.,* 1999a). The newly discovered peptide, called TIP39 (tuberoinfundibular peptide of 39 amino acids), was initially purified from bovine hypothalamus extracts and was shown to be only weakly homologous to PTH and PTHrP (Usdin, 1999). Although TIP39 fails to activate the PTH-1 receptor, it binds to it with moderate affinity (~100 n*M*) (Hoare *et al.,* 2000a). Interestingly, TIP(7-39) and TIP(9-39) bind with higher affinity to the PTH-1 receptor than TIP39 and the truncated peptides function as PTH-1 receptor-specific antagonists (Hoare and Usdin, 2000; Jonsson *et al.,* 2001). Figure 2 illustrates the structural features of the three ligands and their respective binding to and activation of the type 1 and type 2 PTH receptors, as well as to the type 3 PTH receptor identified in zebrafish. The physiological role of TIP39 and the PTH-2 receptor has not yet been identified, but their abundant expression in the central nervous system suggests a possible neuroendocrine function (Usdin, 2000) that is apparently preserved in evolution, as the PTH-2 receptor is found in zebrafish (Rubin and Jüppner, 1999).

Mechanisms of PTH-1 Receptor Function

Role of the Amino-Terminal Receptor Domain

The large glycosylated amino-terminal extracellular domain of the PTH1R contains six highly conserved cysteine residues that are likely to form an intramolecular network of disulfide bonds. A possible arrangement of these disulfide bonds has been suggested from a biochemical study on a recombinant protein corresponding to the amino-terminal domain of the PTH1R (Tyr^{23}-Ile^{191}) that was overproduced

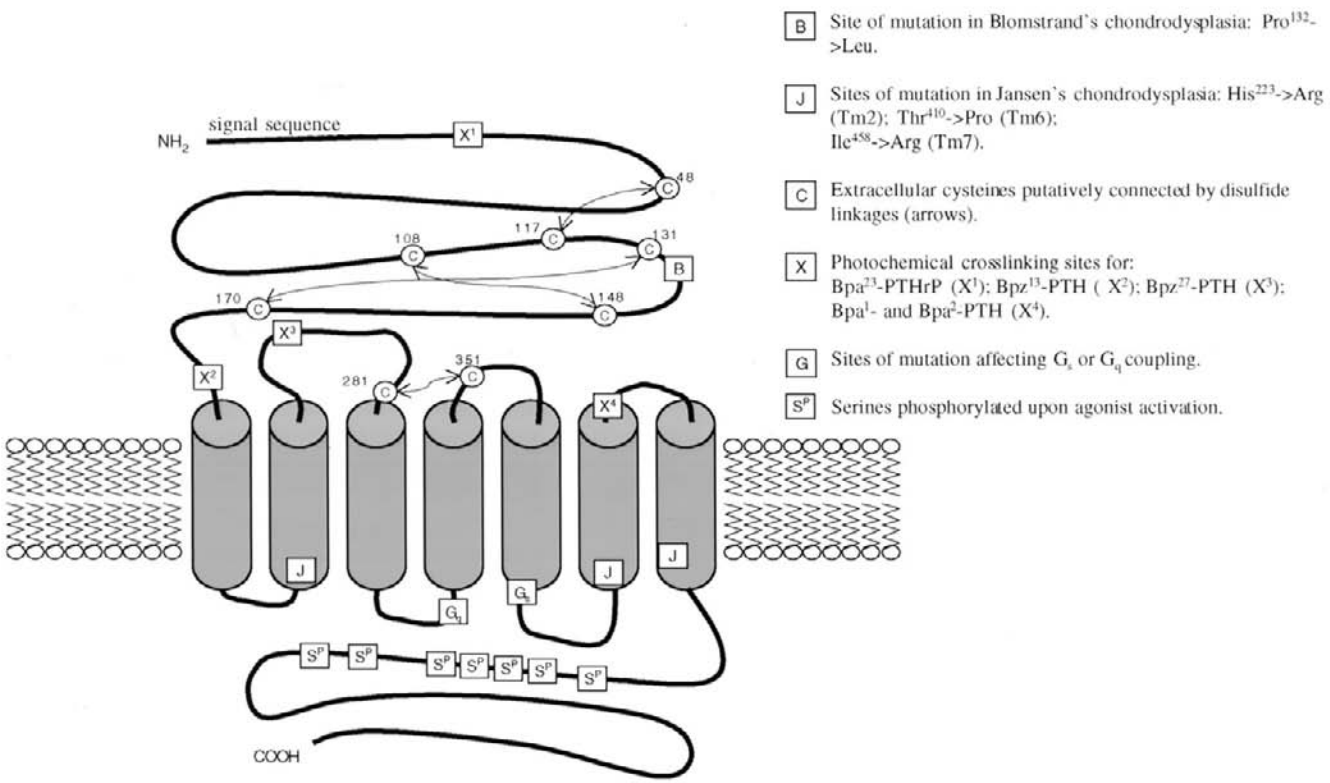

Figure 3 Schematic of the human PTH-1 receptor. The human PTH-1 receptor (591 amino acids) is displayed to illustrate its relative domain organization and the location of selected key residues. A possible arrangement of disulfide bonds involving the eight extracellular cysteines is illustrated by the connecting dotted lines with arrows; this putative arrangement is based on biochemical studies of a purified refolded protein corresponding to the amino-terminal domain of the hPTH1R receptor that was produced in *E.coli* (Grauschopf *et al.,* 2000). As indicated in the figure key, sites of photochemical cross-linking for PTH or PTHrP analogs modified with a benzophenone moiety, either in the form of *p*-benzoyl-*L*-phenylalanine (Bpa) or attached to the ε amino group of a lysine side chain (Bpz), are represened by boxed Xs; sites of mutations in the human skeletal diseases of Blomstrand's chondrodysplasia (receptor inactivity) and Jansen's chondrodysplasia (receptor constitutive activity) are marked by a boxed B and boxed Js, respectively. Serines that are phosphorylated upon agonist activation of the receptor are also shown.

in *Escherichia coli* (Grauschopf *et al.,* 2000). The purified protein was refolded in a glutathione-containing redox buffer system to a homogeneous state and was shown to retain specific, but predictably weak ($K_d \sim 4\ \mu M$), binding affinity for PTH(1-34). The biochemical behavior of this protein suggests that the observed disulfide bond pattern, illustrated in Fig. 3, may faithfully replicate that which occurs in native PTH-1 receptors expressed in eukaryotic cells, although this remains to be verified.

Functional studies on PTH receptor chimeras and mutants generated by site-directed mutagenesis and expressed in COS-7 cells have shown that the amino-terminal domain of the receptor provides important contact sites for at least some residues in the C-terminal-binding domains of PTH(1-34) and PTHrP(1-34) (Bergwitz *et al.,* 1996; Jüppner *et al.,* 1994). The cross-linking studies with [Bpa^{23}]PTHrP(1-36) mentioned earlier support this conclusion (Mannstadt *et al.,* 1998) (see also Chapter 26) Within the 17 amino acid interval identified with the Bpa-23 analog, the specific residues of threonine-33 and glutamine-37 were shown by functional methods to be determinants of PTH(7-34) binding affinity (Mannstadt *et al.,* 1998). A second segment of the amino-terminal extracellular domain involved in ligand interaction maps to the boundary of the amino-terminal domain and the first transmembrane helix. A PTH(1-34) analog having the benzophenone photophore attached to the ε amino group of lysine 13 cross-linked to this region, most likely at Arg-186 (Adams *et al.,* 1998) (Fig. 3), and point mutations at the neighboring hydrophobic residues of Phe-184 and Leu-187 and Ile-190 impaired interaction with PTH(3-34) and PTH(1-14) but not PTHrP(15-36) (Carter *et al.,* 1999b). Residues in this segment of the receptor thus appear to be important interaction determinants for residues in the (3-14) region of the ligand.

Residues in the midportion of the amino-terminal domain of the PTH-1 receptor are also likely to contribute to ligand interaction, but candidate contact points have not been identified. Blomstrand's chondrodysplasia is a human neonatal lethal disorder characterized by dramatically advanced endochondral bone maturation. Investigations into the underlying

molecular defects of this disease revealed that proline-132 in the PTH-1 receptor was mutated on both alleles to leucine (Fig. 3), and the same receptor mutation, when analyzed in transfected COS-7 cells, yielded a loss-of-function phenotype (Karaplis *et al.,* 1998; Zhang *et al.,* 1998). Whether this proline, which is located in the middle of the amino-terminal extracellular domain of the receptor, is directly involved in ligand interaction or provides a more general scaffolding function, as might be inferred from its preservation in all class II receptors, remains to be determined.

Juxtamembrane Region

A number of studies have indicated that interactions between the amino-terminal portion of PTH and the juxtamembrane region of the PTH receptor are important for inducing receptor activation. One such study that utilized PTH-1 receptor/calcitonin receptor chimeras and PTH/calcitonin hybrid ligands showed that efficient functional responses were obtained only when a chimeric receptor was paired with a hybrid ligand such that the amino-terminal portion of the ligand was cognate to the juxtamembrane region of the receptor (Bergwitz *et al.,* 1996). Some specific residues in the juxtamembrane region have been identified as candidate interaction sites for the amino-terminal residues of PTH, such as Ser-370 and Leu-427 at the extracellular ends of transmembrane domain(TM)5 and 6, respectively, which determine the agonist/antagonist response profile observed with [Arg^2]PTH(1-34) (Gardella *et al.,* 1994), and Trp-437 and Gln-440 in the third extracellular loop, at which mutations impair the binding of PTH(1-34) but not PTH(3-34) (implying an interaction site for ligand residues 1 and 2) (Lee *et al.,* 1995). Consistent with these mutational data, the receptor cross-linking sites for [Bpa^1]PTH(1-34) (Bisello *et al.,* 1998) and [Bpa^2]PTHrP(1-36) (Behar *et al.,* 1999) were mapped to the extracellular end of TM6 (Behar *et al.,* 1999). Interestingly, both of these ligands utilized Met-425 for covalent attachment, although [Bpa^2]PTHrP(1-36), an antagonist, utilized an additional second site in TM6, whereas [Bpa^1]PTH(1-34), an agonist, utilized only the methionine. These results raise the possibility that the photochemical cross-linking approach can be used to discern differences in the active and inactive states of the PTH-1 receptor (Behar *et al.,* 1999).

Other receptor residues involved in interactions with the amino-terminal portion of the ligand have been identified in studies aimed at elucidating the molecular basis by which the human PTH-2 receptor discriminates between PTH and PTHrP, an effect that is largely due to the amino acid divergence at position 5 in these ligands (Ile in PTH and His in PTHrP) (Behar *et al.,* 1996; Gardella *et al.,* 1996c). Four PTH-2 receptor residues involved in this specificity were identified at the extracellular ends of several of the TM helices: Ile-244 in TM3, Tyr-318 in TM5, and Cys-397 and Phe-400 in TM7, corresponding to Leu-289, Ile-363, Tyr-443, and Leu-446, respectively, in the human PTH-1 receptor (Bergwitz *et al.,* 1997; Turner *et al.,* 1998). Other residues in the TM domains that have been identified as determinants of PTH(1-34) agonist responsiveness include Ser-229, Arg-233, Ser-236, which may form a hydrophilic surface on TM2 (Turner *et al.,* 1996), and the conserved Gln451 in TM7 (Gardella *et al.,* 1996a).

A series of studies conducted with a truncated PTH-1 receptor (P1R-delNt) that lacks most (residues 24-181) of the amino-terminal extracellular domain has helped discern the role of interactions between the N-terminal residues of PTH and the juxtamembrane region of the receptor in mediating signal transduction. Thus, the modified PTH(1-14) peptides described earlier stimulate cAMP formation with this truncated receptor nearly as effectively as they do with the intact wild-type receptor (Shimizu *et al.,* 2000b, 2001) (Fig 4). The near-full activity of the PTH(1-14) analogs with P1R-delNt stands in dramatic contrast to the markedly (approximately 1000-fold) reduced activity that unmodified PTH(1-34) exhibits with P1R-delNt, as compared to the intact receptor (Fig 4). The weak activity of PTH(1-34) on P1R-delNt highlights the importance of interactions between the (15-34) domain of PTH(1-34) and the N-terminal extracellular domain of the receptor in stabilizing the native hormone–receptor complex (see later), whereas the potent activity of modified PTH(1-14) on P1R-delNt indicates that most, if not all, of the key functional residues in the amino-terminal peptide interact primarily, if not exclusively, with the juxtamembrane region of the recepter.

Studies with conformationally constrained PTH peptides have yielded PTH(1-14) analogs that exhibit full cAMP efficacy and low nanomolar potency on both the intact wild-type PTH-1 receptor and on P1R-delNt (N. Shimizu and T. J. Gardella, unpublished data). Presumably, the modifications in these peptides stabilize a ligand conformation that has high affinity for the juxtamembrane region of the receptor. Most likely, this conformation is α helical, as has been suggested by both nuclear magnetic resource (Rölz *et al.,* 1999) and X-ray crystallographic (Jin *et al.,* 2000) studies of PTH(1-34) and the accompanying computer models of the ligand–receptor complex. At least some of the substitutions in the multisubstituted PTH(1-14) analogs described by Shimizu *et al.* (2000b, 2001), are likely to provide new and favorable interactions with the receptor that compensates for the loss of binding energy that normally derives from residues in the (15-34) domain of PTH, but some may directly facilitate the receptor activation process without affecting binding affinity. One of the goals of the research on these short N-terminal PTH peptides is to discern how individual key residues in the ligand contribute to binding affinity and receptor activation. Such work will most likely require the use of membrane-based pharmacological and kinetic methods, such as those already employed by Hoare and co-workers (1999b, 2001) in their studies on the PTH receptor system.

Data so far on the PTH(1-14) analogs demonstrate that it is possible to achieve full potency and efficacy with a peptide ligand as short as 14 amino acids, and also that

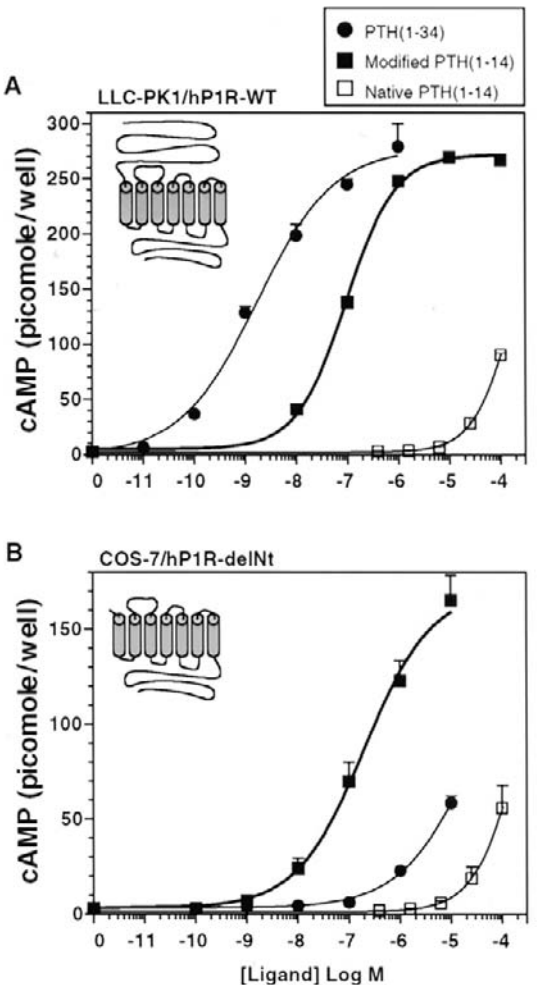

Figure 4 cAMP-stimulating activity of modifed PTH(1-14) and control analogs in cells transfected with wild-type or N-terminally truncated PTH1 receptors. (A) The modified PTH(1-14) analog [$Ala^{3,12}$,Gln^{10},Har^{11},Trp^{14}]-PTH(1-14)amide is shown to stimulate cAMP formation in the porcine kidney cell line LLC-PK1 transfected stably with the wild-type human PTH-1 receptor [HKRK-B28 cells in reference (Takasu *et al.*, 1999b)] with a potency that is 2000-fold greater than that of native PTH(1-14) and only 60-fold weaker than that of PTH(1-34). (B) The same modified PTH(1-14) analog [as well as the native PTH(1-14) peptide] is shown to exhibit nearly the same potency in COS-7 cells transfected transiently with a truncated human PTH1R lacking most of the amino-terminal extracellular domain, hP1R-delNt, as it does with hP1R-WT in A. In contrast, PTH(1-34) is ~20,000-fold weaker with hP1R-delNt than it is with hP1R-WT. The results are excerpted from Shimizu *et al.* (2001) and serve to illustrate three points: (1) the interaction between the N-terminal domain of the intact receptor and the (15-34) domain of the intact ligand is important for the stability of the native hormone–receptor complex; (2) PTH(1-14) peptides can be modified to achieve substantial gains in potency; and (3) full receptor activation can be induced by peptide ligands as short as 14 amino acids that interact solely with the juxtamembrane region of the receptor.

a relatively small agonist ligand can fully activate the PTH receptor by interacting solely with the juxtamembrane region of the receptor. As an extension of these studies, a PTH-1 receptor mutant was constructed in which residues (1-9) or (1-11) of PTH were tethered directly to the juxtamembrane region of the receptor (at Glu-182). When expressed in COS-7 cells, these constructs resulted in basal cAMP levels that closely approached the cAMP level seen with the wild-type PTH-1 receptor fully stimulated with the PTH(1-34) agonist ligand (Shimizu *et al.,* 2000a). A similar result has been reported for corticotropin-releasing factor (CRF) and its class II GPCR (Nielsen *et al.,* 2000).

Two-Site Model of PTH/PTH Receptor Interaction

Combined functional and cross-linking data are consistent with a mechanism for the PTH–PTH receptor interaction that involves two principal components: (1) an interaction between the C-terminal domain of the ligand and the amino-terminal domain of the receptor, which contributes predominantly to binding affinity, and (2) an interaction between the amino-terminal portion of the ligand and the juxtamembrane region of the receptor, which contributes to signaling (Fig. 5). This general interaction model is likely to apply to at least some of the other class II receptors, including those for CRF, calcitonin, secretin, and glucagon (Bergwitz *et al.,* 1996; Nielsen *et al.,* 2000; Stroop *et al.,* 1995; Turner *et al.,* 1998). It has been proposed that the interactions at the two receptor domains occur in a sequential manner (Hoare *et al.,* 2001; Ji *et al.,* 1998) (Fig. 5). There is also the possibility that a higher order of folding is involved. In support of this possibility is the cross-linking study showing that a PTH(1-34) agonist analog having a benzophenone group attached to lysine-27 contacts the first extracellular loop of the PTH-1 receptor (Greenberg *et al.,* 2000) (Fig. 3); thus the amino-terminal extracellular domain of the receptor [and (15-34) portion of the ligand] must be close to the juxtamembrane region of the receptor, at least in the agonist bound state (Piserchio *et al.,* 2000). The model proposed in Fig. 5, based on binding of modified short amino-terminal PTH(1-14) sequences versus the amino-terminally truncated PTH(3-34) antagonist peptide, suggests that the binding steps may even be independent such that the binding of PTH(1-14) analogs to the juxtamembrane regions cannot be blocked by binding of the carboxyl portion of PTH(3-34) to the extracellular domain of the receptor (Hoare *et al.,* 2001). This finding, demonstrated in membrane preparations of receptors, is not seen in cell-based assays that have higher receptor number and differ in other respects (internalization, phosphorylation, etc.) (Shimizu *et al.,* 2000b).

Data seen and the models proposed regarding PTH/PTH-1 receptor interaction mechanisms have clear implications for drug discovery efforts aimed at finding new PTH receptor agonists. Clinical trial data showing that PTH (given as daily subcutaneous injections) can effectively treat osteoporosis (Neer *et al.,* 2000) are likely to heighten interest in developing orally available nonpeptide mimetics for this receptor. So far, however, no such compounds have been reported. Although it is possible that the agonist-dependent activation of the PTH-1 receptor requires multiple ligand contacts to a large and diffuse surface of the receptor,

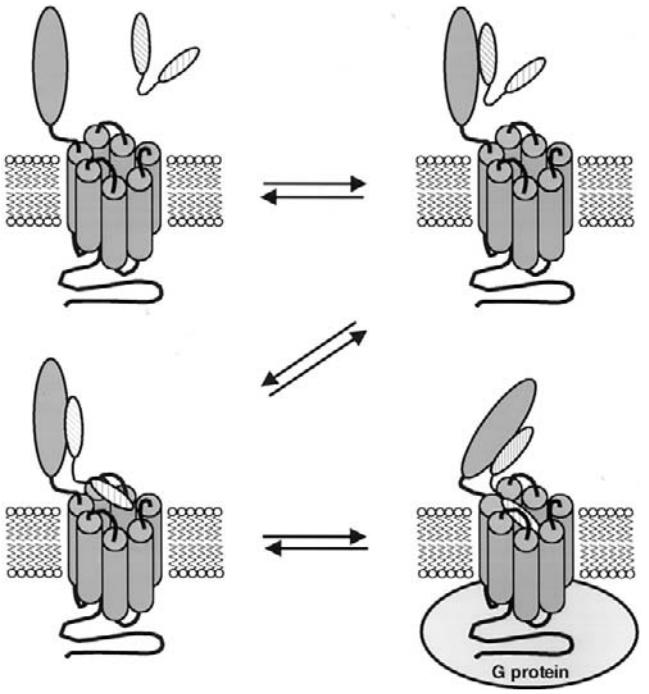

Figure 5 Hypothetical model of the PTH–PTH receptor interaction mechanism. The schematic illustrates current hypotheses regarding the mechanism by which PTH binds to the PTH1 receptor and induces G protein coupling. The interaction with PTH(1-34) (hatched ovals) involves two principal components: (1) binding of the C-terminal domain of PTH(1-34) to the amino-terminal extracellular domain of the receptor and (2) the association of the amino-terminal domain of PTH with the juxtamembrane region of the receptor. These two components of the interaction contribute predominantly to affinity and activation, respectively, and may occur in a sequential manner, as depicted. Upon association of the N-terminal portion of the ligand with the juxtamembrane region, a conformational change occurs, which results in the formation of a "closed" high-affinity ligand–receptor complex that is coupled to G protein. This hypothetical model is adapted from and discussed further in Hoare *et al.* (2001).

including the amino-terminal extracellular domain, the finding that short peptide sequences [modified PTH(1-11) and (1-14), as well as PTH(1-9) in a tethered construct] can activate the receptor (Shimizu *et al.,* 2000a) suggests, as the model in Fig. 5 predicts, that it should be possible for a small nonpeptide molecule that interacts only with the juxtamembrane region of the receptor to function as a potent PTH-1 receptor agonist.

Conformational Changes in the PTH-1 Receptor

As for all GPCRs, the binding of an agonist peptide to the PTH-1 receptor is thought to induce conformational changes in the receptor, including movements of the TM domains that render the cytoplasmic loops more accessible to G proteins (Gether, 2000). The model shown in Fig. 5 deduced from kinetic data (Hoare *et al.,* 2001) that G protein association by a ligand-bound receptor results in a "tightening" of the receptor/ligand complex reflects this proposed conformational change. An agonist-induced movement of TM3 away from TM6 has been demonstrated for the PTH-1 receptor by Sheikh *et al.* (1999), who showed that the chelation of zinc between histidine residues (native or introduced) at the intracellular ends of TM3 and TM6 blocked receptor-mediated G protein activation. An analogous movement was also shown in the β_2-adrenergic receptor, suggesting that the mechanisms of activation for the class I and class II GPCRs are fundamentally similar (Sheikh *et al.,* 1999). Several residues on the cytoplasmic surface of the PTH-1 receptor have been identified within regions that are candidate G protein interaction sites (Fig. 3). In intracellular loop 3, these include Val-384 and Leu-385 (PLC coupling), Thr-387 (AC coupling), and Lys-388 (AC and PLC coupling) (Huang *et al.,* 1996). In intracellular loop 2, Lys-319 has been implicated in PLC signaling (Iida-Klein *et al.,* 1997).

Three different activating mutations in the PTH-1 receptor have been identified in patients with Jansen's metaphyseal chondrodsyplasia, a rare form of dwarfism associated with hypercalcemia (Calvi and Schipani, 2000). These mutations occur at the cytoplasmic termini of TM2 (Arg-233 → His), TM6 (Thr-410 → Pro), and TM7 (Ile-458 → Arg) (Fig. 3) and each results in agonist-independent cAMP signaling. Whether the conformational changes induced by the activating mutations are the same as those that occur in the agonist occupied wild-type PTH receptor is unknown, but the study of these mutant PTH-1 receptors, along with certain peptide ligand analogs, such as [Leu^{11}, D-Trp^{12}]PTHrP(7-34), that behave as inverse agonists with the mutant receptors and depress their basal signaling (Carter *et al.,* 2001; Gardella *et al.,* 1996b), is likely to provide insights into the conformational states that are possible for active and inactive PTH-1 receptors.

PTH-1 Receptor Regulation

The agonist-dependent response capacity of the PTH-1 receptor is diminished markedly within minutes following an initial exposure to agonist (Bergwitz *et al.,* 1994; Fukayama *et al.,* 1992). This desensitization is accompanied by rapid internalization of the PTH–PTH-1 receptor complex (Ferrari *et al.,* 1999; Huang *et al.,* 1999; Malecz *et al.,* 1998). Phosphorylation of other G protein-coupled receptors on cytoplasmic domains is known to play an important role in the internalization/desensitization process (Lefkowitz, 1998). The PTH-1 receptor is phosphorylated on its cytoplasmic tail immediately following agonist activation (Blind *et al.,* 1996), specifically on as many as seven serine residues that cluster to within the midregion of the cytoplasmic tail (Malecz *et al.,* 1998; Qian *et al.,* 1998) (Fig. 3). The second messenger–activated kinases PKA and PKC both appear to contribute to PTH-1 receptor phosphorylation, as both forskolin and phorbol 12-myristate 13-acetate increase PTH-1 receptor phosphorylation (Blind *et al.,* 1995). The inhibitory effect of staurosporine on PTH-1 receptor phosphorylation (Qian *et al.,* 1998) and internalization (Ferrari *et al.,* 1999) supports a role for PKC, and potentially other kinases, in these processes.

Cotransfection experiments have indicated that the G protein receptor kinase-2 (GRK-2) also contributes to PTH-1 receptor phosphorylation (Dicker *et al.,* 1999; Malecz *et al.,* 1998).

With other G protein-coupled receptors, receptor phosphorylation enables the binding of β-arrestin2 (β-Arr2), which then interferes sterically with G protein coupling and, in an adapter role, binds the receptor directly to clatherin (Lefkowitz, 1998). PTH-1 receptor endocytosis occurs largely via a clatherin-coated vesicle-mediated process (Huang *et al.,* 1995). By expressing moderate levels of a phosphorylation-deficient PTH-1 receptor mutant having the clustered serines of the C-tail replaced by alanine, Qian *et al.* (1999) found that agonist-induced internalization in LLC-PK1 cells was reduced markedly in comparison to the wild-type receptor. There does not appear to be a simple relationship between phosphorylation of the C-terminal tail of the PTH-1 receptor and receptor internalization/desensitization, however. Thus, Malecz *et al.* (1998) found that a similar alanine-substituted phosphorylation-deficient PTH-1 receptor mutant expressed at high levels in HEK-293 cells was internalized upon agonist binding just as efficiently as the wild-type receptor; in cotransfection experiments, Dicker *et al.* (1999) found that GRK-2 efficiently cross-linked to, coimmunoprecipitated with, and inhibited agonist-induced PLC signaling by a PTH-1 receptor mutant deleted for the C-terminal tail; and finally, using fluorescent confocal microscopy methods, Ferrari and Bisello (2001) found that a PTH-1 receptor deleted for the C-tail was internalized upon agonist binding just as efficiently as the intact receptor. This latter study also showed that, like the intact receptor, the agonist-occupied C-terminally truncated receptor recruited β-Arr2 tagged with green fluorescent protein (GFP) from the cytosol to the membrane, but where the GFP-β-Arr2 remained associated with the internalized intact receptor, it dissociated rapidly from the internalized truncated receptor. Thus, phosphorylation of the C-terminal tail of the PTH-1 receptor appears to play a role in stabilizing the complex formed with the intracellular trafficking and regulatory proteins, but these proteins must also utilize other cytoplasmic components of the receptor for additional docking interactions.

Studies with the constitutively active mutant PTH receptors of Jansen's disease have begun to shed light on how conformational changes in the receptor might play a role in the internalization process. Both the H223R and the T410P mutant receptors spontaneously recruit β-Arr2 from the cytosol to the membrane (Ferrari and Bisello, 2001), but where the H223R mutant, as well as the wild-type receptor, exhibited little or no internalization of antagonist ligands, the T410P mutant internalized antagonist ligands to levels comparable to those seen with the agonist-occupied wild-type receptor (Carter *et al.,* 2001; Ferrari and Bisello, 2001). Thus, cAMP signaling and β-Arr2 binding by the PTH-1 receptor are not sufficient to induce internalization. In addition, it appears that a specific receptor conformation, which is presumably induced by agonist binding (or in some way mimicked by the T410P mutation), must be accessed for this process to occur. Further investigation with these PTH-1 receptor mutants, new ligand analogs, and fluorescently labeled regulatory proteins should help to elucidate further the molecular mechanisms involved in regulating the PTH-1 receptor-mediated signaling response, a possibly important feature of overall physiological regulation of PTH action and its pharmacological use.

Other Receptors for PTH and/or PTHrP

Receptors for Mid- and Carboxy-Terminal Portions of PTH and PTHrP

There is a substantial amount of pharmacological evidence in the literature for additional nonclassical receptors for PTH and PTHrP (Jüppner *et al.,* 2001). Competition binding studies and other functional assays have suggested that distinct receptors exist for midregional and carboxy-terminal fragments of PTH or PTHrP, although the biological importance of such receptors remains to be established. For example, intact PTH and/or larger carboxyl-terminal PTH fragments have been shown to interact with a novel cell surface receptor (see later). Other receptors appear to mediate the effects of midregional PTHrP on placental calcium transport (Kovacs *et al.,* 1996; Wu *et al.,* 1996) and in the skin (Orloff *et al.,* 1996). Carboxy-terminal portions of PTHrP also have effects on osteoblasts (Cornish *et al.,* 1997, 1999; Fenton *et al.,* 1991a,b), as well as the central nervous system (Fukayama *et al.,* 1995). A novel receptor that interacts with the amino-terminal portion of PTHrP, but not the amino-terminal portion of PTH, has been characterized in the rat supraoptic nucleus, and this PTHrP-selective receptor can regulate the synthesis and release of arginine vasopressin (Yamamoto *et al.,* 1998). Still other receptors that interact with amino-terminal portions of PTH and PTHrP have been identified pharmacologically that signal through changes in intracellular-free calcium but not through increases in cAMP (Gaich *et al.,* 1993; Orloff *et al.,* 1995; Soifer *et al.,* 1992). Clearly there is strong interest in isolating complementary cDNAs, which encode such nonclassical receptors for PTH and PTHrP, but so far none have been identified. Such interest is highlighted by studies on the carboxyl-terminal portion of PTH.

Receptors Specific for Carboxyl-Terminal PTH in Bone (CPTHR)

The traditional view of PTH biology has been that the major biologic actions of PTH on bone, cartilage, and kidney result from activation of PTH1R, which is fully achieved by the N-terminal sequence PTH(1-34) (or the homologous N-terminal portion of PTHrP). This concept has derived largely from a heavy focus in the years following the isolation and structural identification of PTH on the use of cAMP production and of biologic responses now known to be

largely cAMP dependent. These include calcemia and phosphaturia, as indices of PTH action *in vivo* and *in vitro,* although, as reviewed earlier, it is now known that PTH1R can also trigger cAMP-independent signaling events, including activation of PLC, PLD, PLA2, PKC, and increased cytosolic calcium, and that the structural determinants within both the PTH(1-34) ligand and the PTH1R itself that are required for these activities are not fully congruent.

Until very recently, the possibility that the C-terminal portion of the intact PTH(1-84) molecule might be physiologically important has received inadequate attention in part because (a) peptides such as PTH(39-84) or PTH(53-84) cannot be shown to bind or activate PTH1Rs; (b) the expressed PTH1R interacts equivalently with PTH(1-34) and PTH(1-84); (c) even minimal truncation at the N terminus of PTH(1-34) ablates PTH1R activation; and (d) differences in the bioactivity of PTH(1-34) and PTH(1-84) have not been demonstrated consistently in the usual "PTH bioassays" *in vitro* or *in vivo* (Potts and Jüppner, 1998). Moreover, the rapid production of C-terminal PTH fragments via endopeptidic cleavage of intact PTH *in vivo* (mainly in liver and kidney) has been regarded as the major route of metabolic clearance of active PTH, whereby the active N terminus of the molecule is destroyed *in situ* and long-lived C fragments are released back into the circulation (Potts and Jüppner, 1998). However, large portions of the C-terminal sequence of PTH(1-84) have been tightly conserved during evolution, active N-terminal fragments of PTH have not been demonstrated convincingly in blood of normal subjects, and C fragments of PTH (with N termini between residues 24 and 43) are cosecreted with intact hormone by the parathyroid glands in a manner whereby the ratio of intact fragments to C fragments is subject to regulation by blood calcium (Potts and Jüppner, 1998). Uncertainty regarding the potential importance of these observations, vis-à-vis a possible physiologic role for circulating C-terminal PTH peptides (CPTH), resulted mainly from a lack of evidence for specific receptors, distinct from the PTH1R (upon which all major PTH bioassays have been based), at which these peptides might act.

Initial indications that receptors for CPTH (CPTHRs) might exist actually appeared in the 1980s, when techniques were first developed to radiolabel intact PTH(1-84) in a biologically active form. At that time, careful analysis of ^{125}I-bPTH(1-84) binding to renal membranes and intact osteoblastic cells provided clear evidence of a second binding site that had a 10-fold lower affinity than that now known to pertain to the PTH1R and was specifically displaced by CPTH peptides (Demay *et al.,* 1985; Rao and Murray, 1985; Rao *et al.,* 1983). More recently, Inomata *et al.* reported the first direct measurements of CPTHR binding in ROS 17/2.8 rat osteosarcoma cells and rat parathyroid-derived cells, using radioiodinated recombinant peptides ^{125}I-[Tyr^{34}]hPTHrP(19-84) and ^{125}I-[Leu^{8,18}, Tyr^{34}]hPTHrP(1-84) as tracers (Inomata *et al.,* 1995). These two radioligands, which bind minimally, if at all, to the PTH1R, exhibited binding affinity comparable to that of hPTH(1-84) itself. These results suggested that all of the binding determinants of intact PTH(1-84) for the CPTHR reside within the PTH(19-84) sequence. Specific CPTHR binding was observed for hPTH(53-84), hPTH(39-94), and hPTH(1-84) but not hPTH(44-68) or hPTH(1-34); chemical cross-linking demonstrated a predominant 90-kDa receptor band (Inomata *et al.,* 1995). Unequivocal evidence that these CPTHR sites are distinct from the PTH1R subsequently was obtained via the demonstration of specific ^{125}I-[Tyr^{34}]hPTHrP(19-84) binding to clonal osteoblasts and osteocytes in which both PTH1R alleles had been ablated by gene targeting (Divieti *et al.,* 2001). The apparent binding affinity of these CPTHR sites for intact PTH and longer CPTH fragments (K_d = 10–20 n*M*) was 10-fold lower than that of the PTH1R for PTH(1-84) or PTH(1-34) (1-2 n*M*). Interestingly, this difference in affinity of PTH1Rs and CPTHRs for intact PTH and CPTH fragments, respectively, mirrors their relative levels in blood, where evidence suggests a 5- to 10-fold higher concentration of CPTH peptides than intact hormone.

Early work in several laboratories documented increased alkaline phosphatase expression following exposure of rat of human osteosarcoma cells to CPTH peptides, and subsequent research has identified a variety of biologic effects of CPTH fragments, including regulation of collagen and IGFBP-5 mRNA in UMR-106 rat osteosarcoma cells, stimulation of ^{45}Ca uptake in SaOS-2 cells, promotion of osteoclast formation in primary murine bone and marrow cell cultures, regulation of collagen II and X mRNA in primary fetal bovine hypertrophic chondrocytes, induction of cytosolic calcium transients in human primary fetal hypertrophic chondrocytes, and control of dentin and enamel formation in organ-cultured embryonic mouse tooth germ (Erdmann *et al.,* 1996, 1998; Fukayama *et al.,* 1994; Kaji *et al.,* 1994; Murray *et al.,* 1991; Nakamoto *et al.,* 1993; Nasu *et al.,* 1998; Sutherland *et al.,* 1994; Takasu *et al.,* 1996; Tsuboi and Togari, 1998). Most often, these effects of CPTH peptides were different from, if not opposite to, those of PTH(1-34), although mediation by PTH1Rs could not be definitely excluded because all of the cells studied were known, or could be assumed, to express PTH1Rs. More recent work, however, using cells genetically devoid of functional PTH1Rs, has clearly documented biologic responses to CPTHRs that must be distinct from the PTH1R (Divieti *et al.,* 2001). The structural features of the PTH ligand required for CPTHR activation have yet to be fully defined, although the importance of an intact C terminus for some, but not all, responses has been emphasized (Takasu *et al.,* 1996).

It is of interest that CPTHRs have been identified so far primarily in cells of skeletal origin, i.e., marrow stromal cells, osteoblasts, osteocytes, and chondrocytes. However, CPTHRs were also described for cells derived from rat parathyroid cells (rPTs), which have characteristics of fibroblasts (Potts and Jüppner, 1998). The highest levels of CPTHR expression reported to date ($2-3 \times 10^6$/cell) were observed in clonal cell lines, conditionally transformed by

a temperature-sensitive SV40 transgene and isolated from embryonic PTH1R-null mice, with phenotypic features of osteocytes (i.e., a dendritic morphology and abundant expression of osteocalcin and connexin-43 but not of alkaline phosphatase or cbfa-1) (Divieti *et al.,* 2001). In such cells, genetically devoid of PTH1Rs, PTH(1-84) could not elicit cAMP generation and, as expected, no binding of ^{125}I-[Tyr^{34}]hPTHrP(1-36) could be detected (Divieti *et al.,* 2001). Analysis of the structural requirements for CPTHR ligand binding in these cells, using the ^{125}I-[Tyr^{34}]hPTH(19-84) radioligand, demonstrated equivalent affinity of hPTH(1-84), [Tyr^{34}]hPTH(19-84), and hPTH(24-84) (IC_{50} = 20-50 n*M*) that declined substantially with further truncation to hPTH(39-84) (IC_{50} = 20–50 n*M*), indicating the presence of important binding determinants within the sequence hPTH(24-38). Interestingly, hPTH(1-34), which contains most of this region, also weakly displaced the CPTHR radioligand (IC_{50} > 10,000 n*M*). These key features of ligand recognition were shared in common with PTH1R-null chondrocytes, marrow stromal cells, osteoblasts, and osteocytes (P. Divieti, and F. R. Bringhurst, unpublished data), suggesting that these various cell types may express structurally identical CPTHRs.

A possible physiologic role for CPTHRs expressed by cells of the osteoblast lineage was suggested by observations that, in PTH1R-null clonal osteocytes, intact PTH, as well as CPTH fragments, such as hPTH(39-84) and hPTH(53-84), promote apoptosis. This contrasts with the antiapoptotic effect of PTH1R activation in such cells (Jilka *et al.,* 1998) and suggests that the PTH1R and the still-uncloned CPTHR may exert functionally antagonistic actions upon osteoblastic cells *in vivo*. CPTHR activation in clonal osteocytes also modified expression of connexin-43, suggesting a possible role in the regulation of cell-to-cell communication via gap junctions. The signal transduction mechanisms that may underlie these CPTHR effects remain unknown, although, as noted earlier, coupling to G_s is unlikely.

In summary, evidence that receptors with specificity for the carboxyl-terminal portion of intact PTH(1-84) (i.e., CPTHRs) exist in bone is now unequivocal (Divieti *et al.,* 2001). These receptors, most clearly defined *in vitro* using cell systems genetically devoid of PTH/PTHrP receptors (PTH1Rs), can bind and be activated by intact PTH(1–84) and various synthetic CPTH fragments, such as hPTH(19-84), hPTH(39-84), and hPTH(53-84) (Divieti *et al.,* 2001; Murray *et al.,* 1991). Numerous biologic responses to CPTHR activation have been identified, including regulation of calcium transients, alkaline phosphatase, collagen gene expression, osteoclast formation, connexin-43 expression, and apoptosis (Demay *et al.,* 1985; Divieti *et al.,* 2001; Erdmann *et al.,* 1996, 1998; Fukayama *et al.,* 1994; Kaji *et al.,* 1994; Murray *et al.,* 1991; Nakamoto *et al.,* 1993; Nasu *et al.,* 1998; Rao and Murray, 1985; Rao *et al.,* 1983; Sutherland *et al.,* 1994; Takasu *et al.,* 1996; Tsuboi and Togari, 1998). Observations *in vivo* indicate that the fragment hPTH(7-84), which may exist normally *in vivo* but which clearly can bind CPTHRs with high affinity, antagonizes the PTH1R-mediated calcemic effect of PTH *in vivo* (Nguyen-Yamamoto *et al.,* 2000; Slatopolsky *et al.,* 2000). This *in vivo* action is mirrored in *in vitro* studies showing antagonism by hPTH(7-84) but not by hPTHrP(7-34) of calvarial bone resorption induced by PTH(1-34), suggesting a possible role for CPTHR activation in blocking the bone resorption (P. Divieti and F. R. Bringhurst, unpublished data). Finally, evidence that CPTHR activation promotes apoptosis in cells of the osteoblast lineage, an effect opposite that of PTH(1-34) in such cells, points to a potentially important interaction of PTH1Rs and CPTHRs in control of osteoblast and osteocyte number (Divieti *et al.,* 2001).

Carboxyl fragments of intact PTH are secreted in a calcium-regulated manner by the parathyroid glands, are generated rapidly from secreted or injected PTH(1-84) via peripheral metabolism in liver and kidney, circulate normally at molar levels 5- to 10-fold higher than those of intact PTH(1-84), and accumulate to much higher concentrations in renal failure (Potts and Jüppner, 1998). The possibility that CPTHR activation may play a role in modulating osseous responses to full-length (versus N-terminal) PTH administered as a therapeutic for osteoporosis or in the pathogenesis of currently unexplained features of renal osteodystrophy clearly is worthy of further investigation in light of all the accumulating evidence about high concentrations of CPTH fragments, the distinct CPTHR, and the variety of biological effects seen *in vitro* following the interactions of C fragments with the CPTHR.

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CHAPTER 25

Parathyroid Hormone

Molecular Biology

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The Parathyroid Hormone Gene

Chromosomal Location

The human parathyroid hormone (PTH) gene is localized on the short arm of chromosome 11 and is only present once in the genomes of humans, rats, and cows (Antonarakis *et al.,* 1983; Naylor *et al.,* 1983; Mayer *et al.,* 1983). The PTH gene is closely linked to the calcitonin gene. Zabel *et al.* (1985) performed chromosomal *in situ* hybridization studies, which further localized the gene to the region 11p15. Restriction enzyme analysis of the human PTH gene, as well as the use of denaturing gradient gel electrophoresis, demonstrated polymorphism in their cleavage products in different individuals (Antonarakis *et al.,* 1983; Schmidtke *et al.,* 1984; Miric and Levine, 1992). These genetic polymorphisms are useful for genetic analysis and for relating parathyroid disease to structural alterations in the PTH gene.

PTH Gene

Complementary DNA encoding for human (Hendy *et al.,* 1981; Vasicek *et al.,* 1983), bovine (Kronenberg *et al.,* 1979; Weaver *et al.,* 1982), rat (Schmelzer *et al.,* 1987), mouse, pig (Schmelzer *et al.,* 1987), chicken (Khosla *et al.,* 1988; Russell and Sherwood, 1989), dog (Rosol *et al.,* 1995), cat, and horse PTH have all been cloned. Corresponding genomic DNA has also been cloned from human (Vasicek *et al.,* 1983), bovine (Weaver *et al.,* 1984), and rat (Heinrich *et al.,* 1984). The genes all have two introns or intervening sequences and three exons (Kronenberg *et al.,* 1986). The primary RNA transcript consists of RNA transcribed from both introns and exons, and then RNA sequences derived from the introns are spliced out. The product of this RNA processing, which represents the exons, is the mature PTH mRNA, which will then be translated into preproPTH. The first intron separates the 5′-untranslated region of the mRNA from the rest of the gene, and the second intron separates most of the sequence encoding the precursor-specific "pre-pro" region from that encoding mature PTH. The three exons that result are thus roughly divided into functional domains. The large first intron in the human gene (3400 bp) is much larger than that in the rat and bovine. The second intron is about 100 bp in the three species. There is considerable identity among mammalian PTH genes, which is reflected in an 85% identity between human and bovine proteins and 75% identity between human and rat proteins. There is less identity in the 3′-noncoding region. Human and bovine genes have two functional TATA transcription start sites, and the rat only one. The two homologous TATA sequences flanking the human PTH gene direct the synthesis of two human PTH gene transcripts both in normal parathyroid glands and in parathyroid adenomas (Igarashi *et al.,* 1986). The termination codon immediately following the codon for glutamine at position 84 of PTH indicates that there are no additional precursors of PTH with peptide extensions at the carboxyl position.

Tissue-Specific Expression of the PTH Gene

One of the factors that may be related to tissue specificity of the expression of a gene in a particular tissue is decreased methylation of cytosine at particular sites of the gene.

Levine *et al.* (1986) demonstrated that DNA from parathyroid glands is hypomethylated at CpG sequences in the neighborhood of the PTH gene, but not in DNA from control tissues. There was no correlation between the degree of hypomethylation of the PTH gene and the level of parathyroid gland secretory activity. The PTH gene is a typical eukaryotic gene with consensus sequences for initiation of RNA synthesis, RNA splicing, and polyadenylation.

Genetic studies have demonstrated the role of different genes to the development of the parathyroid. Gunther *et al.* (2000) studied Glial cells missing2 (Gcm2), a mouse homologue of *Drosophila* Gcm, a transcription factor whose expression is restricted to the parathyroid glands. They showed that Gcm2-deficient mice lacked parathyroid glands and exhibited hypoparathyroidism, identifying Gcm2 as a master regulatory gene of parathyroid gland development. However, Gcm2-deficient mice were viable and fertile and had only a mildly abnormal bone phenotype. Despite their lack of parathyroid glands, Gcm2-deficient mice had PTH serum levels identical to those of wild-type mice, as did parathyroidectomized wild-type mice. Expression and ablation studies identified the thymus, where Gcm1, another Gcm homologue, is expressed, as the additional, downregulatable source of PTH. Thus, Gcm2 deletion uncovered an auxiliary mechanism for the regulation of calcium homeostasis in the absence of parathyroid glands. It would be interesting to know if these findings are restricted to the mouse or have a wider physiological relevance. A human patient with a defective Gcm B gene, the human equivalent of Gcm[2], exhibited hypoparathyroidism and complete absence of PTH from the bloodstream (Ding *et al.,* 2000).

The thymus, thyroid, and parathyroid glands in vertebrates develop from the pharyngeal region, with contributions both from pharyngeal endoderm and from neural crest cells in the pharyngeal arches. Hoxa3 mutant homozygotes have defects in the development of all three organs and are completely missing parathyroid glands (Manley and Capecchi, 1998). Pax 9 mouse mutants are similarly missing parathyroid glands and other pharyngeal pouch structures (Peters *et al.,* 1998). Humans with one mutated copy of the GATA3 transcription factor exhibit hypoparathyroidism, sensorineural deafness, and renal anomalies (Van Esch *et al.,* 2000). The specific gene causing the hypoparathroidism found in the CATCH-22 syndrome (cardiac defects, abnormal facies, thymic hypoplasia, cleft palate, hypocalcemia, associated with chromosome 22 microdeletion) and the associated DiGeorge syndrome has not yet been identified.

Promoter Sequences

Regions upstream of the transcribed structural gene often determine tissue specificity and contain many of the regulatory sequences for the gene. For PTH, analysis of this region has been hampered by the lack of a parathyroid cell line. Arnold's group has demonstrated, however, that the 4 kb of DNA upstream of the start site of the human PTH gene was able to direct parathyroid gland-specific expression in transgenic mice (Hosokawa *et al.,* 1997). Rupp *et al.* (1990) analyzed the human PTH promoter region up to position -805 and identified a number of consensus sequences by computer analysis. These included a sequence resembling the canonical cAMP-responsive element 5′-TGACGTCA-3′ at position -81 with a single residue deviation. This element was fused to a reporter gene (CAT) and then transfected into different cell lines. Pharmacological agents that increase cAMP led to an increased expression of the CAT gene, suggesting a functional role for the cAMP responsive element (CRE). The role of this possible CRE in the context of the PTH gene in the parathyroid remains to be established.

Several groups have identified DNA sequences that might mediate the negative regulation of PTH gene transcription by 1,25-dihydroxyvitamin D [$1,25(OH)_2D_3$]. Demay *et al.* (1992) identified DNA squences in the human PTH gene that bind the $1,25(OH)_2D_3$ receptor. Nuclear extracts containing the $1,25(OH)_2D_3$ receptor were examined for binding to sequences in the 5′-flanking region of the hPTH gene. A 25-bp oligonucleotide containing sequences from -125 to -101 from the start of exon 1 bound nuclear proteins that were recognized by monoclonal antibodies against the $1,25(OH)_2D_3$ receptor. The sequences in this region contained a single copy of a motif (AGGTTCA) that is homologous to the motifs repeated in the upregulatory $1,25(OH)_2D_3$ response element of the osteocalcin gene. When placed upstream to a heterologous viral promoter, the sequences contained in this 25-bp oligonucleotide mediated transcriptional repression in response to $1,25(OH)_2D_3$ in GH4C1 cells but not in ROS 17/2.8 cells. Therefore, this downregulatory element differs from upregulatory elements both in sequence composition and in the requirement for particular cellular factors other than the $1,25(OH)_2D_3$ receptor (VDR) for repressing PTH transcription (Demay *et al.,* 1992). Farrow *et al.* (1990) and Hawa *et al.* (1994) have identified DNA sequences upstream of the bovine PTH gene that bind the $1,25(OH)_2D_3$ receptor. Russell *et al.* (1999) have shown that there are two negative VDREs in the rat PTH gene. One is situated at -793 to -779 and bound a VDR/RXR heterodimer with high affinity and the other at -760 to -746 bound the heterodimer with a lower affinity. Transfection studies with VDRE-CAT constructs showed that they had an additive effect. Liu *et al.* (1996) have identified such sequences in the chicken PTH gene and demonstrated their functionality after transfection into the opossum kidney (OK) cell line. They converted the negative activity imparted by the a PTH VDRE to a positive transcriptional response through selective mutations introduced into the element. They showed that there was a p160 protein that specifically interacted with a heterodimer complex bound to the wild-type VDRE, but was absent from complexes bound to response elements associated with positive transcriptional activity. Thus, the sequence of the individual VDRE appears to play an active role in dictating transcriptional responses that may be mediated by altering the ability of a vitamin D receptor heterodimer to interact with accessory factor proteins. Further work is needed to demonstrate that any of these differing negative VDREs function in this fashion in parathyroid cells.

Mutations in the PTH Gene

Rare patients have been found with abnormal parathyroid hormone genes that result in hypoparathyroidism. The PTH gene of a patient with familial isolated hypoparathyroidism (Ahn *et al.,* 1986) has been studied by Arnold *et al.* (1990), and a point mutation in the hydrophobic core of the signal peptide-encoding region of preproPTH was identified. This T-to-C point mutation changed the codon for position 18 of the 31 amino acid prepro sequence from cysteine to arginine, and in functional studies the mutant protein was processed inefficiently. The mutation impaired interaction of the nascent protein with signal recognition particle and the translocation machinery, and cleavage of the mutant signal sequence by solubilized signal peptidase was slow (Karaplis *et al.,* 1995). Sunthornthepvarakul *et al.* (1999) reported a novel mutation of the signal peptide of the prepro-PTH gene associated with autosomal recessive familial isolated hypoparathyroidism. The affected members in this family presented with neonatal hypocalcemic seizures. Their intact PTH levels were undetectable during severe hypocalcemia. A replacement of thymine with a cytosine was found in the first nucleotide of position 23 in the 25 amino acid signal peptide. This results in the replacement of the normal Ser (TCG) with a Pro (CCG). Only affected family members were homozygous for the mutant allele, whereas the parents were heterozygous, supporting autosomal recessive inheritance. Because this mutation is at the -3 position in the signal peptide of the prepro-PTH gene, the authors hypothesized that the prepro-PTH mutant might not be cleaved by signal peptidase at the normal position and might be degraded in the rough endoplasmic reticulum. Parkinson and Thakker (1992) studied one kindred with autosomal recessive isolated hypoparathyroidism and identified a G-to-C substitution in the first nucleotide of intron 2 of the parathyroid hormone gene. Restriction enzyme cleavage revealed that the patients were homozygous for mutant alleles, unaffected relatives were heterozygous, and unrelated normals were homozygous for the wild-type alleles. Defects in messenger RNA splicing were investigated by the detection of illegitimate transcription of the PTH gene in lymphoblastoid cells. The mutation resulted in exon skipping with a loss of exon 2, which encodes the initiation codon and the signal peptide, thereby causing parathyroid hormone deficiency. Somatic mutations identified in the PTH gene in some parathyroid adenomas are discussed in Chapter 56.

Regulation of PTH Gene Expression

1,25-Dihydroxyvitamin D

PTH regulates serum concentrations of calcium and phosphate, which, in turn, regulate the synthesis and secretion of PTH. 1,25-Dihydroxyvitamin D or calcitriol has independent effects on calcium and phosphate levels and also participates in a well-defined feedback loop between calcitriol and PTH.

PTH increases the renal synthesis of calcitriol. Calcitriol then increases blood calcium largely by increasing the efficiency of intestinal calcium absorption. Calcitriol also potently decreases transcription of the PTH gene. This action was first demonstrated *in vitro* in bovine parathyroid cells in primary culture, where calcitriol led to a marked decrease in PTH mRNA levels (Silver *et al.,* 1985; Russell *et al.,* 1984) and a consequent decrease in PTH secretion (Cantley *et al.,* 1985; Karmali *et al.,* 1989; Chan *et al.,* 1986). The physiological relevance of these findings was established by *in vivo* studies in rats (Silver *et al.,* 1986). The localization of $1,25(OH)_2D_3$ receptor mRNA (VDR mRNA) to parathyroids was demonstrated by *in situ* hybridization studies of the thyroparathyroid and duodenum. VDR mRNA was localized to the parathyroids in the same concentration as in the duodenum, the classic target organ of calcitriol (Fig. 1) (Naveh-Many *et al.,* 1990). Rats injected with amounts of calcitriol that did not increase serum calcium had marked decreases in PTH mRNA levels, reaching <4% of control at 48 hr (Fig. 2). This effect was shown to be transcriptional both in *in vivo* studies in rats (Silver *et al.,* 1986) and in *in vitro* studies with primary cultures of bovine parathyroid cells (Russell *et al.,* 1986). When 684 bp of the 5′-flanking region of the human PTH gene was linked to a reporter gene and transfected into a rat pituitary cell line (GH4C1), gene expression was lowered by $1,25(OH)_2D_3$ (Okazaki *et al.,* 1988). These studies suggest that $1,25(OH)_2D_3$ decreases PTH transcription by acting on the 5′-flanking region of the PTH gene, probably at least partly through interactions with the vitamin D-binding sequence noted earlier. The effect of $1,25(OH)_2D_3$ may involve heterodimerization with the retinoid acid recptor. This is because 9 *cis*-retinoic acid, which binds to the retinoic acid receptor, when added to bovine parathyroid cells in primary culture, led to a decrease in PTH mRNA levels (MacDonald *et al.,* 1994). Moreover, combined treatment with $1 \times 10^{-6}\,M$ retinoic acid and $1 \times 10^{-8}\,M$ $1,25(OH)_2D_3$ decreased PTH secretion and preproPTH mRNA more effectively than either compound alone (MacDonald *et al.,* 1994). Alternatively, retinoic acid receptors might synergize with VDRs through actions on distinct sequences.

A further level at which $1,25(OH)_2D_3$ might regulate the PTH gene would be at the level of the $1,25(OH)_2D_3$ receptor. $1,25(OH)_2D_3$ acts on its target tissues by binding to the $1,25(OH)_2D_3$ receptor, which regulates the transcription of genes with the appropriate recognition sequences. Concentration of the $1,25(OH)_2D_3$ receptor in $1,25(OH)_2D_3$ target sites could allow a modulation of the $1,25(OH)_2D_3$ effect, with an increase in receptor concentration leading to an amplification of its effect and a decrease in receptor concentration dampening the $1,25(OH)_2D_3$ effect.

Naveh-Many *et al.* (1990) injected $1,25(OH)_2D_3$ into rats and measured the levels of $1,25(OH)_2D_3$ receptor mRNA and PTH mRNA in the parathyrothyroid tissue. They showed that $1,25(OH)_2D_3$ in physiologically relevant

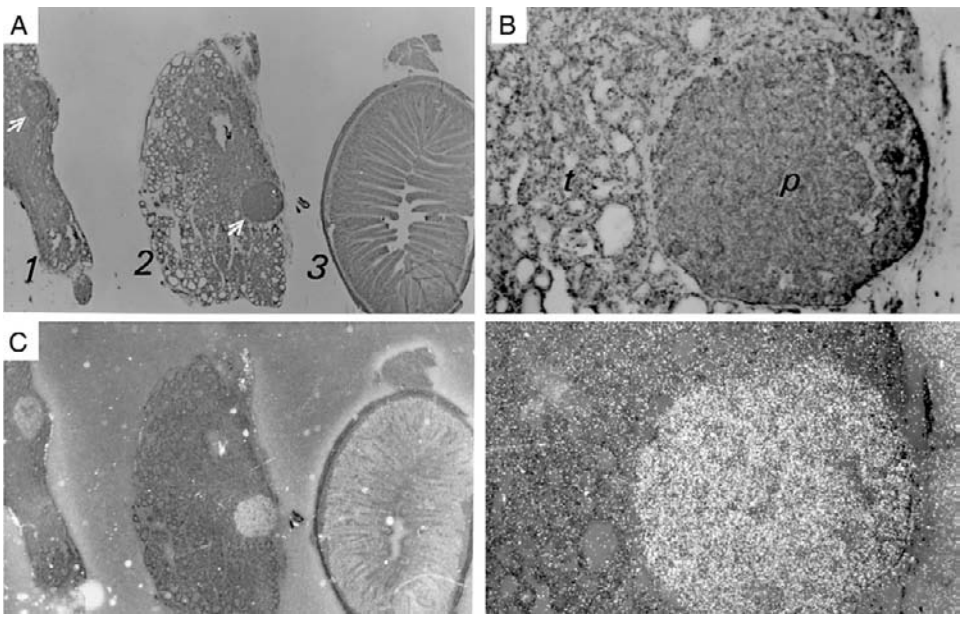

Figure 1 The $1,25(OH)_2$vitamin D receptor (VDR) is localized to the parathyroid in a similar concentration to that found in the duodenum, indicating that the parathyroid is a physiological target organ for $1,25(OH)_2D$. *In situ* hybridization with the VDR probe in rat parathyroid–thyroid and duodenum sections. (A1) Parathyroid–thyroid tissue from a control rat. (A2) Parathyroid–thyroid from a $1,25(OH)_2D_3$-treated rat (100 pmol at 24 hr). (A3) Duodenum from a $1,25(OH)_2D_3$-treated rat. White arrows point to parathyroid glands. (B) A higher power view of A2 that shows the parathyroid gland (*p*) and thyroid follicles (*t*). Top figures were photographed under bright-field illumination, whereas bottom figures show dark-field illumination of the same sections. Reproduced with permission from Naveh-Many *et al.* (1990).

doses led to an increase in VDR mRNA levels in the parathyroid glands in contrast to the decrease in PTH mRNA levels (Fig. 2). This increase in VDR mRNA occurred after a time lag of 6 hr, and a dose response showed a peak at 25 pmol. Weanling rats fed a diet deficient in calcium were markedly hypocalcemic at 3 weeks and had very high serum $1,25(OH)_2D_3$ levels. Despite the chronically high serum $1,25(OH)_2D_3$ levels, there was no increase in VDR mRNA levels; furthermore, PTH mRNA levels did not fall and were increased markedly. The low calcium may have prevented the increase in parathyroid VDR levels, which may partially explain PTH mRNA suppression. Whatever the mechanism, the lack of suppression of PTH synthesis in the setting of hypocalcemia and increased serum $1,25(OH)_2D_3$ is crucial physiologically because it allows an increase in both PTH and $1,25(OH)_2D_3$ at a time of chronic hypocalcemic stress. Russell *et al.* (1993) studied the parathyroids of chicks with vitamin D deficiency and confirmed that $1,25(OH)_2D_3$ regulates PTH and VDR gene expression in the avian parathyroid gland. Chicks in this study were fed a vitamin D-deficient diet from birth for 21 days and had established secondary hyperparathyroidism. These hypocalcemic chicks were then fed a diet with different calcium contents (0.5, 1.0, and 1.6%) for 6 days. The serum calciums were all still low (5, 6, and 7 mg/dl) with the expected inverse relationship between PTH mRNA and serum calcium. There was also a direct relationship between serum calcium and VDR mRNA levels. This result suggests either that VDR mRNA was not upregulated in the setting of secondary hyperparathroidism or that calcium directly regulates the VDR gene. Brown *et al.* (1995) studied vitamin D-deficient rats and confirmed that calcitriol upregulated parathyroid VDR

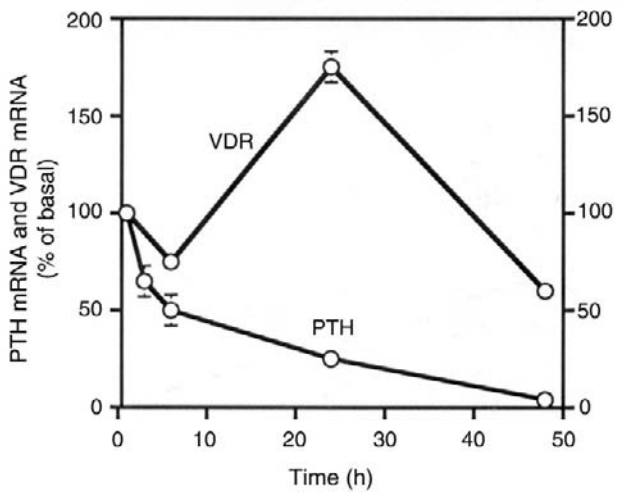

Figure 2 Time course for the effect of $1,25(OH)_2D_3$ on mRNA levels for PTH and the $1,25(OH)_2D_3$ receptor (VDR) in rat thyroparathyroid glands. Rats were injected with a single dose of either 100 or 50 pmol $1,25(OH)_2D_3$ at 0 and 24 hr. Data represent the mean ± SE of four rats. From Naveh-Many *et al.* (1990). By copyright permission of the American Society for Clinical Investigation.

mRNA and that in secondary hyperparathyroidism with hypocalcemia, PTH mRNA was upregulated without a change in VDR mRNA (Naveh-Many *et al.,* 1990).

All these studies show that $1,25(OH)_2D_3$ increases the expression of its receptor's gene in the parathyroid gland, which would result in increased VDR protein synthesis and increased binding of $1,25(OH)_2D_3$ (Fig. 3). This ligand-dependent receptor upregulation would lead to an amplified effect of $1,25(OH)_2D_3$ on the PTH gene and might help explain the dramatic effect of $1,25(OH)_2D_3$ on the PTH gene.

The use of calcitriol is limited by its hypercalcemic effect, and therefore a number of calcitriol analogs have been synthesized that are biologically active but are less hypercalcemic than calcitriol. These analogs usually involve modifications of the calcitriol side chain, such as 22-oxa-$1,25(OH)_2D_3$, which is the chemical modification in oxacacitriol (Nishii *et al.,* 1991), or a cyclopropyl group at the end of the side chain in calcipotriol (Kissmeyer and Binderup, 1991; Evans *et al.,* 1991). Brown *et al.* (1989b) showed that oxacalcitriol *in vitro* decreased PTH secretion from primary cultures of bovine parathyroid cells with a similar dose response to that of calcitriol. *In vivo* the injection of both vitamin D compounds led to a decrease in rat parathyroid PTH mRNA levels (Brown *et al.,* 1989b). However, detailed *in vivo* dose–response studies showed that *in vivo* calcitriol is the most effective analog for decreasing PTH mRNA levels, even at doses that do not cause hypercalcemia (Naveh-Many and Silver, 1993). Oxacalcalcitriol and calcipotriol are less effective for decreasing PTH RNA levels but have a wider dose range at which they do not cause hypercalcemia; this property might be useful clinically. The marked activity of calcitriol analogs *in vitro* as compared to their modest hypercalcemic actions *in vivo* probably reflects their rapid clearance from the circulation (Bouillon *et al.,* 1991). There much interest in the development and marketing of new calcitriol analogs to decrease PTH gene expression and serum PTH levels without causing hypercalcemia, but there have been few rigorous comparisons of their biological effects compared to those of calcitriol itself (Brown, 1998; Verstuyf *et al.,* 1998). The ability of calcitriol to decrease PTH gene transcription is used therapeutically in the management of patients with chronic renal failure. They are treated with calcitriol in order to prevent the secondary hyperparathyroidism of chronic renal failure. The poor response in some patients who do not respond may well result from poor control of serum phosphate, decreased vitamin D receptor concentration (Fukuda *et al.,* 1993), an inhibitory effect of a uremic toxin(s) on VDR-VDRE binding (Patel *et al.,* 1995), or tertiary hyperparathyroidism with monoclonal parathyroid tumors (Arnold *et al.,* 1995).

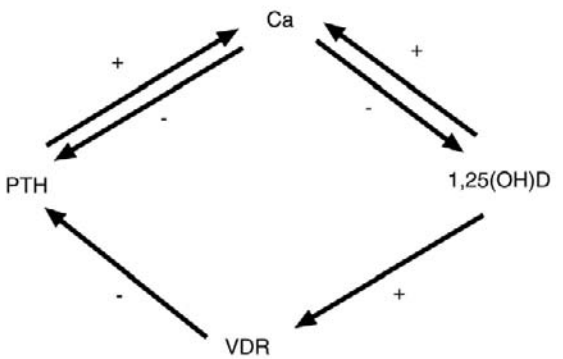

Figure 3 Interrelationships of calcium, $1,25(OH)_2D_3$, the vitamin D receptor (VDR), and PTH.

Calreticulin and the Action of $1,25(OH)_2D_3$ on the PTH Gene

Another possible level at which $1,25(OH)_2D_3$ might regulate PTH gene expression involves calreticulin. Calreticulin is a calcium-binding protein present in the endoplasmic reticulum of the cell and may also have a nuclear function. It regulates gene transcription via its ability to bind a protein motif in the DNA-binding domain of nuclear hormone receptors of sterol hormones. It has been shown to prevent vitamin D's binding and action on the osteocalcin gene *in vitro* (Wheeler *et al.,* 1995). Sela-Brown *et al.* (1998) showed that calreticulin might inhibit the action of vitamin D on the PTH gene. Both rat and chicken VDRE sequences of the PTH gene were incubated with recombinant VDR and retinoic acid receptor (RXR) proteins in a gel retardation assay and showed a clear retarded band. Purified calreticulin inhibited binding of the VDR–RXR complex to the VDREs in gel retardation assays. This inhibition was due to direct protein–protein interactions between VDR and calreticulin. OK cells were transiently cotransfected with calreticulin expression vectors (sense and antisense) and either rat or chicken PTH gene promoter–CAT constructs. The cells were then assayed for $1,25(OH)_2D_3$-induced CAT gene expression. $1,25(OH)_2D_3$ decreased PTH promoter–CAT transcription. Cotransfection with sense calreticulin, which increases calreticulin protein levels, completely inhibited the effect of $1,25(OH)_2D_3$ on the PTH promoters of both rat and chicken. Cotransfection with the antisense calreticulin construct did not interfere with the effect of vitamin on PTH gene transcription. Sense calreticulin expression had no effect on basal CAT mRNA levels. In order to determine a physiological role for calreticulin in regulation of the PTH gene, levels of calreticulin protein were determined in the nuclear fraction of rat parathyroids. The rats were fed either a control diet or a low calcium diet, which leads to increased PTH mRNA levels, despite high serum $1,25(OH)_2D_3$ levels that would be expected to inhibit PTH gene transcription (Sela-Brown *et al.,* 1998). It was postulated that high calreticulin levels in the nuclear fraction would prevent the effect of $1,25(OH)_2D_3$ on the PTH gene. In fact, hypocalcemic rats had increased levels of calreticulin protein, as measured by Western blots, in their parathyroid nuclear faction. This may help explain why hypocalcemia leads to increased PTH gene expression, despite high serum $1,25(OH)_2D_3$

levels, and may also be relevant to the refractoriness of the secondary hyperparathyroidism of many chronic renal failure patients to $1,25(OH)_2D_3$ treatment. These studies, therefore, indicate a role for calreticulin in regulating the effect of vitamin D on the PTH gene and suggest a physiological relevance to these studies (Sela-Brown *et al.,* 1998).

Calcium

In Vitro Studies

A remarkable characteristic of the parathyroid is its sensitivity to small changes in serum calcium, which leads to large changes in PTH secretion. This calcium sensing is also expressed at the levels of PTH gene expression and parathyroid cell proliferation. *In vitro* and *in vivo* data agree that calcium regulates PTH mRNA levels, but data differ in important ways. *In vitro* studies with bovine parathyroid cells in primary culture showed that calcium regulated PTH mRNA levels (Russell *et al.,* 1983; Brookman *et al.,* 1986), with an effect mainly of high calcium to decrease PTH mRNA. These effects were most pronounced after more prolonged incubations, such as 72 hr. The physiologic correlates of these studies in tissue culture are hard to ascertain, as the parathyroid calcium sensor might well have decreased over the time period of the experiment (Mithal *et al.,* 1995). This may explain why the dose response differs from *in vivo* data, but the dramatic difference in time course suggests that *in vivo* data reflect something not seen in cultured cells.

In Vivo Studies

Calcium and phosphate both have marked effects on the levels of PTH mRNA *in vivo.* The major effect is for low calcium to increase PTH mRNA levels and low phosphate to decrease PTH mRNA levels (Fig. 4). Naveh-Many *et al.* (1989) studied rats *in vivo.* They showed that a small decrease in serum calcium from 2.6 to 2.1 mmol/liter led to large increases in PTH mRNA levels, reaching threefold that of controls at 1 and 6 hr. A high serum calcium had no effect on PTH mRNA levels even at concentrations as high as 6.0 mmol/liter. Interestingly, in these same thyroparathyroid tissue RNA extracts, calcium had no effect on the expression of the calcitonin gene. Thus, while a high calcium is a secretagogue for calcitonin, it does not regulate calcitonin gene expression. Yamamoto *et al.* (1989) also studied the *in vivo* effect of calcium on PTH mRNA levels in rats. They showed that hypocalcemia induced by a calcitonin infusion for 48 hr led to a seven-fold increase in PTH mRNA levels. Rats made hypercalcemic (2.9–3.4 m*M*) for 48 hr had the the same PTH mRNA levels as controls that had received no infusion (2.5 m*M*); these levels were modestly lower than those found in rats that had received a calcium-free infusion. In further studies, Naveh-Many *et al.* (1992b) transplanted Walker carcinosarcoma 256 cells into rats. Serum calciums increased to 18 mg/dl at day 10 after transplantation. There was no change in PTH mRNA levels in these rats with marked chronic hypercalcemia (Naveh-Many *et al.,* 1992b). Differences between *in vivo* and *in vitro* results probably reflect the instability of the *in vitro* system, but it is also impossible to eliminate the possibility that *in vivo* effects are influenced by indirect effects of a high or low serum calcium or by other variables changed in the *in vivo* protocols. Nevertheless, the physiological conclusion is that common causes of hypercalcemia *in vivo* do not importantly decrease PTH mRNA levels; these results emphasize that the gland is geared to respond to hypocalcemia and not hypercalcemia.

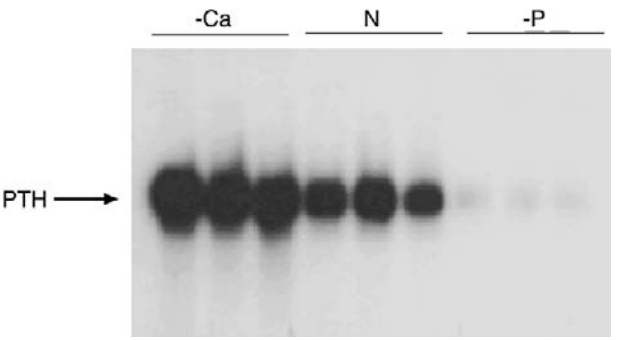

Figure 4 The effect of dietary phosphate and calcium on PTH mRNA levels. PTH mRNA levels are shown for individual rats fed diets for 3 weeks containing low phosphate; control; high phosphate; low calcium.

Mechanisms of Regulation of PTH mRNA by Calcium

The mechanism whereby calcium regulates PTH gene expression is particularly interesting. Changes in extracellular calcium are sensed by a calcium sensor that then regulates PTH secretion (Brown *et al.,* 1993). Signal transduction from the CaSR involves activation of phospholipase C, D, and A_2 enzymes (Kifor *et al.,* 1997). It is not known what mechanism transduces the message of changes in extracellular calcium leading to changes in PTH mRNA. Okazaki *et al.* (1992) identified a negative calcium regulatory element (nCaRE) in the atrial natiuretic peptide gene, with a homologous sequence in the PTH gene. They identified a redox factor protein (ref1), which was known to activate several transcription factors via alterations of their redox state, which bound a nCaRE, and the level of ref1 mRNA and protein were elevated by an increase in extracellular calcium concentration (Okazaki *et al.,* 1994). They suggested that ref1 had transcription repressor activity in addition to its function as a transcriptional auxiliary protein (Okazaki *et al.,* 1994). Because no parathyroid cell line is available, these studies were performed in nonparathyroid cell lines, so their relevance to physiologic PTH gene regulation remains to be established.

We have performed *in vivo* studies on the effect of hypocalcemia on PTH gene expression. The effect is posttranscriptional *in vivo* and involves protein–RNA interactions at the 3′-untranslated region of the PTH mRNA (Moallem *et al.,* 1998). A similar mechanism is involved in the effect of phosphate on PTH gene expression so the

mechanisms involved will be discussed after the independent effect of phosphate on the PT is considered.

Phosphate

A Specific Parathyroid Cell Sodium–Phosphate Cotransporter

The rat parathyroid harbors a type III Na^+-P_i cotransporter whose mRNA was increased by a low P_i diet and increased by vitamin D treatment (Tatsumi *et al.,* 1998). This transporter may contribute to the effects of P_i and vitamin D on parathyroid function.

Phosphate Regulates the Parathyroid Independently of Calcium and $1,25(OH)_2D_3$

The demonstration of a direct effect of high phosphate on the parathyroid *in vivo* has been difficult. One of the reasons is that the various maneuvers used to increase or decrease serum phosphate invariably lead to a change in the ionized calcium concentration. In moderate renal failure, phosphate clearance decreases and serum phosphate increases; this increase becomes an important problem in severe renal failure. Hyperphosphatemia has always been considered central to the pathogenesis of secondary hyperparathyroidism, but it has been difficult to separate the effects of hyperphosphatemia from those of the attendant hypocalcemia and decrease in serum $1,25(OH)_2D_3$ levels. In the 1970s, Slatopolsky and Bricker (1973) showed in dogs with experimental chronic renal failure that dietary phosphate restriction prevented secondary hyperparathyroidism. Clinical studies (Portale *et al.,* 1984) demonstrated that phosphate restriction in patients with chronic renal insufficiency is effective in preventing the increase in serum PTH levels (Lucas *et al.,* 1986; Portale *et al.,* 1984; Lafage *et al.,* 1992; Combe and Aparicio, 1994; Aparicio *et al.,* 1994). The mechanism of this effect was not clear, although at least part of it was considered to be due to changes in serum $1,25(OH)_2D_3$ concentrations. *In vitro* (Tanaka and DeLuca, 1973; Condamine *et al.,* 1994) and *in vivo* (Portale *et al.,* 1984; Portale *et al.,* 1989) phosphate directly regulated the production of $1,25(OH)_2D_3$. A raised serum phosphate decreases serum $1,25(OH)_2D_3$ levels, which then leads to decreased calcium absorption from the diet and eventually a low serum calcium. The raised phosphate complexes calcium, which is then deposited in bone and soft tissues and, thereby, decreases serum calcium. However, a number of careful clinical and experimental studies suggested that the effect of phosphate on serum PTH levels was independent of changes in both serum calcium and $1,25(OH)_2D_3$ levels. In dogs with experimental chronic renal failure, Lopez-Hilker *et al.* (1990) have shown that phosphate restriction corrected their secondary hyperparathyroidism independent of changes in serum calcium and $1,25(OH)_2D_3$ levels. They did this by placing the uremic dogs on diets deficient in both calcium and phosphate. This led to lower levels of serum phosphate and calcium, with no increase in the low levels of serum $1,25(OH)_2D_3$. Despite this, there was a 70% decrease in PTH levels. This study suggested that, at least in chronic renal failure, phosphate affected the parathyroid cell by a mechanism independent of its effect on serum $1,25(OH)_2D_3$ and calcium levels (Lopez-Hilker *et al.,* 1990). Therefore, phosphate plays a central role in the pathogenesis of secondary hyperparathyroidism, both by its effect on serum $1,25(OH)_2D_3$ and calcium levels and possibly independently.

Kilav *et al.* (1995) were the first to establish that the effects of serum phosphate on PTH gene expression and serum PTH levels were independent of any changes in serum calcium or $1,25(OH)_2D_3$. In a particularly informative experiment, they bred second-generation vitamin D-deficient rats and then placed the weanling vitamin D-deficient rats on a diet with no vitamin D, low calcium, and low phosphate. After 1 night of this diet, serum phosphate had decreased markedly with no changes in serum calcium or $1,25(OH)_2D_3$. These rats with isolated hypophosphatemia had marked decreases in PTH mRNA levels and serum PTH. However, the very low serum phosphates in these *in vivo* studies may have no direct relevance to possible direct effects of high phosphate in renal failure. It is necessary to separate nonspecific effects of very low phosphate from true physiologic regulation. To establish that the effect of serum phosphate on the parathyroid was indeed a direct effect, *in vitro* confirmation was needed, which was provided by three groups. Rodriguez was the first to show that increased phosphate levels increased PTH secretion from isolated parathyroid glands *in vitro;* the effect required maintainenance of tissue architecture (Almaden *et al.,* 1996). The effect was found in whole glands or tissue slices but not in isolated cells. This result was soon confirmed by Slatopolsky *et al.* (1996). Olgaard's laboratory provided elegant further evidence of the importance of cell–cell communication in mediating the effect of phosphate on PTH secretion (Nielsen *et al.,* 1996). The requirement for intact tissue suggests either that the sensing mechanism for phosphate is damaged during the preparation of isolated cells or that the intact gland structure is important to the phosphate response.

Parathyroid responds to changes in serum phosphate at the level of secretion, gene expression, and cell proliferation, although the mechanism of these effects is unknown. The effect of high phosphate to increase PTH secretion may be mediated by phospholipase A_2-activated signal transduction. Bourdeau *et al.* (1992, 1994) showed that arachidonic acid and its metabolites inhibit PTH secretion. Almaden *et al.* (2000) showed *in vitro* that a high phosphate medium increased PTH secretion, which was prevented by the addition of arachidonic acid. When dog parathyroid tissue was cultured in a high calcium and normal phosphate medium, there was an increase in arachidonic acid production at 30 and 45 min, returning to baseline at 60 min. A high phosphate medium prevented the increase in arachidonic acid production at 30 and 45 min, and there was a modest increase in PTH secretion only after 2 and 3 hr incubation. These results suggest that phosphate decreases the production of

arachidonic acid in the parathyroid and that arachidonic acid decreases PTH secretion, but it is less clear to what extent the effect of phosphate on PTH secretion is dependent upon this pathway. The use of inhibitors of the phospholipase A_2 pathway may help clarify this question. We can now provide some of the answers for the effect of phosphate and calcium on PTH gene expression.

Protein–RNA Interaction at PTH mRNA

Protein–RNA Binding to the PTH mRNA 3′-Untranslated Region (*3′-UTR*) and Its Regulation by Calcium and Phosphate

The clearest rat *in vivo* models for effects of calcium and phosphate on PTH gene expression are diet-induced hypocalcemia with a large increase in PTH mRNA levels and diet-induced hypophosphatemia with a large decrease in PTH mRNA levels. In both instances the effect was post-transcriptional, as shown by nuclear transcript run-on experiments. Parathyroid cytosolic proteins were found to bind *in vitro*-transcribed PTH mRNA, with three bands at about 50, 60, and 110 kDa (Moallem *et al.,* 1998). Interestingly, this binding was increased with parathyroid proteins from hypocalcemic rats (with increased PTH mRNA levels) and decreased with parathyroid proteins from hypophosphatemic rats (with decreased PTH mRNA levels). Proteins from many tissues bound to PTH mRNA, but this binding is regulated by calcium and phosphate only with parathyroid proteins. Intriguingly, binding requires the presence of the terminal 60 nucleotides of the PTH transcript.

PTH mRNA Is Degraded *in Vitro* by Parathyroid Cytosolic Proteins, Which Is Regulated by Calcium and Phosphate

Naveh-Many and colleagues utilized an *in vitro* degradation assay to study the effects of hypocalcemic and hypophosphatemic parathyroid proteins on PTH mRNA stability (Moallem *et al.,* 1998). In this assay, control rats' parathyroid cytosolic proteins led to the degradation of a radiolabeled PTH transcript in about 40–60 min. Hypocalcemic parathyroid proteins degraded the transcript only in 180 min, whereas hypophosphatemic parathyroid proteins degraded the transcript within 5 min. Moreover, the rapid degradation of PTH mRNA by hypophosphatemic proteins was totally dependent on an intact 3′-untranslated region (UTR) and, in particular, on the terminal 60 nucleotides (Fig. 5). Proteins from other tissues in these rats were not regulated by calcium or phosphate. Therefore, calcium and phosphate change the properties of parathyroid cytosolic proteins, which bind specifically to the PTH mRNA 3′-UTR and determine its stability (Fig. 5). What are these proteins?

Identification of AUF1 as a PTH mRNA 39-UTR Binding Protein That Determines PTH mRNA Stability

Sela-Brown *et al.* (2000) have utilized affinity chromatography to isolate these RNA-binding proteins. The proteins, which bind the PTH mRNA, are also present in other tissues, such as brain, but only in the parathyroid is their binding regulated by calcium and phosphate. The function of PTH mRNA 3′-UTR binding proteins was studied using the *in vitro* degradation assay. Competition for parathyroid-binding proteins by excess unlabeled 3′-UTR destabilized the full-length PTH transcript in this assay, indicating that these proteins protect the RNA from RNase activity (Fig. 6). The PTH RNA 3′-UTR binding proteins were purified by PTH 3′-region RNA affinity chromatography of rat brain S-100 extracts. The eluate from the column was enriched in PTH RNA 3′-UTR binding activity. Addition of eluate to the *in vitro* degradation assay with parathyroid protein extracts stabilized the PTH

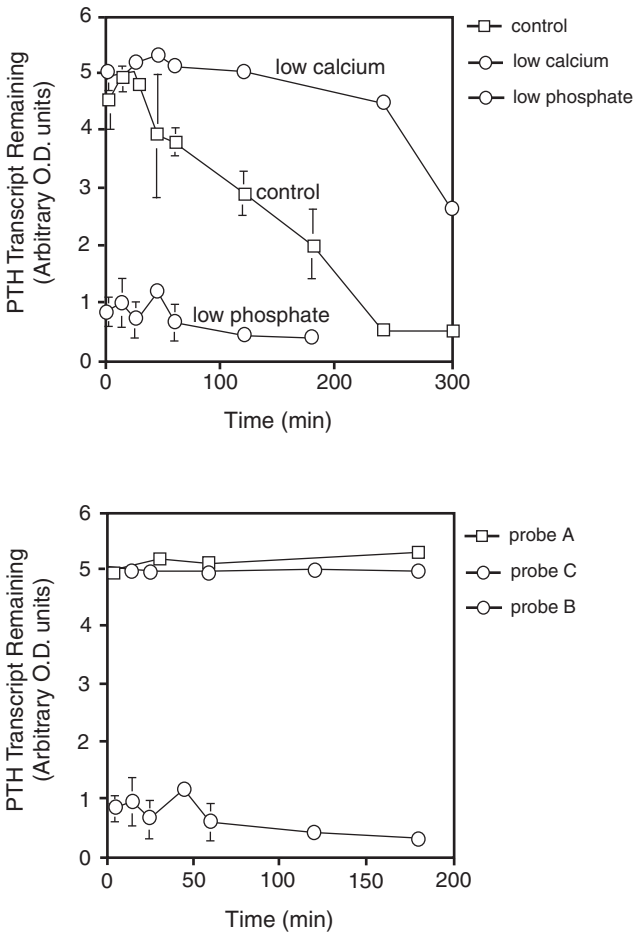

Figure 5 *In vitro* degradation of PTH mRNA by parathyroid cytosolic proteins. (Top) Time – response curves of intact full-length PTH mRNA after incubation with parathyroid cytosolic proteins. Each point represents the mean ± SE of three to four different experiments, apart from -Ca at 240 and 300 min, which is the mean of two experiments. At some points the SE is less than the size of the graphic symbols. The PTH transcript was degraded very rapidly by proteins from -P rats and remained intact for a longer time period with proteins from -Ca rats. (Bottom) Mapping a region in the PTH 3′-UTR that mediates degradation by proteins from -P rats. PTH mRNA probes used were intact PTH mRNA (probe A), without the 3′-UTR (probe C), and without the 3′-terminal 60 nucleotides of the 3′-UTR (probe B). Reproduced with permission from Moallem *et al.* (1998).

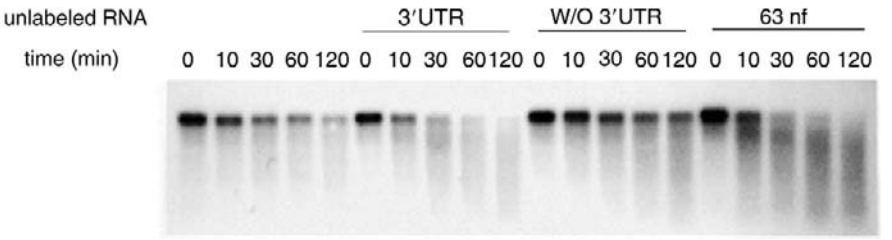

Figure 6 Competitor PTH RNA 3′-UTR accelerates PTH RNA degradation *in vitro*. The full-length PTH mRNA transcript was incubated with cytosolic parathyroid protein extracts (10 μg). At timed intervals, samples were extracted, run on agarose gels, and autoradiographed to measure the intact transcript remaining. The degradation assay was performed in the presence of PT extracts without competitor, with 25 ng of unlabeled PTH RNA 3′-UTR, with unlabeled PTH RNA excluding the 3′-UTR (without 3′-UTR), or with a 63 nucleotide unlabeled transcript comprising the sequences necessary for binding of proteins to the PTH RNA 3′-UTR. Excess 3′-UTR or the 63 nucleotide transcripts led to an accelerated decay of the full-length PTH transcript by PT proteins. The transcript that did not contain 3′-UTR had no effect. Reproduced with permission from Sela-Brown *et al.* (2000).

transcript. A major band from the eluate at 50 kDa was sequenced and was identical to AU-rich binding protein (AUF1). Recombinant AUF1 bound the full-length PTH mRNA and the 3′-UTR. Added recombinant AUF1 also stabilized the PTH transcript in the *in vitro* degradation assay. These results showed that AUF1 is a protein that binds to the PTH mRNA 3′-UTR and stabilizes the PTH transcript. This observation should now be supplemented by identification of the other protein(s) (60 and 110 kDa), which forms the protein complex on the PTH mRNA 3′-UTR. It might then be possible to understand how the different messages of changes in serum calcium and phosphate are transduced to the parathyroid cytosol. These proteins then bind to the PTH mRNA 3′-UTR and, in particular, the terminal 60 nucleotides and determine its stability. A stable transcript, e.g., after hypocalcemia, would then be translated into the hormone and available for rapid secretion. An unstable transcript, such as after hypophosphatemia, would be degraded rapidly and less PTH translated and secreted.

In vitro studies by Hawa *et al.* (1993) have also suggested a posttranscriptional effect of calcium on PTH gene expression. They incubated bovine parathyroid cells for 48 in 0.4 m*M* calcium. This did not increase PTH mRNA levels as compared to controls, but did increase the membrane-bound polysomal content of PTH mRNA twofold. It remains to be determined whether this *in vitro* translational effect involves the binding of parathyroid cytosolic proteins to the PTH mRNA 5′-UTR.

A Conserved Sequence in PTH mRNA 3′-UTR Binds Parathyroid Cytosolic Proteins and Determines RNA Stability in Response to Changes in Calcium and Phosphate

Kilav *et al.* (2001) have identified the minimal sequence for protein binding in the PTH mRNA 3′-UTR and determined its functionality. RNA transcripts of different regions in the 3′-UTR were assayed for their binding to parathyroid proteins by REMSA assays, and the specificity was determined by competition experiments. A minimum sequence of 26 nucleotides was sufficient for PTH RNA–protein binding and competition. Binding interference with antisense DNA oligonucleotides to different regions of the conserved RNA element further identified this 26 nucleotide element. Significantly, this sequence was preserved among species. To study the functionality of the sequence in the context of another RNA, a 63-bp cDNA PTH sequence consisting of the 26 nucleotide and flanking regions was fused to growth hormone (GH) cDNA. There is no parathyroid cell line and therefore an *in vitro* degradation assay was used. In this assay the effect of parathyroid cytosolic proteins on the stability of RNA transcripts for PTH, GH, and a chimeric GH-PTH 63 nucleotide was studied. The PTH transcript was stabilized by parathyroid proteins from rats fed a low calcium diet and destabilized by proteins from rats fed a low phosphate diet, correlating with PTH mRNA levels *in vivo*. The GH transcript was more stable than PTH RNA and was not affected by parathyroid proteins from rats fed the different diets. The chimeric GH transcript was stabilized by low calcium parathyroid proteins and destabilized by low phosphate parathyroid proteins, similar to the PTH full-length transcript. Therefore, the conserved PTH RNA protein-binding region confers responsiveness to calcium and phosphate and determines PTH mRNA stability and levels.

Dynein Light Chain (M_r 8000) Binds the PTH mRNA 3′-Untranslated Region and Mediates Its Association with Microtubules

To isolate other proteins binding to the 3′-UTR of parathyroid hormone, mRNA expression cloning was used. Epstein *et al.* (2000) screened an expression library for proteins that bound the PTH mRNA 3′-UTR, and the sequence of one clone was identical to dynein light chain (M_r 8000) (LC8). LC8 is part of the cytoplasmic dynein complexes that function as molecular motors that translocate along microtubules. Recombinant LC8 bound PTH mRNA 3′-UTR

by the RNA mobility shift assay. PTH mRNA colocalized with polymerized microtubules in the parathyroid gland, as well as with a purified microtubule preparation from calf brain, and this was mediated by LC8 (Fig. 7). This was the first report of a dynein complex protein binding a mRNA. Dynein light chain is also involved in targeting Swallow and *bicoid* RNA to the anterior pole of *Drosophila* oocytes (Schnorrer *et al.,* 2000). Therefore, the dynein complex may be the motor for the transport and localization of mRNAs in the cytoplasm and the subsequent asymmetric distribution of translated proteins in the cell.

Secondary Hyperparathyroidism and Parathyroid Cell Proliferation

Chronic changes in the physiologic milieu often lead to changes in both parathyroid cell proliferation and parathyroid hormone gene regulation. In such complicated settings, the regulation of PTH gene expression may well be controlled by mechanisms that differ from those in nonproliferating cells. Further, the effects of change in cell number and activity of individual cells can be complicated to dissect. Nevertheless, such chronic changes represent commonly observed clinical circumstances that require examination. Secondary parathyroid hyperplasia is a complication of chronic renal disease (Castleman and Mallory, 1932, 1937; Drueke, 1995) or vitamin D deficiency and may lead to disabling skeletal complications. The expression and regulation of the PTH gene have been studied in two models of secondary hyperparathyroidism: (1) rats with experimental uremia due to 5/6 nephrectomy and (2) rats with nutritional secondary hyperparathyroidism due to diets deficient in vitamin D and/or calcium.

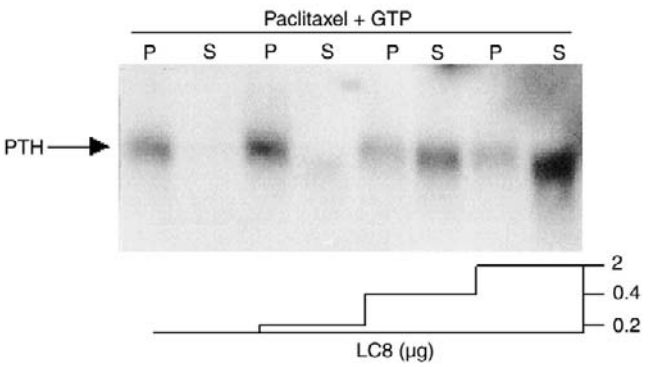

Figure 7 PTH mRNA colocalizes with microtubules. Purified calf brain microtubule preparations, which contained microtubule associated proteins (MAPs), were assembled in the presence of paclitaxel and GTP and incubated with total RNA from rat thyroparathyroid tissue. Polymerized microtubules were sedimented, and the RNA, which was extracted from both supernatants (S) and pellets (P), was assayed for PTH mRNA by Northern blot. PTH mRNA was present in the polymerized microtubule pellet and not in the supernatant. When increasing concentrations of LC8 were added to the RNA-microtubule preparation there was a shift of PTH mRNA from the pellet to the supernatant, indicating that LC8 mediates the association of PTH mRNA to microtubules. From Epstein *et al.* (2000). By copyright permission of the American Society for Clinical Investigation.

5/6 nephrectomy rats had higher serum creatinines and also appreciably higher levels of parathyroid gland PTH mRNA (Shvil *et al.,* 1990). Their PTH mRNA levels decreased after single injections of $1,25(OH)_2D_3$, a response similar to that of normal rats (Shvil *et al.,* 1990). Interestingly, secondary hyperparathyroidism was characterized by an increase in parathyroid gland PTH mRNA but not in VDR mRNA (Shvil *et al.,* 1990). This suggests that in 5/6 nephrectomy rats there was relatively less VDR mRNA per parathyroid cell or a relative downregulation of the VDR, as has been reported in VDR-binding studies (Brown *et al.,* 1989a,b; Korkor, 1987; Merke *et al.,* 1987). Yalcindag *et al.* (1999) showed by the *in vitro* degradation assay that the increase in PTH mRNA levels in uremic rats was due to a decrease in degradation by uremic parathyroid proteins. These results suggested that a decrease in parathyroid cytosolic endonuclease activity in uremia resulted in a more stable PTH transcript. Fukagawa *et al.* (1991) also studied 5/6 nephrectomized rats and confirmed that calcitriol decreased PTH mRNA levels, as did the calcitriol analog 22-oxacalcitriol.

The second model of experimental secondary hyperparathyroidism studied was that due to dietary deficiency of vitamin D (-D) and/or calcium (-Ca), as compared to normal vitamin D (ND) and normal calcium (NCa) (Naveh-Many and Silver, 1990). These dietary regimes were selected to mimic secondary hyperparathyroidism in which stimuli for the production of hyperparathyroidism are the low serum levels of $1,25(OH)_2D_3$ and ionized calcium. Weanling rats were maintained on these diets for 3 weeks and then studied. Rats on diets deficient in both vitamin D and calcium (-D-Ca) had a 10-fold increase in PTH mRNA as compared to controls (NDNCa), together with much lower serum calciums and also lower serum $1,25(OH)_2D_3$ levels. Calcium deficiency alone (-Ca ND) led to a 5-fold increase in PTH mRNA levels, whereas a diet deficient in vitamin D alone (-DNCa) led to a 2-fold increase in PTH mRNA levels.

Because renal failure and prolonged changes in blood calcium and $1,25(OH)_2D_3$ can affect both parathyroid cell number and the activity of each parathyroid cell, a change in parathyroid cell number must be assessed in each model to understand the various mechanisms of secondary hyperparathyroidism. Parathyroid cell number was determined in thyroparathyroid tissue of normal rats and -D-Ca rats. To do this, the tissue was digested enzymatically into an isolated cell population, which was then passed through a flow cytometer (FACS) and separated by size into two peaks. The first peak of smaller cells contained parathyroid cells as determined by the presence of PTH mRNA, and the second peak contained thyroid follicular cells and calcitonin-producing cells, which hybridized positively for thyroglobulin mRNA and calcitonin mRNA, but not PTH mRNA. There were 1.6-fold more cells in -D-Ca rats than in normal rats, as compared to the 10-fold increase in PTH mRNA. Therefore, this model of secondary hyperparathyroidism is characterized by increased gene expression per cell, together with a smaller increase in cell number (Naveh-Many and Silver,

1990). Wernerson has studied parathyroid cell number in dietary secondary hyperparathyroidism (ND-Ca) using stereoscopic electron microscopy and has shown that the cells are markedly hypertrophic without an increase in cell number (Wernerson *et al.,* 1989; Svensson *et al.,* 1988). Thus, in models of secondary parathyroid enlargement such as this one, parathyroid cell hypertrophy can precede the development of parathyroid cell hyperplasia. Further studies by Naveh-Many *et al.* (1995) have clearly demonstrated that hypocalcemia is a stimulus for parathyroid cell proliferation. They studied parathyroid cell proliferation by staining for proliferating cell nuclear antigen (PCNA) and found that a low calcium diet led to increased levels of PTH mRNA (Fig. 4) and a 10-fold increase in PT cell proliferation (Fig. 8) (Naveh-Many *et al.,* 1995). The secondary hyperparathyroidism of 5/6 nephrectomized rats was characterized by an increase in both PTH mRNA levels and PCNA-positive PT cells. Therefore, hypocalcemia and uremia induce PT cell proliferation *in vivo.* $1,25(OH)_2D_3$ decreases PT cell proliferation (Szabo *et al.,* 1989; Nygren *et al.,* 1988). Wada *et al.* (1997) showed that a calimimetic drug (NPS R-568) that acts on the parathyroid CaSR was able to suppress parathyroid cell proliferation in rats with experimental uremia, indicating a role for CaSR in the parathyroid hyperplasia of chronic renal failure. These findings emphasize the importance of normal calcium in the prevention of parathyroid cell hyperplasia, as well as the role of $1,25(OH)_2D_3$ in the management of parathyroid cell proliferation. The important role of calcium in regulating parathyroid cell number was also illustrated in the vitamin D receptor knockout mouse. These mice become hypocalcemic after weaning, with large parathyroid glands. The increase in cellular proliferation and the increase in parathyroid gland size are completely prevented when these mice are treated with a diet that maintains normal calcium and phosphate levels (Li *et al.,* 1998). Thus, these mice, which cannot respond to $1,25(OH)_2$ vitamin D_3, have parathyroid glands of normal size when levels of blood calcium are controlled.

A further mechanism by which calcium might regulate parathyroid cell number is by inducing apoptosis. This has been studied in the parathyroids of hypocalcemic rats, as well as rats with experimental uremia fed different diets (Naveh-Many *et al.,* 1995). Apoptosis was determined by the TUNEL method, which detects nuclear DNA fragmentation *in situ.* In no situation were apoptotic cells detected in the parathyroids. Wang *et al.* (1996a) studied mature rats by the TUNEL method and also could not detect apoptotic cells. In the absence of detectable apoptosis, it is unlikely that PT cells are normally proliferating. However, in human PT adenomas, apoptotic cells were demonstrated and this apoptosis correlated with the number of cells proliferating, as measured by Ki 67 immunoreactivity (Wang *et al.,* 1996b). Moreover, in a study of parathyroids of uremic patients with secondary hyperparathyroidism, convincing evidence of apoptosis was documented (Zhang *et al.,* 2000; Drueke, 2000). Therefore, it is likely that PT cells have the latent ability not only to proliferate, but also to apoptose.

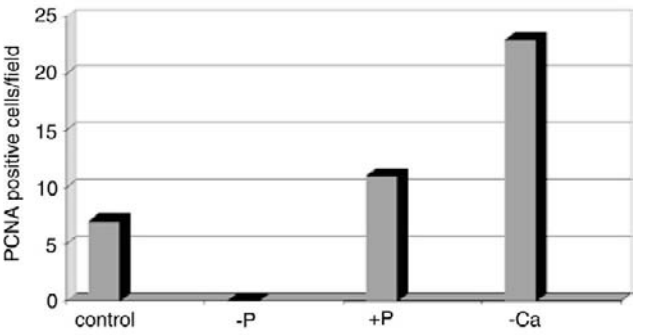

Figure 8 Dietary phosphate and calcium regulate parathyroid cell proliferation. Weanling rats were fed different diets for 10 or 21 days, and the number of proliferating cells was determined by PCNA staining. Diets were control, low calcium (0.02%), low phosphate (0.02%), or high phosphate (1.2%). Results are expressed as PCNA-positive cells per microscope field, as mean ± SE for four different rats and compared to rats fed the control diet.* $p < 0.05$; ** $p < 0.01$. From Naveh-Many *et al.* (1995). By copyright permission of the American Society for Clinical Investigation.

These experimental findings are relevant to the management of patients with secondary hyperparathyroidism. Increased transcription of the PTH gene is readily reversible, but the regulated reversibility of an increased number of parathyroid cells by accelerating cell death has not yet been demonstrated.

Sex Steroids

PTH is anabolic to bone and is an effective means of treating postmenopausal osteoporosis (Finkelstein *et al.,* 1994; Dempster *et al.,* 1993). In postmenopausal women with osteoporosis time series, analysis has shown that there is a loss in the periodicity of PTH secretion (Prank *et al.,* 1995; Fraser *et al.,* 1998). This suggests that estrogens may have an effect on the parathyroid. Estradiol and progesterone both increased the secretion of PTH from bovine parathyroid cells in primary culture (Greenberg *et al.,* 1987). However, transdermal estrogen did not increase serum PTH levels in postmenopausal patients (Prince *et al.,* 1990). Estrogen receptors were not detected in parathyroid tissue by a hormone-binding method (Prince *et al.,* 1991), but were detected by immunohistochemistry and PCR for the estrogen receptor mRNA (Naveh-Many *et al.,* 1992a). *In vivo* in ovariectomized rats, both estrogen and progestins regulated PTH gene expression.

Rats were ovariectomized and after 2 weeks were treated with estradiol or vehicle. PTH gene expression was increased markedly by 17β-estradiol given as a single injection of 37,73, and 145 nmol or by osmotic minipumps at a dose of 12 pmol/day for 7 or 14 days (Naveh-Many *et al.,* 1992a). Serum Ca^{2+} and vitamin D levels did not change. Another parameter of estrogen activity, namely uterus weight, was measured in these rats. Ovariectomy led to a large decrease in uterus weight. One and seven daily injections of 73 nmol 17β-estradiol increased uterus weight.

The much smaller dose of 12 pmol/day by constant infusion for 7 or 14 days did not increase uterus weight, despite its marked effect on PTH mRNA levels. This small dose successfully separated an estrogen effect on the Ca^{2+}-regulating hormones from that on the uterus. This may be important in searching for estrogen analogs or dose regimes that will affect these hormones and through them bone and not other organs, such as breast and uterus. Estrogen receptor mRNA and protein were shown to be present in the parathyroid. These results demonstrate that the ER gene and protein are expressed in parathyroid cells, suggesting that these are target organs for estrogen.

Further studies were performed on the effect of progestins on PTH gene expression (Epstein *et al.,* 1995). The 19-nor progestin R5020 given to weanling rats or mature ovariectomized rats led to a twofold increase in thyroparathyroid PTH mRNA levels. In addition, *in vitro,* in primary cultures of bovine parathyroid cells, progesterone increased PTH mRNA levels. The progesterone receptor (PR) mRNA was demonstrated in rat parathyroid tissue by *in situ* hybridization and in human parathyroid adenoma by immunohistochemistry. Changes in PTH mRNA levels during the rat estrous cycle were also studied. At proestrus and estrus, PTH mRNA levels were increased significantly by three- and fourfold as compared to diestrus (Epstein *et al.,* 1995). These results confirm that the parathyroid gland is a target organ for the ovarian sex steroids estrogen and progesterone and are of physiological relevance, as shown by the changes during estrus.

Therefore, *in vitro* progesterone has been shown to increase PTH mRNA levels, but the same effect *in vitro* has not been shown for estrogen. *In vivo,* both estrogens and progestins increased PTH mRNA levels.

Protein Kinase A and C

CaSR activates phospholipase A_2, C, and D pathways, which are relevant to the regulation of PTH secretion (Kifor *et al.,* 1997). Moallem *et al.* (1995) showed that protein kinases A and C regulate PTH mRNA levels *in vitro* in dispersed bovine parathyroid cells. Activation of protein kinase A by cholera toxin increased PTH mRNA levels about twofold after 3 and 7 hrs of incubation. Incubation with pertussis toxin increased PTH mRNA at low (0.7 m*M*) (four fold increase) and normal (1.25 m*M*) (four fold increase) extracellular Ca^{2+} concentrations and reversed the decrease in PTH mRNA levels caused by high Ca^{2+} (2.0 m*M*), leading to a large increase in PTH mRNA. Inhibition of protein kinase C both by staurosporine and by prolonged incubation with the phorbol ester phorbol 12-myristate 13-acetate (PMA), which then also decreases protein kinase C, decreased PTH mRNA levels at 24 hrs, reaching approximately 40 and 5% of control, respectively. A short-term incubation with PMA (3 hr), which stimulates the protein kinase C pathway, had no effect on PTH mRNA levels. These results show that both protein kinases A and C are involved in the regulation of PTH gene expression. Stimulators of protein kinase A increased PTH mRNA levels. Hypocalcemia is the major stimulus to increased PTH mRNA, and the marked increase after pertussis toxin raises the question as to whether G_i is involved in the signal transduction of hypocalcemia. Downregulation of protein kinase C activity decreased PTH mRNA levels, suggesting that protein kinase C may be necessary for normal PTH gene expression.

Summary

The PTH gene is regulated by a number of factors. Calcitriol acts on the PTH gene to decrease its transcription, and this action is used in the management of patients with chronic renal failure. The main effect of calcium on PTH gene expression *in vivo* is for hypocalcemia to increase PTH mRNA levels, and this seems likely to be posttranscriptional, but an *in vitro* transcriptional mechanism has been described whose physiological relevance remains to be determined. Phosphate also regulates PTH gene expression *in vivo,* and this effect appears to be independent of the effect of phosphate on serum calcium and $1,25(OH)_2D_3$. The effect of phosphate is posttranscriptional. *trans*-acting parathyroid cytosolic proteins bind to a defined *cis* element in the PTH mRNA 3′-UTR, which determine the degradation of PTH mRNA by degrading enzymes and thereby the PTH mRNA half-life. The posttranscriptional effects of calcium and phosphate are the result of changes in the balance of these stabilizing and degrading factors on PTH mRNA. PTH mRNA binds to LC8, a member of the dynein family, which mediates the binding of mRNA to the microtubule apparatus of the parathyroid and may be important to the localization of PTH mRNA in the cell. Estradiol and progestins increase PTH mRNA levels, and their specific receptors are present in the parathyroids. There are changes in PTH mRNA levels during the rat estrous cycle that suggests a physiological relevance for these findings.

In diseases such as chronic renal failure, secondary hyperparathyroidism involves abnormalities in PTH secretion and synthesis. An understanding of how the parathyroid is regulated at each level will help devise rational therapy for the management of such conditions, as well as treatment for diseases, such as osteoporosis, in which alterations in PTH may have a role.

Acknowledgments

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CHAPTER 26

Parathyroid Hormone–Receptor Interactions

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Introduction

Investigating structure–function–conformation relations is driven by the desire to advance our understanding of the fundamental nature of ligand–receptor interactions and the molecular recognition events leading to signal transduction. For the parathyroid hormone (PTH)–receptor system, the growing interest in providing osteoporosis patients with a true anabolic therapeutic agent is another driving force in stimulating research in this area. Currently, the field of bone biology is experiencing a period of growth and integration among biology, chemistry, molecular biology, pharmacology, and medicine. Many researchers share the belief that disease-oriented research must be approached in a collaborative and interdisciplinary manner.

The PTH–PTH receptor field is studied today in a wider context than before. Additional PTH receptor subtypes have been discovered and novel cognate ligands identified for these receptors. The family of class II G protein-coupled receptors (GPCRs) is growing steadily. Understanding of ligand–receptor interactions is advancing simultaneously for several hormone–receptor systems belonging to this class. As a result, we can generate hybrid ligands and chimera receptors, thus expanding our repertoire of tools to study functional as well as structural relatedness. Molecular biology is instrumental in providing powerful techniques for the study of receptors: cloning and expressing, adding tags to macromolecular entities, and labeling signaling and adapter molecules with green fluorescent protein (GFP). This "tagging" of proteins allows monitoring of the temporal changes in expression levels and cellular distribution of receptors and their ligands.

This chapter summarizes PTH/PTH-related protein (PTHrP)–receptor interactions in a broad sense. It addresses the traditional approaches, which focus on either the ligand or the receptor, and the more recent integrated approach that examines directly the ligand–receptor bimolecular complex. We provide information on PTH2 and PTH3 receptors (PTH2- and PTH3-Rc), which are structurally related to the classical PTH1 receptor, knowing that they may have no role in normal bone physiology. Nevertheless, their interactions with ligands recognized by the PTH1-Rc make them important tools for gaining important insight into structure–function relations. For the same reason, we also discuss the tuberoinfidibular peptide of 39 amino acid residues (TIP39). In some cases, when there is more than one way of studying a problem or different interpretations of similar results, we present the controversy so that the reader may use his/her own judgement. Such is the case with the debate whether PTH and PTHrP have a stable tertiary structure and its relevance to the biology of the hormones.

Recent results and conclusions from investigations of PTH–receptor interactions are reviewed. Delineation of contact sites, sometimes in atomic detail, between ligand and receptor allow for the first time the generation of an experimentally based model for the PTH–PTH1-Rc complex. This model serves as a testing ground to understand functional consequences of structural modifications in the ligand and the receptor. Moreover, it provides insight for understanding disease-related mutations in the receptor and related intracellular signaling events. Development of signaling-selective PTH analogs not only opens new opportunities for studying the heterogeneous signaling pathway system,

but also generates leads to test new therapeutic paradigms for osteoporosis. Minimization of the size of a ligand and better understanding of its dynamic mode of interaction with the receptor in either the G protein-coupled or -uncoupled states are concepts also described in this chapter.

The Ligand: New Insights into Structure and Function

Early work on the structure–activity relations of PTH and PTHrP has been covered in extensive reviews (Chorev and Rosenblatt, 1994, 1996; Potts *et al.,* 1997). It established that the N-terminal 1-34 amino acid sequence of both calciotropic hormones is sufficient to induce the entire spectrum of *in vitro* and *in vivo* PTH1-Rc-mediated activities (Dempster *et al.,* 1993; Whitfield and Morley, 1995). Both PTH-(1-34) and PTHrP-(1-36) have similar affinities to PTH1-Rc and are equipotent in stimulating adenylyl cyclase (AC) and intracellular Ca^{2+} transients in cells expressing PTH1-Rc. Significant sequence homology between PTH and PTHrP is limited to the first 13 residues, of which 8 are identical (Fig. 1). The molecular architecture consists of two functional domains. The "activation domain" is assigned to the homologous N-terminal sequences, and the principal "binding domain," located at the structurally divergent mid- and C-terminal sequences, contains the "binding domain" assigned to residues 14-34 (Abou-Samra *et al.,* 1989; Caulfield *et al.,* 1990; Gardella *et al.,* 1993; Nussbaum *et al.,* 1980). Truncation of 2-6 residues from the N terminus converts the 1-34/36 agonists into potent antagonists (Rosenblatt, 1986; Rosenblatt *et al.,* 1993, 1997). The architecture similarity of the functional domains in two hormones that also share a common receptor has promoted the hypothesis that they have similar conformations, despite their limited sequence homology (Caulfield *et al.,* 1990; Mierke *et al.,* 1997).

The new challenges facing structural studies of PTH- and PTHrP-derived ligands originate from advances along several avenues of PTH-related biology. Subtype receptor multiplicity raised the need for receptor subtype- or target-specific ligands. Exploring the multiplicity of signaling pathways and their relevance to physiological and pathophysiological processes required the development of signaling selective analogs. The interest in developing a more clinic-friendly PTH-like drug with a better therapeutic window than PTH, i.e., a more favorable ratio of catabolic to anabolic activities, has intensified markedly. There is still a strong feeling that knowing the bioactive conformation will help in understanding structure–function relations and guide the rational design of novel PTH/PTHrP analogs and nonpeptide PTH mimetics. Moreover, for the purpose of drug development, reducing ligand size by introducing affinity and potency enhancing substitutions is a new field of growing interest.

Probing the Primary Structure

A wealth of corroborating data has been generated by exhaustively scanning either the entire sequence of PTH-(1-34/36) or portions of it. These studies employ both synthetic and biosynthetic methodologies to generate new insights into the tolerance and significance of certain residues with regard to bioactivity (Gardella *et al.,* 1991; Gombert *et al.,* 1996; Oldenburg *et al.,* 1996). Both Gardella and Oldenburg and their coworkers used recombinant DNA methodologies to generate analogs, either by randomly mutating codons coding for positions 1-4 in hPTH (Gardella *et al.,* 1991) or by replacing individual codons with [(A/G)(A/G)G] (coding for Lys, Arg, Glu, or Gly) (Oldenburg *et al.,* 1996). Conversely, Gombert *et al.* (1995) used a parallel multisynthesis approach to generate D-Ala, L-Ala and D-Xxx scans of hPTH-(1-36), where D-Xxx is the enantiomeric form of the native amino acid residue in the particular position.

The D-Ala scan reveals segments 2-8 and 20-28 to be the least tolerant. Substitutions with D-Ala within these segments result in large decreases in binding affinity and are accompanied by a large loss in ligand-stimulated AC activity. The latter is most pronounced for substitutions of Arg^{20}, Trp^{23}, Leu^{24}, and Leu^{28} in the sequence (20-28), which overlaps with the principal binding domain (Gombert *et al.,* 1995). A high correlation between binding and AC activation was observed in the L-Ala scan. The largest loss in AC activity occurs when the substitutions are

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                    1         10        20        30        40
Human PTH           SVSEIQLMHNLGKHLNSMERVEWLPKKLQDVHNFVALGAP~~~
Human PTHrP         AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHT-AEIRAT~~~
Bovine PTH          AVSEIQFMHNLGKHLSSMERVEWLPKKLQDVHNFVALGAS~~~
Bovine TIP39     SLALADDAAFRERARLLAALERRHWLNSYM---HKLLVLDAP
                 1         10        20        30        39
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Figure 1 Alignment of human PTH-(1-40) with human PTHrP-(1-39), bovine PTH-(1-40), and bovine TIP39 sequences. Amino acid residues in bold indicate direct homology. Gaps in the sequences of PTHrP and TIP are introduced to maximize homology. Numbering at the top and bottom refers to mature PTH and mature TIP39, respectively. The wavy line indicates C-terminal truncated sequences. It can be appreciated that the highest homology observed is between human and bovine PTH. There is much less homology between human PTH and human PTHrP. The homology among TIP39 and PTH or PTHrP is very limited.

within the activation domain (residues 2-8) (Gombert *et al.,* 1995). Only substituting L-Ala for Lys^{13}, Asn^{16}, or Glu^{19} yielded slightly more active analogs. The D-Xxx scan resulted in an overall loss of affinity and efficacy. The most affected region was the putative amphiphilic helical domain (residues 23-29) and, to a lesser extent, the C-terminal segment (32-36) (Gombert *et al.,* 1995).

Gardella and co-workers (1991) focused on the evolutionarily conserved, first four N-terminal residues in PTH. Residues Glu^4 and Val^2 are less tolerant to substitution than the other two positions, suggesting an important role in receptor binding and activation (Gardella *et al.,* 1991). The most intriguing finding of this study was the divergent activity displayed by [Arg^2,Tyr^{34}]PTH-(1-34)NH_2 in two different cell lines expressing the wild-type PTH1-Rc: ROS 17/2.8, a rat osteosarcoma cell line, and OK, an opossum kidney cell line (Gardella *et al.,* 1991). This analog is a weak partial agonist for stimulation of AC in ROS 17/2.8 cells, whereas it is a full agonist for cAMP increases in the OK cell system (Gardella *et al.,* 1991). Gardella and co-workers (1994) further analyzed the activities of [Arg^2,Tyr^{34}]PTH-(1-34)NH_2 in COS-7 cells transfected with rat or opossum PTH1-Rc or rat/opossum PTH1-Rc chimeras. They demonstrated that the differences in activity in ROS and OK cells are due to differences in the interaction of the receptors (rat vs opossum) with the amino terminus of the ligand and not to tissue-specific (bone vs kidney) effects.

Cohen and co-workers (1991) reported that the highly conserved PTH and PTHrP residues, Ser^3 and Gln^6, make important contributions to the binding to and activation of PTH1-Rc. Substituting of Phe or Tyr for Ser^3, and Phe or Ser for Gln^6, generates partial agonists. Both [Phe^3]hPTH-(1-34) and [Phe^6]hPTH-(1-34) competitively inhibit bPTH-(1-34)- and PTHrP-(1-34)-stimulated AC activity. Taken together, the finding that specific substitutions within the "activation domain" may convert full agonists into partial antagonists provides new tools for designing potent, full-length antagonists (1-34). It also suggests that structural perturbation of ligand–receptor bimolecular interactions at the N terminus of PTH-(1-34) can interfere preferentially with the induction of conformational changes, and therefore inhibit intracellular signaling. Such conformational changes are required for the effective coupling of the ligand-occupied receptor to G proteins, but are not important for ligand recognition and binding.

Oldenburg and co-workers (1996) conducted an extensive study in which they introduced single, nonconservative point mutations in PTH-(1-34) and characterized them in UMR106, rat osteosarcoma cells. These mutations, which span the mid- and C-terminal regions, namely, the 11-34 sequence, generated several analogs more potent than the parent peptide. One of the more interesting analogs is [$Arg^{19,22,30}$,Lys^{29},Hse^{34}]hPTH-(1-34), which is equipotent to bPTH (EC_{50} ~ 0.9 and K_d ~ 1.5 n*M*). The high potency of this analog is attributed to the presence and disposition of the seven positive charges in the C-terminal helix and not to its amphiphilic nature. The positive charges may play a critical role in intramolecular, ligand–receptor or ligand–lipid interactions (Oldenburg *et al.,* 1996).

Although amino-terminal fragments of PTH and PTHrP shorter than 1-27 were previously reported devoid of biological activity (Azarani *et al.,* 1996; Kemp *et al.,* 1987; Rosenblatt, 1981; Tregear *et al.,* 1973), the efforts of Gardella and co-workers focused on the isolated activation domain represented by the amino terminus, PTH-(1-14) (Carter and Gardella, 1999; Luck *et al.,* 1999; Shimizu *et al.,* 1999). In the search for small peptide and nonpeptide PTH-mimicking molecules as potential therapies for bone metabolic disorders, the marginally active PTH-(1-14) was used as the starting point for structural manipulations in an effort to optimize its activity. Numerous studies employing site-directed mutagenesis and chimera studies of PTH1-Rc (Bergwitz *et al.,* 1996; Gardella *et al.,* 1994; Juppner *et al.,* 1994; Lee *et al.,* 1995a; Turner *et al.,* 1996), as well as photoaffinity cross-linking studies between photoreactive PTH and PTHrP analogs and PTH receptors (Adams *et al.,* 1995; Behar *et al.,* 1999, 2000; Bisello *et al.,* 1998; Mannstadt *et al.,* 1998; Zhou *et al.,* 1997), support the paradigm that the activation domain of PTH interacts with the extracellular loops (ECLs) and the juxtamembrane portions of the TMs. These receptor sites are different from those involved in interacting with the binding domain of PTH, which are located primarily within the N-terminal extracellular domain of the receptor (N-ECD). Similar observations were reported for secretin (Dong *et al.,* 1999; Holtmann *et al.,* 1996), vasoactive intestinal peptide (VIP) (Holtmann *et al.,* 1996a,b), calcitonin (CT) (Stroop *et al.,* 1996), and CT/glucagon chimera (Stroop *et al.,* 1995) receptors, all belonging to class II GPCRs.

Luck and co-workers (1999) reported that PTH-(1-14) is equipotent in stimulating increased cAMP levels (EC_{50} 100 μM) via the intact rat (r) PTH1-Rc and the *N*-terminal truncated receptor (rΔNt) missing N-ECD residues 26-181, both transiently expressed in COS-7 cells. In contrast, PTH-(1-34) is two orders of magnitude less potent in stimulating AC via the rΔNt than through the intact rPTH1-Rc (Luck *et al.,* 1999). In addition, the "Ala scan" of PTH-(1-14) revealed that the first nine of the N-terminal residues forming the critical activation domain are involved in ligand–receptor interaction, rather than in an intramolecular interaction with the C-terminal domain PTH(15-34) as suggested previously (Cohen *et al.,* 1991; Gardella *et al.,* 1995; Marx *et al.,* 1995). This study reinforced the paradigm that the N terminus of PTH interacts with binding determinants within both ECLs and the juxtamembranal portions of the TMs of the PTH1-Rc.

Shimizu and co-workers (1999) identified some substitutions in the 10–14 sequence of the hormone that are not only compatible with function, but also result in more potent peptides. The peptides [$Ala^{3,10,12}$,Arg^{11}]PTH-(1-14) and [$Ala^{3,10}$,Arg^{11}]PTH-(1-11) are, respectively, 100- and 500-fold more active than PTH-(1-14) (Fig. 2). In addition, insertion of His, a "Zn^{2+} switch," into some positions in the 10-13 sequence of PTH-(1-14) led to increases in ligand-stimulated cAMP levels in the presence of Zn^{2+} (Carter and

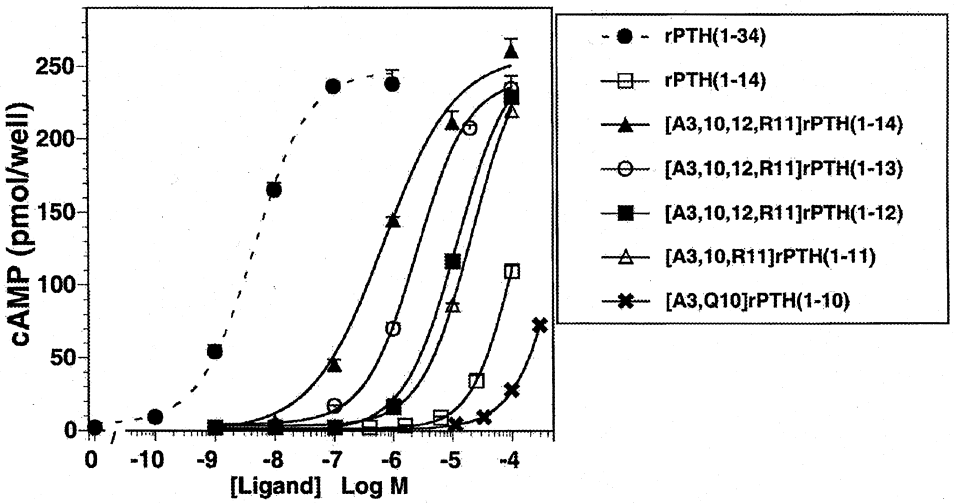

Figure 2 Dose–response analysis of PTH C-terminal truncated sequences in LLC-PK1 cells stably transfected with PTH1-Rc. Dose–response curves for AC activity by control peptide, [Nle^{8,21},Tyr^{34}]rPTH-(1-34)NH_2 [rPTH(1-34)] and the native or modified amino-terminal fragment analogs of rPTH in HKRK-B7 cells (Shimizu *et al.,* 2000b).

Gardella, 1999). This result was interpreted by Carter and co-workers (1999) to suggest that the C-terminal portion of PTH-(1-14) contributes important interactions with the ECLs and TM domains. A particular ternary metal–ligand–receptor complex, in which the histidine residues in the ligand and the receptor participate in the coordination sphere around the Zn^{2+}, stabilizes these interactions. However, in the absence of demonstrable specific binding, it is unclear whether the extremely low levels of ligand-induced cAMP increases result from nonspecific interactions between PTH-(1-14) and PTH1-Rc or from interactions at sites different from the ones used by the 1-34 sequence.

Receptor Subtype Specificity Switch

The finding that the N-truncated sequence, PTHrP-(7-34), can bind and weakly activate the PTH2-Rc, which selectively binds PTH but not PTHrP (Behar *et al.,* 1996b; Usdin *et al.,* 1995), suggested to Behar and co-workers (1996a) that the N-terminal sequence 1-6 of PTHrP must contain a structural element that disrupts the PTHrP-(1-34)–PTH2-Rc interaction. The single-point hybrid ligands, [His^5,Nle^{8,18},Tyr^{34}]bPTH-(1-34)NH_2 and [Ile^5]PTHrP-(1-34)NH_2, were generated by swapping the nonconserved residues in position 5 between PTH-(1-34), which binds and activates both PTH1- and PTH2-Rc, and PTHrP-(1-34) (Behar *et al.,* 1996a). Indeed, in HEK293 cells stably transfected with either hPTH1-Rc or hPTH2-Rc, the receptor specificity of these point-hybrid ligands is reversed when compared with their parent compounds. Therefore, His^5, a single residue within the activation domain of the ligands, acts as a specificity "switch" between these two highly homologous receptor subtypes (Behar *et al.,* 1996a).

A similar study conducted by Gardella and colleagues (1996a) in COS-7 cells transiently expressing PTH1-Rc and PTH2-Rc arrived at a somewhat different conclusion. According to their study, two sites are responsible for the divergent specificity of PTH and PTHrP: position 5 determines signaling and position 23 determines receptor-binding affinity. Simultaneously swapping the residues in positions 5 and 23 between PTH and PTHrP results in [His^5,Phe^{23},Tyr^{34}]PTH-(1-34)NH_2 ($IC_{50} > 10{,}000$ n*M* for both PTH1- and PTH2-Rc, and $EC_{50} = 1.18$ and > 1000 n*M* for PTH1- and PTH2-Rc, respectively) and [Ile^5, Trp^{23},Tyr^{36}]PTHrP-(1-36)NH_2 ($IC_{50} = 16$ and 10 n*M,* and $EC_{50} = 0.21$ and 0.5 n*M* for PTH1- and PTH2-Rc, respectively). In their hands, [Trp^{23},Tyr^{36}]PTHrP-(1-36)NH_2 is an antagonist for the PTH2-Rc, but a full agonist for the PTH1-Rc.

Studies by Behar and Gardella agree in identifying position 5 as a signaling switch between PTH1- and PTH2-Rc (Behar *et al.,* 1996a; Gardella *et al.,* 1996a). They differ in the assigning of the affinity switch to two different residues. Whereas Behar and co-workers (1996a) do not identify distinct affinity determinants unique to each of the receptor subtypes, Gardella and colleagues (1996a) attribute the affinity switch to a distinct residue at position 23. These discrepancies may be related to differences in the experimental systems employed, as they vary in a number of ways: the use of stable vs transient transfections, homologous vs heterologous receptor–cell systems, and radioligands (rat- vs bovine-derived peptides).

TIP39, a Putative Endogenous Ligand of PTH2-Rc

Usdin and co-workers (1999b) have purified an endogenous ligand selective for PTH2-Rc, a tuberoinfundibular peptide of 39 amino acids (bTIP39), from bovine hypothalamic extracts. A homology search reveals that 9 out of the 39 residues of bTIP39 are identical to bPTH (Fig. 1).

Interestingly, TIP39 does not appreciably activate AC in COS-7 cells transfected with either human or rat PTH1-Rc (Usdin *et al.,* 1999b), although it binds to the receptor with moderate affinity [IC_{50} = 59 n*M,* displacing ^{125}I-bPTH-(3-34)] (Hoare *et al.,* 2000a). Jonsson and co-workers (2001) reported that TIP39 binds with weak affinity (~200 n*M*) to hPTH1-Rc stably expressed in $LLCPK_1$ cells (HKrk-B7 cells) and failed to stimulate AC activity. The truncated peptides, TIP-(3-39) and TIP-(9-39), which display 3- and 5.5-fold higher affinity than the intact peptide, are similarly devoid of any AC activity. Moreover, while TIP39 is a weak antagonist, both of the TIP39-derived N-terminal truncated peptides inhibit PTH-(1-34)- and PTHrP-(1-36)-stimulated AC with efficiencies similar to a highly potent PTH1-Rc antagonist, [Leu^{11},D-Trp^{12},Trp^{23}, Tyr^{36}]PTHrP-(7-36)NH_2 (Jonsson *et al.,* 2001).

Additional work with the hybrid PTHrP/TIP39 peptide and PTH1-Rc/PTH2-Rc chimeras provides some insight into TIP39–receptor interactions (Hoare *et al.,* 2000; Jonsson *et al.,* 2001). Jonsson and co-workers (2001) reported that the hybrid peptide PTHrP-(1-20)-TIP39-(23-39) binds effectively (IC_{50} = 8–11 n*M*) and stimulates cAMP efficaciously in HKrk-B7 and SaOS-2 cells (EC_{50} = 1.4 and 0.38 n*M,* respectively). Hoare and co-workers (2000) reported that the juxtamembrane domains (JMD) of the PTH2-Rc, which include ECL's 1-3, determine binding and signaling selectivity of TIP39 for PTH2-Rc over PTH1-Rc. This was established by studying TIP39 interactions with the reciprocal chimeric PTH1-Rc/PTH2-Rc, in which the N-ECD domains were exchanged. TIP39 fully activated [N-ECD]PTH1-Rc/[JMD]PTH2-Rc (EC_{50} = 2 n*M*) and bound to it with affinity equal to wild-type PTH2-Rc (IC_{50} = 2.3 and 2 n*M,* respectively) (Hoare *et al.,* 2000). However, the reciprocal chimeric receptor, ([N-ECD]PTH2-Rc/[JMD]PTH1-Rc), is not activated by TIP39 and binds it with affinity similar to that of PTH1-Rc. Truncation of the first six amino acid residues from the N terminus of TIP39 results in a 10-fold increase in binding affinity for PTH1-Rc (IC_{50} = 6 n*M*), making it a potent, selective antagonist of this receptor (Hoare *et al.,* 2000). At the same time, TIP39-(7-39) does not activate PTH2-Rc and has a 70-fold lower affinity to it than the intact hormone (IC_{50} = 370 and 5.2 n*M,* respectively). Jonsson and colleagues (2001) concluded from their studies with amino-terminal truncated TIP39 and PTHrP/TIP39 hybrid peptides (see earlier discussion) that the carboxyl-terminal portion of TIP39 interacts with PTH1-Rc very similarly to the analogous domains in PTH-(1-34) and PTHrP-(1-36). At the same time, the amino-terminal portion has destabilizing interactions with this receptor that result in poor affinity and are therefore unproductive (Jonsson *et al.,* 2001).

Taken together, these studies suggest that the dominating molecular determinants of the binding selectivity of TIP39 to PTH2-Rc are different from those identified for PTH and PTHrP binding to PTH1-Rc. While in the TIP39/PTH2-Rc system, binding specificity is assigned to the JMD and the N-terminal of the ligand (Hoare *et al.,* 2000), the binding selectivity in the PTH-PTHrP/PTH1-Rc system is specified by the N-ECD in the receptor and the C-terminal portion of the ligands (Bergwitz *et al.,* 1996; Gardella *et al.,* 1994, 1996a; Rosenblatt *et al.,* 1978). Although these findings are of major significance for understanding bimolecular ligand–receptor interactions, the physiological role of the TIP39/PTH2-Rc system remains to be established (Usdin *et al.,* 2000).

Dual Intracellular Signaling Pathways

Activation of PTH1-Rc by either PTH or PTHrP stimulates multiple intracellular signaling pathways via coupling to multiple G proteins (Abou-Samra *et al.,* 1992; Bringhurst *et al.,* 1993; Juppner *et al.,* 1991; Lee *et al.,* 1995b; McCuaig *et al.,* 1994; Pines *et al.,* 1994, 1996; Schwindinger *et al.,* 1998; Segre and Goldring, 1993; Segre, 1996; Takasu *et al.,* 1999b). PTH increases intracellular cAMP via G_s. PTH also activates phospholipase C (PLC), leading to the accumulation of IP_3 (Schneider *et al.,* 1994), which stimulates the release of Ca^{2+} from intracellular stores (Pines *et al.,* 1996) via pertussis toxin-insensitive G_q, as well as the accumulation of diacylglycerols, which stimulate membrane-associated protein kinase C (PKC) (Gagnon *et al.,* 1993; Janulis *et al.,* 1992, 1993; Jouishomme *et al.,* 1992; Massry, 1983). While the cAMP–protein kinase A (PKA) signaling pathway seems to be responsible for almost all of the calciotropic and skeletal actions of PTH and PTHrP, the physiological role of the PKC signaling pathway is not yet fully understood (Goltzman, 1999). For example, PTH/PTHrP regulation of mineralization or alkaline phosphatase activity in hypertrophic chondrocytes is PLC independent (Guo *et al.,* 2001). It has been suggested that the relative intensity of AC and PLC signaling via the PTH1-Rc is differentially regulated by the density of receptors expressed on the cell surface (Schwindinger *et al.,* 1998; Takasu *et al.,* 1999). In LLC-PK1 (porcine renal epithelial cells), maximal AC stimulation is achieved at levels of stably expressed recombinant hPTH1-Rc (≥90,000 Rc/cell) that are much lower than those required for detectable activation of PLC (≥600,000 Rc/cell) (Takasu *et al.,* 1999b). According to Schwindinger and co-workers (1998), the differential coupling efficacy of PTH1-Rc to different G protein α subunits may provide some explanation to the lower response of the PLC pathway. PLC activation was observed in COS-7 cells transiently transfected with PTH1-Rc (Schipani *et al.,* 1995; Schneider *et al.,* 1994), but was barely detected in stably transfected HEK-293 cells (Jobert *et al.,* 1997; Pines *et al.,* 1996; Tong *et al.,* 1996). PTH-(1-34)-induced release of free intracellular Ca^{2+} in parental HEK-293 cells (Jobert *et al.,* 1997) or in cells stably transfected with recombinant PTH1-Rc (Pines *et al.,* 1996; Seuwen and Boddeke, 1995) may involve PTH1-Rc-mediated, PLC-independent PKC activation (Tong *et al.,* 1996), such as stimulation of PLD (Friedman *et al.,* 1999; Singh *et al.,* 1999) or phospholipase A_2 (Ribeiro *et al.,* 1994). Alternatively, receptor subtypes other

than PTH1-Rc may mediate these PKC activities. These putative yet unidentified receptors, for example, may fail to increase cAMP in response to PTH and PTHrP in otherwise AC-competent cells (Atkinson *et al.,* 1987; Orloff *et al.,* 1992, 1995) or may display PTHrP-(1-34)- but not PTH-(1-34)-stimulated release of vasopressin from the rat supraoptic nucleus cells, *in vitro* (Yamamoto *et al.,* 1997).

One interesting proposition is that cell surface density of the PTH1-Rc is a key determinant, independent of ligand concentration, for both AC and PLC signaling pathways in response to PTH-(1-34), with the latter functioning only at high receptor density. It may imply the dissociation between structural determinants in PTH1-Rc involved in agonist recognition and signal transduction and those involved in G protein–effector interaction (Takasu *et al.,* 1999b). The potential physiological relevance of this hypothesis is supported by findings such as the wide range in expression levels of the endogenous PTH1-Rc in different cell systems, e.g., rat calvaria cells and chondrogenically differentiating mouse embryonic carcinoma-derived clonal cells (Bos *et al.,* 1996; Shukunami *et al.,* 1996), and the desensitization/downregulation observed in response to prolonged or repetitive exposure to PTH (Bellorin-Font *et al.,* 1995; Fukayama *et al.,* 1994; Mitchell and Goltzman, 1990; Shukunami *et al.,* 1996).

Downstream Signaling Activities

Mitogen-activated protein kinases (MAPKs) are a group of protein serine and threonine kinases that are important regulators of cell growth and differentiation. Many of the MAPKs are regulated by agonist-activated GPCRs and growth factor receptor tyrosine kinases (RTKs). Several studies have reported that PTH regulates the activity of some members of the MAPK family, such as p44/ERK2, p42/ERK1, and p38, in a cell-specific and G protein type-dependent manner (Chaudhary and Avioli, 1998; Cole, 1999; Sneddon *et al.,* 2001; Verheijen and Defize, 1995, 1997; Zhen *et al.,* 2001). Sneddon and co-workers (2001) observed that PTH activates MAPK in a PKC-dependent manner, causing phosphorylation and activation of extracellular-regulated kinase 2 (ERK2) in both distal and proximal renal tubule cells. In contrast to distal renal tubule cells, where Ca^{2+} transients are ERK2 activation dependent and therefore depend on MAPK activation, the increase in $[Ca^{2+}]_i$ in proximal renal tubule cells is independent of ERK2 phosphorylation (Sneddon *et al.,* 2001). This differential signaling pathway mediated by a common PTH1-Rc results in inhibition of phosphate absorption by the proximal tubules, while stimulating calcium transport by the distal tubules (Sneddon *et al.,* 2001). In OK cells, PTH activation of p42 and p44 MAPKs (ERK1 and ERK2, respectively) is mediated by both PKA and PKC in a time- and concentration-dependent manner (Cole, 1999). MAPK activation is preceded by PTH-induced phosphorylation of the EGFR, suggesting that the latter is involved in PTH signaling (Cole, 1999). PTH regulation of chondrocyte differentiation from prehypertrophic to hypertrophic condrocytes is mediated by PKC inhibiting p38 MAPK, leading to upregulation of Bcl-2, an antiapoptotic molecule, and delayed endochondral ossification (Zhen *et al.,* 2001).

Interestingly, PTH has an inhibitory EGF-induced activation of p42 MAPK in osteoblastic UMR 106 osteosarcoma cells in a PKA-dependent manner (Verheijen and Defize, 1995), and it also inhibits bFGF- and PDGF-induced activation of ERK2 in osteoblastic cells (Chaudhary and Avioli, 1998). At the same time, however, it transiently activates MAPK in CHO-R15 stably transfected with rPTH1-Rc in a PKA-dependent and Ras-independent manner (Verheijen and Defize, 1997). Swarthout and co-workers (2001) reported activation of ERK1 and ERK2 and proliferation of UMR106-01 following continuous treatment with very low concentrations of PTH (10^{-12}–10^{-11} *M*). This proliferation is PKC dependent, but stimulation of MAPKs does not require the activation of small G protein Ras or phosphorylation of epidermal growth factor receptor (EGF). The biphasic effect of PTH on DNA synthesis in OK cells (Cole, 1999) and the opposing effects of PTH on MAPK activity in UMR 106 and CHO-R15 cells (Verheijen and Defize, 1995, 1997) suggest that the effect of cAMP increase on MAPK-induced proliferation and differentiation is cell-signaling dependent. Alternatively, sustained activation of MAPK by PTH-dependent PKC activation will induce proliferation, whereas short-lived activation of MAPK by PTH-dependent PKA activation may result in growth arrest and inhibition of cellular differentiation (Cole, 1999).

PTH regulation of cell growth, proliferation, and differentiation of osteoblasts is due to the modulation of downstream activities (Majeska and Rodan, 1981; Nijweide *et al.,* 1986). PTH affects osteoclasts indirectly through its direct action on osteoblasts (McSheehy and Chambers, 1986). Subcutaneous administration of PTH results in an immediate and transient expression of c-fos mRNA in PTH1-Rc-bearing cells (chondrocytes, osteoblasts, and spindle-shaped stromal cells), followed by a delayed expression in the majority of stromal cells and osteoclasts (Lee *et al.,* 1994b). This observation provides further support for the indirect action of PTH on osteoclasts, which may be mediated by osteoblasts and/or a subpopulation of stromal cells. In UMR cells, PTH rapidly and dose dependently induces transcription of c-fos (Clohisy *et al.,* 1992; Kano *et al.,* 1994). Pearman and colleagues (1996) reported that the cAMP response element (CRE) in the c-fos promoter is required for PTH induction of c-fos in UMR cells and that the CRE-binding protein (CREB) binds to this site, apparently as a homodimer, and is phosphorylated in a PTH-inducible fashion at Ser^{133}. Therefore, c-fos appears to have pleiotropic and essential effects in bone. These include mitogenesis and/or differentiation in the skeletal system, as well as inhibition of osteocalcin expression, which is achieved by binding to the AP-1 site in the osteocalcin promoter and thereby suppressing the mature osteoblast phenotype (Owen *et al.,* 1990). PTH-induced c-fos promoter activity was completely

inhibited in a dose-dependent manner by transfection of a heat-stable inhibitor of PKA (Tyson *et al.,* 1999). This finding provides strong evidence that PKA is the enzyme responsible for the phosphorylation of CREB at Ser^{133} in response to PTH and that PKA activity is required for PTH-induced c-fos expression.

Amling and co-workers (1997) tested the hypothesis that accelerated chondrocyte differentiation in the growth plate of PTHrP knockout mice could be due to an increase in apoptosis. They reported that Bcl-2, a programmed cell death inhibitor, and Bax, a programmed cell death inducer, both members of the Bcl-2 family of proteins whose function involves the regulation of programmed cell death, are expressed in chondrocytes *in vivo.* Both proteins show a characteristic distribution within the developing growth plate. In addition, accelerated endochondrial bone maturation is observed in *bcl-2* knockout mice, whose phenotype resembles that of PTHrP knockout mice (Amizuka *et al.,* 1994). Moreover, using transgenic mice that overexpress PTHrP targeted to chondrocytes, they reported *in vitro* and *in vivo* evidence that Bcl-2 is downstream of PTHrP in a signaling pathway important for normal skeletal development (Amling *et al.,* 1997). Taken together, this study offers a strong link between PTHrP-mediated signaling and apoptosis.

Contrary to numerous reports that have consistently shown PTH treatment to increase the osteoclastogenensis of functional osteoclasts (Cosman *et al.,* 1998; Dempster *et al.,* 1993), Jilka and co-workers (1999) reported that intermittently administered PTH had antiapoptotic activity in mice. The increase in bone formation was accompanied by an increase in the life span of mature osteoblasts. In this study, apoptosis was a rare event, affecting less than 2% of the cells in control experiments. Interesting results were reported by Stanislaus and co-workers (2000), who studied the effect of intermittent PTH treatment on apoptosis in bone cells of the distal metaphysis of young male rats. They observed a 40–60% transient increase in the number of apoptotic osteoblasts in the proliferating zone and apoptotic osteocytes in the terminal trabecular zone within 2–6 days of PTH treatment. This effect subsided to control levels after 21–28 days of treatment. According to Turner and colleagues (2000), PTH-mediated apoptotic activity can also be observed in HEK293 cells stably transfected with oPTH1-Rc. This activity was PKC and caspase dependent. Overexpression of the antiapoptotic protooncogene bcl-2, which acts upstream to caspase 3, did not prevent PTH-induced apoptosis. Taken together, PTH-modulated apoptosis may indeed be one of its downstream signaling activities. Nevertheless, the physiological relevance of apoptosis in osteoblasts and bone metabolism, as well as its role in PTH-induced *in vitro* and *in vivo* activities, remains to be elaborated further.

Signaling-Selective Ligands

The activation of PTH1-Rc evokes dual signaling pathways, increasing both AC/PKA via $G_s\alpha$ and PLC/IP_3-DAG/cytosolic transients of $[Ca^{2+}]_i$/PKC via G_q (Abou-Samra *et al.,* 1992; Bringhurst *et al.,* 1993; Juppner *et al.,* 1991; Lee *et al.,* 1995b; McCuaig *et al.,* 1994; Pines *et al.,* 1994; Schneider *et al.,* 1993; Smith *et al.,* 1996) in homologous as well as heterologous receptor/cell systems. Currently, the relationship between these signaling pathways and the cellular and *in vivo* responses to PTH is not fully established. In general, the role of cellular processes such as receptor inactivation, internalization, trafficking, and recycling in bone metabolism is only beginning to be elucidated. One of the open questions in the development of PTH-based, anabolic anti osteoporotic therapies focuses on understanding the mechanism responsible for catabolic vs anabolic actions of PTH induced by continuous vs intermittent administration of hormone, respectively. The linkage of one or both of these signaling pathways to the anabolic activity of PTH remains to be established.

The development of signaling-selective PTH/PTHrP-derived agonists to dissociate the two major signaling pathways is of great significance for understanding the role of the different pathways in cellular metabolic processes. Studies carried out using osteoblastic cells and organ cultures suggest that PTH residues 1-7 form the cAMP/PKA activation domain (Fujimori *et al.,* 1991), whereas PTH residues 28-34 comprise the PKC activation domain (Jouishomme *et al.,* 1992, 1994). The latter encompasses the region also associated with PTH mitogenic activity in cultured osteoblast-like cells (residues 30-34) (Schluter *et al.,* 1989; Somjen *et al.,* 1990). Cyclic AMP appears to be involved in the bone formation (Rixon *et al.,* 1994) and resorption activities of PTH (Tregear *et al.,* 1973). Although PTH stimulation of bone resorption *in vitro* is mediated primarily through the cAMP-dependent activation of PKA (Kaji *et al.,* 1992), it may not be the sole second messenger pathway involved (Herrmann-Erlee *et al.,* 1988; Lerner *et al.,* 1991). At the same time, stimulation of TE-85 human osteosarcoma cell proliferation by PTH-(1-34) is not associated with an increase in intracellular cAMP (Finkelman *et al.,* 1992). Taken together, PTH analogs that stimulate increases in cAMP levels have been shown to either inhibit (Kano *et al.,* 1991; Reid *et al.,* 1988; Sabatini *et al.,* 1996) or stimulate (McDonald *et al.,* 1986; Sabatini *et al.,* 1996; Van der Plas *et al.,* 1985) osteoblastic cell proliferation, depending on species, cell models used, and experimental conditions.

The search for selective anabolic agents explores N-terminally truncated PTH fragments, e.g., PTH-(3-34) and PTH-(7-34), that selectively activate PKC without affecting cAMP (Chakravarthy *et al.,* 1990; Fujimori *et al.,* 1991, 1992) and are also mitogenic for osteoblastic cells (Somjen *et al.,* 1991). Because these truncated fragments do not stimulate bone resorption (Tregear *et al.,* 1973), they may be more effective "anabolic" agents than peptides with an intact N terminus. Truncation of two amino acids from the N terminus of PTH-(1-34) generates PTH-(3-34), which displays reduced AC activity without significantly affecting PKC activation or the mitogenic response *in vitro* (Fujimori *et al.,* 1992). Therefore, if stimulation of bone formation *in vivo* is related only to the

mitogenic response *in vitro,* the bone formation response should be retained in these amino-truncated PTH fragments that are inactive in stimulating bone resorption *in vitro.* However, N-terminal truncated PTH analogs, such as PTH-(3-34), PTH-(7-34), PTH-(13-34), and PTH(8-84), although capable of stimulating PKC activity, were devoid of any bone anabolic *in vivo* activity (Armamento-Villareal *et al.,* 1997; Hilliker *et al.,* 1996; Jouishomme *et al.,* 1992; Rixon *et al.,* 1994; Schneider *et al.,* 1994; Whitfield *et al.,* 1996; Whitfield and Morley, 1995). Moreover, desamino-hPTH-(1-34), which stimulates PKC as potently as hPTH-(1-34) (Rixon *et al.,* 1994) and is a weak stimulator of AC [at 100 n*M* it has only 40% of maximal AC stimulation by hPTH-(1-34)] (Fujimori *et al.,* 1992; Rixon *et al.,* 1994), is also devoid of any anabolic activity on bone in the OVX rat model (Rixon *et al.,* 1994).

The failure to demonstrate bone-forming activity *in vitro* and bone anabolic effects *in vivo* with PTH analogs in which the capacity to activate AC is severely compromised or completely eliminated turned attention to AC-selective analogs. The AC-selective analog hPTH-(1-31)NH_2 (Ostabolin) is equipotent to PTH-(1-34) in stimulating cAMP production in ROS 17/2 (Jouishomme *et al.,* 1994; Neugebauer *et al.,* 1995) and a potent stimulator of cortical and trabecular bone growth in OVX rats (Armamento-Villareal *et al.,* 1997; Hilliker *et al.,* 1996; Rixon *et al.,* 1994; Whitfield *et al.,* 1996, 2000). By truncating the C terminus up to residue 31, Jouishomme and co-workers (1994) were able to compromise the putative PKC-signaling motif, Gln^{28}-His^{32}, and generate hPTH-(1-31)NH_2, a PKA-selective analog. Whitfield and colleagues (1997) developed *c*[Glu^{22},Lys^{26},Leu^{27}]hPTH (1-31)NH_2, a second-generation PKA signaling-selective analog, in which the helical nature of the C terminus is enhanced by formation of a side-chain to side-chain lactam ring and the introduction of a hydrophobic residue at position 27. The replacement of Lys^{27} with Leu improves the amphiphilicity of the C-terminal helical domain, which interacts with L^{261} in ECL3 of PTH1-Rc (Greenberg *et al.,* 2000; Piserchio *et al.,* 2000a). This analog is only a 1.4- to 2-fold stronger stimulator of femoral trabecular bone formation than the linear parent analog. Both hPTH-(1-31)NH_2 and *c*[Glu^{22},Lys^{26},Leu^{27}]hPTH (1-31)NH_2 have been reported to prevent loss of vertebral and trabecular bone and to raise vertebral and trabecular bone volume and thickness over those of control, vehicle-injected, sham-operated rats (Whitfield *et al.,* 2000). The action of these analogs on vertebral bone was as effective as that of hPTH-(1-34)NH_2. However, unlike hPTH-(1-34)NH_2, their effect on pelvic BMD was equivocal.

Takasu and co-workers (1999a) offered an alternative view regarding the structural determinants associated with signaling pathway activation. They showed that replacement of Glu^{19}→Arg, a receptor-binding and affinity-enhancing modification, generates [Arg^{19}]PTH-(1-28), which is a potent and full stimulator of AC and PKC. Interestingly, they find that substitution of Ala^1 for Gly generates [Gly^1,Arg^{19}]hPTH-(1-28), which is a PKA-selective agonist (Takasu *et al.,* 1999a). This study concludes that the extreme N terminus of hPTH constitutes a critical activation domain for coupling to PLC. The C-terminal region, especially hPTH-(28-31), contributes to PLC activation through receptor binding, but this domain is not required for full PLC activation. Therefore, they suggest that the N-terminal determinants for AC and PLC activation in hPTH(1-34) overlap but are not identical and that subtle modifications in this region may dissociate activation of these two effectors.

In the course of designing photoreactive PTHrP analogs for mapping the bimolecular ligand–receptor interface, Behar and co-workers (2000) generated [Bpa^1,Ile^5,$Arg^{11,13}$,Try^{36}] PTHrP(1-36)NH_2. This analog binds and stimulates AC equipotently to the parent analog [Ile^5,$Arg^{11,13}$,Try^{36}]PTHrP (1-36)NH_2 in HEK-293/C-21 cells overexpressing the recombinant human PTH1-Rc (~ 400,000 receptors/cell), but does not elicit intracellular calcium transients. Moreover, it does not stimulate the translocation of β-arrestin2–GFP fusion protein, an effect that is PKC dependent (Ferrari *et al.,* 1999). It will be very interesting to test this PKA-selective analog for its anabolic activity in animal models of osteoporosis.

Target-Specific Ligands

The wide distribution of PTH1-Rc in tissues other than bone and kidney (Urena *et al.,* 1993), the classical target tissues for PTH, and its physiological activation by locally secreted PTHrP (Philbrick *et al.,* 1996) raise concerns about potential side effects following parenteral administration of therapeutic doses of exogenous PTH. Bone-selective PTH/PTHrP-derived analogs may reduce the activation of PTH1-Rc in kidney and nonosseous tissues and provide better bone anabolic drugs.

To this end, Cohen and co-workers reported that [His^3]- and [Leu^3]hPTH-(1-34) are partial agonists of AC in a kidney cell line (50 and 20%, respectively), but full agonists in UMR-106 rat osteosarcoma cells (Cohen *et al.,* 1991; Lane *et al.,* 1996). *In vivo,* however, both analogs were less potent than PTH in the induction of bone formation.

In summary, the development of an effective and safe therapeutic modality that would stimulate the formation of new, mechanically competent bone and possibly reconstitute trabecular architecture in osteoporotic patients continues to be a worthy goal. This goal may be approached by analogs that interact with the PTH1-Rc in a signaling-selective manner or are targeted specifically to bone to achieve a more favorable therapeutic window.

Putative Bioactive Conformation

Elucidation of the bioactive conformation of peptide ligands, namely, the conformation that is recognized by, binds to, and activates the cognate GPCR, is a major objective in structural biology. The ligand–receptor complex is the definitive system for studying the putative bioactive conformation. Unfortunately, for GPCRs, this is currently

an unattainable goal, as no hormone–GPCR complex has been crystallized, probably because of its size and its location as an integral part of the cell membrane. Inooka and co-workers (2001) reported the first nuclear magnetic resonance (NMR)-based structure of GPCR-bound ligand. They analyzed the conformation of the peptide ligand PACAP-(1-21)NH_2 bound to its cognate GPCR. Because recognition, binding, and signal transduction are carried out by membrane-embedded GPCRs, Schwyzer (1991, 1992, 1995) hypothesized that the initial conformations adapted by a ligand are induced by nonspecific interactions with the membrane. Only some of these membrane-induced conformations are recognized by the membrane-embedded GPCR. Therefore, the study of conformations in the presence of membrane mimetic milieu, like the micellar environment, is probably the best available approximation of the natural state.

Secondary structure prediction methods suggest that the N-terminal 1-34 sequences of both PTH and PTHrP assume helical structures at their N- and C-terminal domains (Chorev *et al.,* 1990; Cohen *et al.,* 1991; Epand *et al.,* 1985). These helical sequences span residues 1-9 and 17-31 in PTH and 1-11 and 21-34 in PTHrP (Cohen *et al.,* 1991). A good correlation between receptor-binding affinity and the extent of helicity was established by circular dichroism (CD), a method spectroscopic that can assess the average conformation of a peptide (Neugebauer *et al.,* 1995). Assessment of helical content by CD estimated PTH-(1-34) to have on the average fewer than 8 residues in helical conformation, which is lower than that estimated for PTHrP-(1-34) (Cohen *et al.,* 1991; Epand *et al.,* 1985; Neugebauer *et al.,* 1992; Willis and Szabo, 1992; Zull *et al.,* 1990). In the presence of 45% trifluoroethanol (TFE), a solvent that promotes secondary structure, the total helical content of bPTH-(1-34) and hPTHrP-(1-34) is about 73% (Cohen *et al.,* 1991). Nevertheless, the relevance of the conformation in TFE to the bioactive conformations is still debated.

According to ^{1}H-NMR studies in water, the structure of PTH-(1-34) is mostly random, except for a short ordered region encompassing residues 20-24 (Bundi *et al.,* 1976, 1978; Lee and Russell, 1989). Pellegrini and co-workers (1998b) reported that hPTH-(1-34) in water is highly flexible, with some evidence of transient helical loops spanning the sequences 21-26 and 7-8. This CD and NMR study was carried out in aqueous solutions, with variable pHs and salt concentrations, and in dodecylphosphocholine (DPC) micelles. The subsequent distance geometry calculations, generated conformations that were refined by molecular dynamic simulations explicitly incorporating solvent (H_2O) (Pellegrini *et al.,* 1998b). This study generated high-resolution conformational preferences of hPTH-(1-34), which were later used in the construction of an experimentally based model of the hormone–receptor complex. Both in aqueous solution and in the presence of DPC micelles, Pellegrini and co-workers (1998b) observed fast conformational averaging on the NMR time scale. As anticipated, the two helical domains observed in aqueous solution — the N-terminal helix, comprising residues 6-14, and the C-terminal helix, comprising residues 19-23 — are extended (4-17 and 21-33, respectively) and stabilized in the presence of DPC micelles. A region of flexibility, which is centered around residues 15-16 in aqueous solutions and around residues 18-19 in the micellar system, separates both helices (Fig. 3). Therefore, in solution, the two helical domains adopt a range of different spatial orientations, none of which corresponds to a tertiary structure in which helix–helix interactions can be observed (Pellegrini *et al.,* 1998b). This observation is in complete accord with conformational studies of lactam-containing PTHrP analogs (Mierke *et al.,* 1997), point-mutated and segment PTH-PTHrP hybrids (Peggion *et al.,* 1999; Schievano, 2000), and a model amphiphilic α-helix-containing PTHrP analog (Pellegrini *et al.,* 1997a).

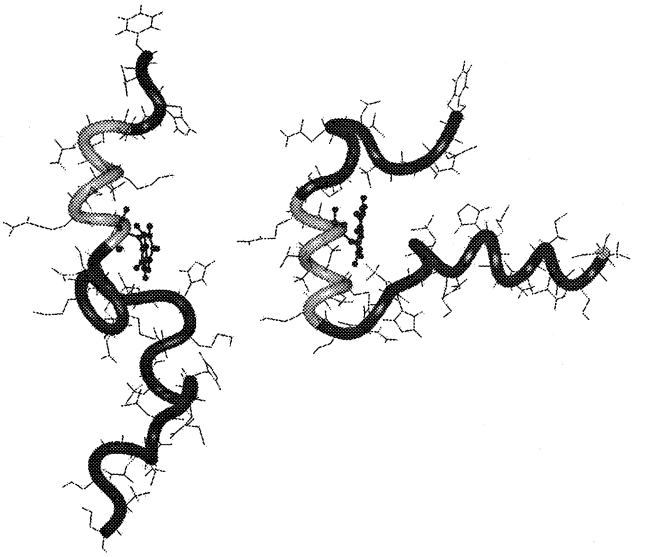

Figure 3 Ribbon diagram of two conformations of hPTH-(1-34) resulting from ensemble-based calculations. Averaging over these two-member ensembles fulfills all of the CD- and NMR-based experimental observations. The different locations and extends of the α helices are highlighted in gray; the side chain of Trp^{23}, used to align the conformations, is shown as a ball-and-stick structure (Pellegrini *et al.,* 1998b).

Weidler and co-workers (1999) studied PTHrP-(1-34) by CD and NMR in what they define as near physiological solution (50 m*M* potassium phosphate, pH 5.1, 250 m*M* NaCl). According to their studies, PTHrP-(1-34) contains two helical domains, His^5-Leu^8 and Glu^{16}-Leu^{27}, which are connected by a flexible linker (Weidler *et al.,* 1999). Similar to Pellegrini and co-workers (1998b), who studied PTH-(1-34), they also could not detect any tertiary structure in PTHrP-(1-34).

The more hydrophobic and amphiphilic C-terminal sequence in PTHrP has a higher propensity for forming a helix than the N-terminal domain, as demonstrated by the higher percentage of TFE needed for nucleation of an N-terminal helix (Mierke *et al.,* 1997). Similar conclusions were reached by adding lipids (Epand *et al.,* 1985; Neugebauer *et al.,* 1992; Willis, 1994) or DPC micelles (Pellegrini

et al., 1997a, 1998b) to PTH- and PTHrP-derived sequences. Taken together, the conformational analyses allow us to postulate a dynamic model for ligand–receptor binding. Binding is initiated by complementary hydrophobic interactions between the hydrophobic face of the amphiphilic C-terminal helical domain of the ligand, including the principal binding domain, and the hydrophobic membrane. This hydrophobic ligand–membrane interaction allows the propagation of the C-terminal helix and the formation of specific interactions with extracellular portions of the PTH receptor. In the membrane environment, nucleation of the N-terminal helix occurs either cooperatively with (in the antagonist) or independently of (in the agonist) the previously formed C-terminal helical domain. Consequently, in the case of PTH/PTHrP agonists, the flexibility around hinges 12-13 and 19-20 allows the membrane-induced "message domain" to be positioned correctly within the receptor. Specific message–receptor interactions leading to the conformational changes required for signal transduction can thus occur. In the case of PTH/PTHrP antagonists, which lack most of the "message" sequence, no conformational change in the receptor occurs and no signal transduction event is triggered. Absence of the critical hinge around positions 19-20, or a shift in register of the hinge region, results in reduced binding affinity and efficacy, as observed for the cyclic PTHrP analogs (Mierke *et al.,* 1997) and point-mutated PTH/PTHrP hybrids (Peggion *et al.,* 1999). This proposed dynamic model for ligand–membrane–receptor interaction provides a testable paradigm for future experiments.

A much debated issue is whether PTH and PTHrP fold into a tertiary structure, in which secondary structural elements interact specifically with each other to form a more stable and higher ordered structure. Cohen and co-workers (1991) suggested that in TFE, the amphiphilic helices located at the N and C termini of bPTH-(1-34) and hPTHrP-(1-34) interact to form a U-shaped tertiary structure, with the hydrophobic residues facing inward to form a hydrophobic core. As a result, the hydrophilic residues are oriented outward, exposing them to the polar solvent (Cohen *et al.,* 1991). However, in light of the lack of compelling spectroscopic evidence for long-range interactions between the two N- and C-terminal helices in both hPTH-(1-34) and PTHrP-(1-34) (Klaus *et al.,* 1991; Strickland *et al.,* 1993; Wray *et al.,* 1994), the notion of a U-shaped tertiary structure remains unsupported. Interestingly, Gronwald and co-workers (1996) reported that in aqueous TFE, the long-range proton–proton correlations (Val^2-to-Trp^{23} and Ile^5-to-Asn^{10}) between the two N-terminal helices (sequences 1-10 and 17-27) in the full-length, recombinant hPTH are dependent on interactions provided by residues in the middle and C-terminal portion of the molecule (sequences 30-37 and 57-62, respectively). They cautiously suggested that the molecule shows a tendency toward tertiary structure. It should be noted that in TFE, the low dielectric constant, which helps stabilize helices, is also supposed to shield the side chains from hydrophobic interactions between the helices and, therefore, destabilizes alleged U-shaped tertiary structures.

Marx and co-workers (1995) suggested that hPTH-(1-37) assumes a U-shaped structure in aqueous solution containing a high salt concentration. However, their reported long-range, proton–proton correlations are limited to side chains of Leu^{15} and Trp^{23} located close to the bend forming the putative U-shaped structure, leaving, therefore, too much flexibility to define a stable U-shaped structure. Others have also assigned a tertiary folded structure to PTH-(1-39), PTH-(1-34), and the osteogenic PTH sequence PTH-(1-31) (Chen *et al.,* 2000; Marx *et al.,* 2000). The same researchers identify a loop region around His^{14}-Ser^{17}, stabilized by hydrophobic interactions, and long-range proton–proton correlations between Leu^{15} and Trp^{23}, which are also found in hPTH-(1-37) and in N-truncated analogs hPTH-(2-37), -(3-47), and -(4-37) (Marx *et al.,* 1998).

Other studies of PTHrP analogs describe interactions between N- and C-terminal helical domains, in the presence of TFE, thus offering support for the U-shaped structure (Barden and Kemp, 1989, 1994, 1995, 1996; Barden *et al.,* 1997). Barden and Kemp mentioned the presence of a hinge at Arg^{19}-Arg^{20} in [Ala^9]PTHrP-(1-34)NH_2 and attributed to it a functional role in signal transduction. They also postulate long-range interactions between side chains located on both sides of the turn, Gln^{16}-Arg^{19}, implicating the presence of a tertiary structure (Barden and Kemp, 1996). The question of tertiary structure in PTHrP-(1-34) was also addressed by Gronwald and co-workers (1997), who studied it in water and in 50% TFE. In the presence of TFE, they observed two stable α-helical regions spanning residues 3 to 12 and 17 to 33, which are connected by a flexible linker. Based on their CD and NMR study, Gronwald and co-workers (1997) concluded that there is no evidence of a stable tertiary structure in PTHrP-(1-34). Taken together, the current and prevailing view is that both PTH- and PTHrP-derived linear peptides in solution do not form an appreciable component of stable tertiary structure. This understanding runs counter to the notion of a folded or U-shaped structure as the predominant bioactive conformation.

The lack of tertiary structure in PTHrP-(1-34) in either aqueous solutions or in the presence of TFE is not limited to the linear parent peptide (Barbier *et al.,* 2000; Maretto *et al.,* 1997; Mierke *et al.,* 1997). Similar conformational behavior was observed in a series of side-chain to side-chain-bridged mono- and bicyclic lactam-containing PTHrP analogs. These conformationally constrained analogs are obtained through cyclization of side-chain pairs, Asp^{13} to Lys^{17}, Asp^{22} to Lys^{26}, Lys^{25} (replacing Arg) to Glu^{29}, and Lys^{26} to Asp^{30}, located at the putative N- and C-terminal helical domains (Barbier *et al.,* 2000; Bisello *et al.,* 1997; Chorev *et al.,* 1991, 1993; Maretto *et al.,* 1997, 1998; Mierke *et al.,* 1997). This i-to-i + 4 side-chain to side-chain cyclization is known to stabilize helical structures in other peptide systems. Bioactivity in the agonist (1-34) and antagonist (7-34) series of lactam-containing analogs requires well-defined N- and C-helical domains that are linked by two flexible hinges located around residues 12-13 and 19-20 (Maretto *et al.,* 1997; Mierke *et al.,* 1997).

Two separate studies suggested that the bioactive conformation of PTH forms an extended helix (Condon *et al.,* 2000; Jin *et al.,* 2000). In a CD study, Condon and co-workers (2000) analyzed a series of lactam-containing PTH-(1-31) analogs that include the tricyclo (Lys^{13}Asp^{17},Lys^{18}Asp^{22},Lys^{26}Asp^{30})-[Ala^{1},Nle^{8},Lys^{18}, Asp^{17,22}, Leu^{27}]hPTH-(1-31)NH_2, a highly potent (EC_{50} = 0.14 n*M*) analog. This analog forms an extended helix-spanning residues 13-30 in aqueous solution and is fully helical in 40% TFE. This tricyclic analog includes a lactam bridge, Lys^{18}-to-Asp^{22}, which engulfs Arg^{19}, a putative hinge site in the bioactive conformation. They concluded, therefore, that PTH binds to its cognate receptor in an extended helical conformation (Fig. 4, see also color plate). A similar conclusion was reached by Jin and co-workers (2000), who reported the crystal structure of hPTH-(1-34). The PTH crystallized in a slightly bent (residues 12-21), long helical antiparallel dimer (Fig. 5, see also color plate). In general, solid-phase structures of short peptides can be very much affected by the intermolecular packing forces stabilizing the crystal structure. In particular, formation of the dimer, as in the PTH crystal, can override other intramolecular interactions. In addition, there is no resemblance between the crystal environment and a membrane-mimicking milieu in which the bimolecular ligand–GPCR interaction takes place. We do not think that these studies contradict previous findings obtained with both PTH and PTHrP in solution, or with analogs more flexible than the tricyclic one. Conformations of the tricyclic analog of PTH in solution or PTH-(1-34) in the solid state may represent only a fraction of the ensemble of fast equilibrating conformations that can generate the putative bioactive conformation. At any rate, the tricyclic analog may retain sufficient structural flexibility to allow optimal interaction with the receptor.

Piserchio and colleagues (2000b) studied bTIP39, the recently identified endogenous ligand for PTH2-Rc, using both high-resolution NMR and CD in the presence of DPC micelles and computer simulation in a water/decane simulation cell. They reported a molecular architecture consisting of two stable α helices, Ala^{5}-Leu^{20} and Leu^{27}-Val^{35}, separated by an unstructured region. This architecture is reminiscent of the structure of PTH-(1-34), with which TIP39 shares only limited sequence homology (Fig. 1). The N-terminal helix in TIP39, important for activation of PTH2-Rc, has a spatial distribution of polar and hydrophobic amino acid residues almost identical to PTH, making it only moderately amphiphilic. Interestingly, in molecular modeling, Asp^{7} in TIP39 is homologous to Ile^{5} in PTH and His^{5} in PTHrP, both of which are crucial for ligand selectivity toward the PTH2-Rc (Behar *et al.,* 1996a; Gardella *et al.,* 1996a). Thus, the putative steric and repulsive interaction between His^{5} in PTHrP and H^{384} in the bottom of the narrow binding pocket of PTH2-Rc, which is responsible for this receptor–ligand selectivity (Rolz *et al.,* 1999), is replaced by an attractive Coulombic interaction and smaller side chain presented by Asp^{7} in TIP39. This complementarity may favorably accommodate the interaction between TIP39 and its cognate PTH2-Rc. Compared to PTH, the N-terminal helix in TIP39 is longer by five residues (Leu^{16}-Leu-Ala-Ala-Leu^{20}), causing a shift in the relative location of the flexible region 21-26. The C-terminal helices in TIP39 and PTH-(1-34) are only slightly amphiphilic and share a lower degree of homology than the one observed for N-terminal helices. Some unique structural features related to the C-terminal portion of TIP39 may explain its weaker

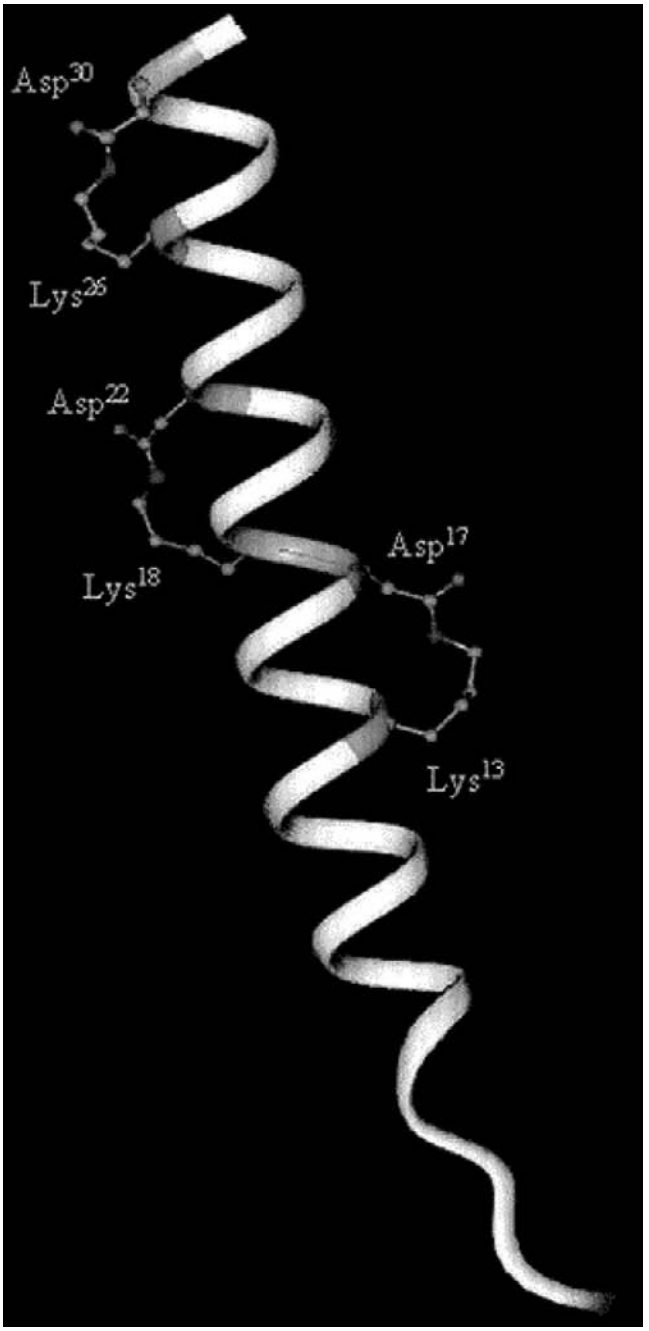

Figure 4 Schematic representation of the tricyclo (Lys^{13}Asp^{30}, Lys^{18}Asp^{22},Lys^{26}Asp^{30})-[Ala^{1},Nle^{8},Lys^{18},Asp^{17,22},Leu^{27}]hPTH-(1–31) NH_2. The peptide backbone is shown as a white ribbon. The three lactam bridges are indicated in orange (Condon *et al.,* 2000). (See also color plate.)

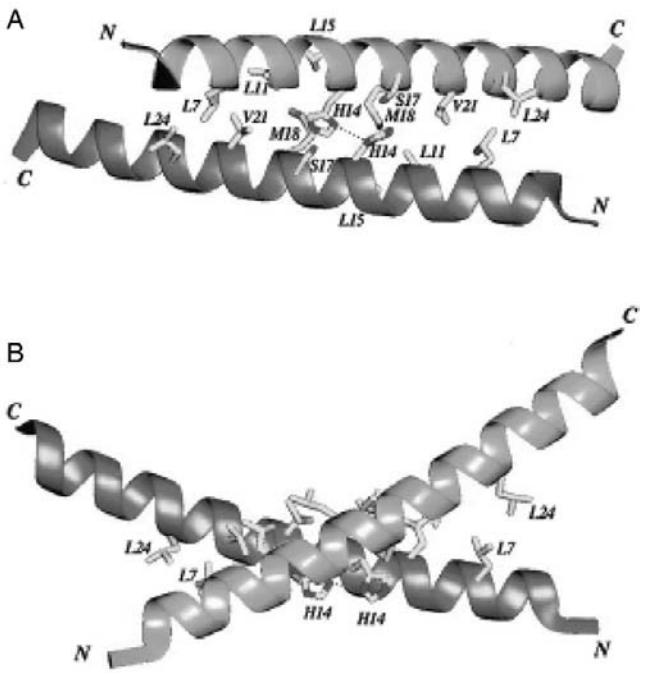

Figure 5 Overall structure of hPTH-(1-34) in the solid state. The monomeric chain is a slightly bent helix presented as green ribbons. At the crossing point of the amphiphilic helices in the dimer, His^{14} from each chain forms a hydrogen bond shown as a dotted line. The dimer interface is mainly hydrophobic. A and B are two different views of the dimer (Jin *et al.,* 2000). (See also color plate.)

interaction with the N-ECD of PTH2-Rc, and therefore its diminished role in recognition and binding (Hoare *et al.,* 2000). Compared to the C-terminal helix in PTH-(1-34), the amphiphilicity of the C-terminal helix of TIP39 is compromised by two factors: the presence of charged residues within the hydrophobic face and the location of Trp^{25} in the unordered region separating the N- and C-helices instead of within the C-terminal helix, as Trp^{23} is in PTH. These experimental and computational results reiterate the significance and relevance of studying structurally related, but functionally distinct ligand–receptor systems to the PTH/PTHrP–PTH1-Rc system.

Conformation-Based Design of PTH/PTHrP Analogs

Enhancing receptor-favored conformations by restricting conformational freedom in a local or global manner may preclude a wide range of nonproductive conformations and will result in more potent analogs. Indeed, one of the goals of searching for the bioactive conformation is to identify structural elements essential for bioactivity and devise ways to stabilize them. Furthermore, enhancing of complementary topological features in the ligand–receptor interface may also contribute to increased potency. For example, enhancement of the amphiphilicity of a helical segment may stabilize a favored bimolecular interaction and lead to a more productive receptor interaction. To this end, several studies have incorporated structural modifications that stabilize an element important in the putative bioactive conformation (Barbier *et al.,* 1997; Bisello *et al.,* 1997; Chorev *et al.,* 1991, 1993; Leaffer *et al.,* 1995; Mierke *et al.,* 1997; Surewicz *et al.,* 1999).

Much attention has been drawn to the amphiphilic nature of the C-terminal helix comprising residues 20-34 of hPTH-(1-34) and its role in receptor binding (Gardella *et al.,* 1993; Neugebauer *et al.,* 1992). A Lys27Leu substitution in PTH-(1-34)NH_2 and PTH-(1-31)NH_2 improved the amphiphilic character of the C-terminal helical sequence and increased AC activity over the corresponding nonsubstituted sequences (Barbier *et al.,* 1997; Surewicz *et al.,* 1999).

Vickery and co-workers (1996) reported a more extensive enhancement of the amphiphilicity of the C-terminal helix of PTHrP. Substitution in PTHrP-(1-34)NH_2 of the sequence 22-31 with a model amphipathic peptide (MAP; Glu^1-Leu-Leu-Glu-Lys-Leu-Leu-Glu-Lys-Leu-Lys^{10}), which is highly α helical when incorporated into short peptides (Krstenansky *et al.,* 1989), generates [$(MAP_{1\text{-}10})^{22\text{-}31}$] hPTHrP-(1-34)$NH_2$ (RS-66271). In this analog, important structural features, such as $Leu^{24 \text{ and } 27}$, are maintained, and $Ile^{22 \text{ and } 31}$ are substituted conservatively by Leu. In aqueous buffer, RS-66271 displays 8- to 9-fold higher helicity than the parent peptide. A detailed conformational analysis of RS-66271 in water, employing CD and ^1H NMR spectroscopy, confirmed the presence of an extensive helical structure encompassing residues 16-32 (Pellegrini *et al.,* 1997a). Nevertheless, the absence of a hinge element around Arg^{19}, considered to contribute to high biological activity, may explain the 6-fold lower AC activity and 10-fold lower binding affinity in ROS17/2.8 cells of RS-66271, compared to the more flexible and less helical PTHrP-(1-34) (Krstenansky *et al.,* 1989, 1994). Importantly, the preservation of significant *in vitro* potency, despite the multitude of substitutions, validates the rationale behind the design of RS-66271.

The discrepancy between the low binding affinity of RS-66271 compared to that of PTH-(1-34), and its PTH-(1-34)-like *in vivo* anabolic activity and *in vitro* activity measured by bone resorption, cAMP accumulation, and inositol phosphate assays, is very intriguing (Frolik *et al.,* 1999; Krstenansky *et al.,* 1994; Vickery *et al.,* 1996). According to Usdin and co-workers, RS-66271 binds selectively to the G protein-coupled PTH1-Rc with high affinity. Therefore, if the PTH1-Rc population in the intact cell is predominantly uncoupled from G protein, the binding affinity of RS-66271 will be markedly low (Hoare *et al.,* 1999b; Hoare and Usdin, 1999, 2000). Using a membrane-based binding assay, they demonstrated that hPTH-(1-34) binds with high affinity ($IC_{50} < 10$ n*M*) to the PTH1-Rc, whether or not it is coupled to G protein (Hoare *et al.,* 1999b). However, RS-66271 binds with high affinity ($IC_{50} = 16$ p*M*) to the coupled receptor, but with much lower affinity ($IC_{50} > 100$ n*M*) to the uncoupled receptor (Hoare and Usdin, 1999). Interestingly, His^5 in RS-66271, which has been implicated previously in specifying the signaling and binding of PTHrP to PTH1-Rc but not to PTH2-Rc (Behar *et al.,* 1996a; Gardella *et al.,* 1996a), is also

implicated as a determinant in G protein-coupled- vs-uncoupled PTH1-Rc selectivity (Hoare *et al.,* 2001). Replacement of His^5 in RS-66271 with Ile reduced selectivity toward the high-affinity G protein-coupled PTH1-Rc by 17-fold and increased the affinity to the uncoupled receptor by 160-fold. The ability of [Ile^5]RS-66271 to restore AC activation in N-ECD-truncated PTH1-Rc, in which RS-66271 failed to stimulate cAMP production, suggests that the residue in position 5 affects receptor selectivity through interactions with the ECLs and the ectopic portions of the TMs (Hoare *et al.,* 2001).

It is generally accepted that PTH-(1-34) and PTHrP-(1-34) contain two helical domains spanning sequences 13-18 and 20-34 (Barden and Cuthbertson, 1993; Barden and Kemp, 1993; Strickland *et al.,* 1993; Wray *et al.,* 1994). Introduction of side-chain to side-chain cyclizations via lactam bond formation between residues that are 4 amino acids apart and located across a single helical pitch (residue i-to-residue i+4) has been demonstrated to be an effective way to stabilize a helical structure (Bouvier and Taylor, 1992; Danho *et al.,* 1991; Felix *et al.,* 1988a,b; Madison *et al.,* 1990). Therefore, we undertook replacement of a potential ion pair participating in α-helical stabilization by a covalent lactam bridge in an attempt to further stabilize the helices in these regions. The initial application of this approach generated *c*[Lys^{13}-Asp^{17}]PTHrP-(7-34)NH_2, which was about 10-fold more potent than the linear parent antagonist (K_b = 18 and 170 n*M* K_i = 17 and 80 n*M,* respectively, in Saos2/B10 cells) (Chorev *et al.,* 1991). Rigidification of the C-terminal helix in *c*[Lys^{26}-Asp^{30}]PTHrP-(7-34)NH_2 did not improve antagonist potency (Bisello *et al.,* 1997), but a combination of two 20-membered lactam bridges, in both N- and C-terminal helices, generated *c*[Lys^{13}-Asp^{17},Lys^{26}-Asp^{30}]PTHrP(7-34)NH_2, a potent (K_b = 95 n*M* and K_i = 130 n*M* in Saos2/B10 cells) (Bisello *et al.,* 1997), highly conformationally constrained, PTHrP-derived antagonist, and a valuable tool for conformational studies (Maretto *et al.,* 1997).

The same approach applied to the agonist PTHrP-(1-34)NH_2 yielded the mono- and bicyclic analogs *c*[Lys^{13}-Asp^{17}]PTHrP-(1-34)NH_2 and *c*[Lys^{13}-Asp^{17},Lys^{26}-Asp^{30}]PTHrP-(1-34)NH_2, which were equipotent to the linear parent compound (K_b = 3.2, 2.1 and 1 n*M,* K_m = 0.17, 0.22, and 0.57 n*M,* respectively, in Saos2/B10 cells) (Bisello *et al.,* 1997). A similar approach was also applied to signaling-selective analogs, hPTH-(1-31)NH_2, and the more potent [Leu^{27}]hPTH-(1-31)NH_2. Both of these analogs stimulate the AC, but not the PLC/PKC signaling pathway (Barbier *et al.,* 1997). Whereas i-to-i+4 lactam bridge formation between Glu^{22} and Lys^{26}, as in *c*[Glu^{22}-Lys^{26},Leu^{27}]hPTH-(1-31)NH_2, results in about a four fold increase in AC activity compared to the linear parent peptide (EC_{50} = 3.3 and 11.5 n*M,* respectively, in ROS 17/2 cells), similar cyclization between Lys^{26} and Asp^{30} or i-to-i+3 lactam bridge formation between Lys^{27} and Asp^{30} results in cyclic analogs less potent than the corresponding linear parent peptides (Barbier *et al.,* 1997).

Interestingly, the higher AC activity *in vitro* observed for *c*[Glu^{22}-Lys^{26},Leu^{27}]hPTH-(1-31)NH_2 compared to the linear peptide results in a higher anabolic effect on trabecular bone growth in ovariectomized rats (Whitfield *et al.,* 1997) and more effective protection than hPTH-(1-34) affords against loss of femoral trabeculae in the same animal model (Whitfield *et al.,* 1998).

The retention of full PKC activity (in ROS 17/2 cells) by the extensively N-terminally truncated linear fragment [Lys^{27}]hPTH-(20-34)NH_2 and the structurally related lactam-bridged analog *c*[Lys^{26}-Asp^{30}]hPTH-(20-34)NH_2 was consistent with the stabilization of the amphiphilic helix at the C terminus and implicated the helix as an important functional motif for binding to the PTH1-Rc (Neugebauer *et al.,* 1994). Taken together, these studies provide important insights regarding the structural nature of the hormones PTH-(1-34) and PTHrP-(1-34) and help to better characterize conformational features important for PTH binding and bioactivity.

PTH Receptors

The physiological and pathophysiological activities of PTH and PTHrP are mediated predominantly by PTH1-Rc. PTH1-Rc is encoded by a single-copy gene expressed primarily in kidney, intestine, and bone, the target tissues for PTH, and the PTH–PTH1-Rc interaction is essential for maintaining mineral ion homeostasis (Schipani *et al.,* 1993). The discovery of receptors for PTH and PTHrP, their functional properties, and biological importance are summarized in some excellent reviews (Juppner, 1995, 1999; Mannstadt *et al.,* 1999).

The expanding pharmacological evidence for actions of PTH on targets other than bone and kidney and the divergence of signaling pathways suggest the presence of a distinct subfamily of cognate receptors for PTH, PTHrP, and other related peptides. Homology-based screen and the exploration of molecular evolution of PTH receptors in various species yielded cDNAs encoding three distinct PTH receptor subtypes (Fig. 6) (Rubin and Juppner, 1999). The potential role of these new receptors in physiological processes and in disease, and their usefulness in drug screening and design drive the search for novel receptor subtypes and their cognate ligands.

PTH1 Receptor

The ~87-kDa N-glycosylated PTH1-Rc is a member of the class II hepta-helical transmembrane domain G protein-coupled receptors. Class II comprises receptors recognizing peptide hormones ranging in size from 27 to 173 amino acid residues and it includes receptors for secretin, glucagon, calcitonin, growth hormone-releasing hormone, corticotropin-releasing hormone, vasoactive intestinal peptide, pituitary AC-activating peptide, gastric inhibitory peptide, and glucagon-like peptide 1 (Juppner, 1994, 1995; Segre and Goldring, 1993). The putative hepta-helical structure, which defines the extracellular, TM, and cytoplasmic

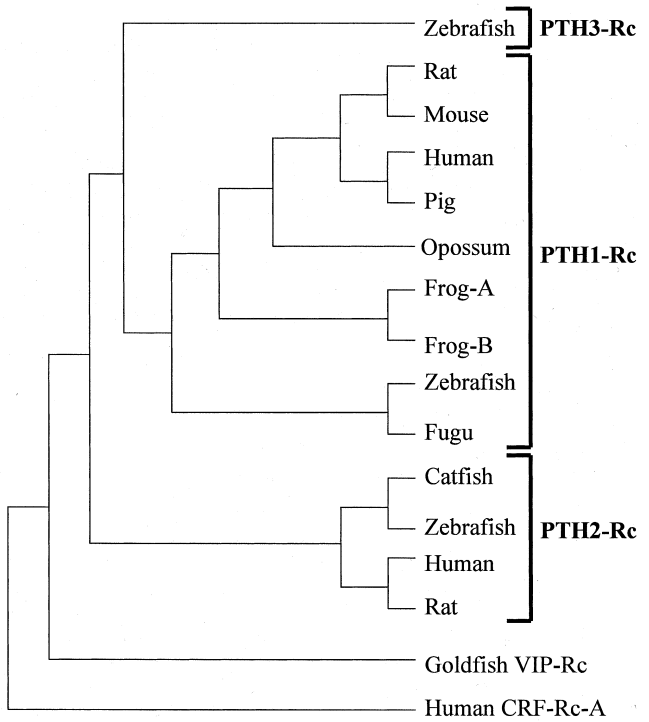

Figure 6 A phylogenic dendogram of PTH receptor subtypes. Analysis was reported by Rubin and Juppner (1999). VIP-Rc, vasoactive intestinal polypeptide receptor; CRF-Rc-A, corticotropin-releasing factor receptor A

domains, was derived from structural homology studies (Abou-Samra *et al.,* 1992; Juppner *et al.,* 1991) and confirmed by epitope tag mapping of the extracellular and cytoplasmic domains (Xie and Abou-Samra, 1998). Class II GPCRs have no significant sequence identity (<12%) with other GPCRs. In addition, only 50 amino acid residues are strictly conserved, indicating their early emergence in evolution. The conserved residues are located predominantly in the N-ECD and the TM domains and must play an important structural and functional role.

The distinct features of the class II GPCRs include an N-ECD of ~160 residues, which is intermediate in length between those of the glycoprotein hormone (~400 residues) and aminergic receptors (~35 residues); a highly conserved pattern of 8 cysteines (6 in the N-ECD, 1 in the ECL1, and 1 in the ECL2); and multiple potential N-glycosylation sites located within the N-ECD.

PTH1-Rc homologues from different mammalian species, such as rat, opossum, pig, human, and mouse, are most divergent within the N-ECD, the ECL1, and the carboxyl-terminal intracellular tail (Abou-Samra *et al.,* 1992; Juppner *et al.,* 1991; Kong *et al.,* 1994; Schipani *et al.,* 1993; Smith *et al.,* 1996). Evidently, the divergence in the response of different PTH1-Rc homologues to truncated sequences and analogs of PTH and PTHrP is instrumental in defining important functional domains within the PTH1-Rc. For example, despite their 91% homology and similar apparent dissociation constant of ~10 n*M* for PTH-(1-34), rat and human PTH1-Rc have very different affinities for the antagonist PTH-(7-34) (K_d = 14 and 4385 n*M,* respectively) (Juppner *et al.,* 1994).

Studying the structure–function relationship of the PTH1-Rc is an important indirect approach to gaining insight into the ligand–receptor bimolecular recognition process and the signal transduction mechanisms. This approach, however, focuses on only one component of the hormone–receptor complex, the receptor, and therefore it is "blind" to the structural information of the ligand. In addition, many of these point mutated, truncated, and hybrid receptors can be affected by long-range structural consequences that are removed from the site of modification, thus making the interpretation of stucture–function relationships quite difficult.

PTH Receptor Subtypes

Two novel PTH receptor subtypes, PTH2- and PTH3-Rc, have been identified (Rubin and Juppner, 1999; Usdin *et al.,* 1995). Their designation as PTH receptor subtypes is based on their high level of sequence homology with PTH1-Rc, their interaction with PTH- and/or PTHrP-derived peptides, and their capacity to stimulate both PKA- and PKC-dependent signaling pathways. However, this designation does not reflect any physiological or functional relationship to the PTH/PTHrP–PTH1-Rc system. Therefore, they are very valuable as natural structure–function experiments that can provide insights into ligand–receptor bimolecular interactions.

PTH2 Receptor Subtype and TIP39

PTH2-Rc, which has been cloned from rat and human cDNA libraries, selectively binds PTH, but not PTHrP (Usdin *et al.,* 1995, 1999). *In situ* hybridization studies have identified a high level of expression of PTH2-Rc in brain and lower levels in the exocrine pancreas, epididymis, artrial and cardiac endothelium, vascular smooth muscle, lung, placenta, and vascular pole of renal glomeruli (Usdin *et al.,* 1995, 1999a). However, little is known about its physiological role in these tissues. While the tissue distribution, and particularly the lack of PTH2-Rc expression in kidney and bone, suggests a limited physiologic role in mineral metabolism, the distinct ligand specificity of PTH2-Rc has provided insight into the current model of PTH ligand–receptor interactions.

Usdin *et al.* (1999b) isolated a novel peptide from bovine hypothalamus, tuberofundibular peptide 39 (TIP39), whose binding affinity and stimulating activity for the PTH2-Rc are similar to those of PTH (Usdin *et al.,* 1995, 1999). In contrast to PTH, TIP39 does not appreciably activate AC in COS-7 cells transiently transfected with either human or rat PTH1-Rc (Usdin *et al.,* 1999b), but instead binds to them with moderate affinity (Hoare *et al.,* 2000). A homology search reveals that 9 out of the 39 residues of TIP39 are

identical to bPTH, and most of them are located in the midregion of the molecule. The physiological role of the TIP39–PTH2-Rc system remains to be established.

Several lines of evidence suggest that PTH is unlikely to be a physiologically important endogenous ligand for PTH2-Rc. These include (1) different ligand rank order of intrinsic activity of a series of PTH analogs in the human and rat PTH2-Rc, (2) considerably lower intrinsic activities and relative potencies of PTH-like ligands at the rPTH2-Rc than at the hPTH2-Rc, (3) the partial agonist effect of PTH-based peptides when compared to bovine hypothalamic extracts (Hoare *et al.,* 1999a), and, last but not least, (4) the discovery that TIP39, a peptide distantly related to either PTH or PTHrP, is a potent and selective activator of the PTH2-Rc (Usdin *et al.,* 2000).

PTH3 Receptor Subtype

Two PTH receptor alleles, one highly homologous to human PTH1-Rc and the other a novel PTH3-Rc, have been cloned by genomic PCR from zebrafish (z) DNA. While these receptors exhibited 69% similarity (61% identity) with each other, neither of them exhibited as great a degree of homology with zPTH2-Rc (Rubin *et al.,* 1999). Zebrafish PTH1-Rc and zPTH3-Rc showed 76 and 67% amino acid sequence similarity with hPTH1-Rc, respectively, but similarity with hPTH2-Rc was 63 and only 59% for both teleost receptors (Rubin and Juppner, 1999). Recombinant zPTH3-Rc, transiently expressed in COS-7 cells, exhibited more efficient AC activity when stimulated by [Ala^{29},Glu^{30},Ala^{34},Glu^{35},Tyr^{36}]fugufish PTHrP-(1-36)NH_2 and [Tyr^{36}]hPTHrP-(1-36)NH_2 (EC_{50} = 0.47 and 0.45, respectively) than by [Tyr^{34}]hPTH-(1-34)NH_2 (EC_{50} = 9.95 n*M*). In addition, zPTH3-Rc showed higher affinity to the PTHrP analogs than to the PTH analog. Finally, zPTH1-Rc activated the inositol phosphate (IP) pathway but zPTH3-Rc did not (Rubin and Juppner, 1999).

When these results are taken together, compared to the nondiscriminatory interaction of PTH1-Rcs with PTH and PTHrP and the selectivity of PTH2-Rc for PTH, zPTH3-Rc emerges as the preferential target for PTHrP-derived agonists. Interestingly, some compelling findings suggest the presence of a PTHrP-selective receptor in rat supraoptic nucleus, which is distinct from PTH1-Rc, whose activation leads to the release of Arg-vasopressin (Yamamoto *et al.,* 1997, 1998). We may speculate that since PTH1-Rc from all known species appear to have similar structural and functional properties, it is likely that the mammalian homologue of zPTH3-Rc, when identified, will interact preferentially with PTHrP.

Receptor Chimera

PTH receptor subtype chimeras, deletion mutation, and point mutations have been used to explore the functional domains in the receptor involved in ligand binding and signal transduction (Gardella *et al.,* 1994; Juppner *et al.,* 1994; Lee *et al.,* 1994a). Rat/human and rat/opossum PTH1-Rc chimeras revealed that the N-ECD plays an important role in binding of the amino-truncated PTH-(1-34)-derived peptides, such as the antagonist PTH-(7-34) (Juppner *et al.,* 1994). Chimeras with the N-ECD of hPTH1-Rc have considerably higher binding affinity for PTH-(7-34), PTH-(10-34), and PTH-(15-34) than the reciprocal chimera where the N-ECD is from the rPTH1-Rc. In addition, deletion of the sequence 61-105 (encoded by exon E2) from the N-ECD did not affect the binding of either PTH-(1-34) or PTH-(7-34) (Juppner *et al.,* 1994). Therefore, this region, which is much more variable among the human, rat, and opossum receptor species than the rest of the N-ECD, does not contribute to the difference in binding affinity of PTH-(7-34) in the rat and human PTH1-Rc. Interestingly, the ectopic regions of TM5 (residues S^{370} and V^{371}) and TM6 (residue L^{427}), which provide important interactions with the extreme amino-terminal residues of PTH and PTHrP, have been found to participate in the binding and signaling of [Arg^2]PTH-(1-34) (Gardella *et al.,* 1994). This analog is a weak partial agonist for cAMP stimulation through rPTH1-Rc and full agonist for cAMP stimulation through the opossum PTH1-Rc (Gardella *et al.,* 1991). Reciprocal specific point mutations of residues in rPTH1-Rc with residues from oPTH1-Rc (S370A, V371I, and L427T) increased binding affinity to the mutated rPTH1-Rc to the level observed for the wild-type opossum receptor, yet without affecting the binding of PTH-(1-34). Only one of these mutations in the rPTH1-Rc (S370A) conferred agonist activity to [Arg^2]PTH-(1-34) (Gardella *et al.,* 1994). The tolerance for the deletion of residues 61-105, which are located in the N-ECD, was utilized to replace it with an epitope tag derived from *Haemophilus influenza* hemagglutinin (HA) without affecting receptor functions, thus generating a powerful tool for monitoring receptor expression levels (Lee *et al.,* 1994a). However, deletions of residues 31-47 near the amino terminus and residues 431-440 in the ECL3 were both detrimental to the efficient binding of PTH-(1-34) (Lee *et al.,* 1994a).

The prevailing paradigm for the global topological organization of the PTH–PTH1-Rc complex suggests two major interdomain interactions. The first is between the N-ECD of PTH1-Rc and the principal binding domain in the ligand, located at its C-terminal portion. The second is between ECLs and juxtamembrane regions of the TMs of the receptor and the activation domain in the ligand, located at the N-terminal portion of the sequence. In line with this paradigm, Luck and co-workers (1999) reported that the amino-terminal fragment PTH-(1-14), which encompasses the principal activation domain, is equally potent in stimulating AC in rPTH1-Rc and in the N-ECD-truncated rPTH1-Rc (Carter *et al.,* 1999b; Luck *et al.,* 1999). In contrast, PTH-(1-34) was ~100-fold weaker in potency with N-ECD-truncated rPTH1-Rc than PTH-(1-14). An alanine scan identified R^{186} in the PTH1-Rc as critical for the cAMP response only in the case of PTH-(1-14) but not for PTH-(1-34) (Luck *et al.,* 1999).

Lack of photocross-linking of fully biologically active ^{125}I-Lys^{13}(*p*Bz$_2$)-PTH-(1-34) to [R186A/K]hPTH1-Rc mutants (Adams *et al.,* 1998) suggests that a contact site in the proximity of R^{186} contributes bimolecular interactions with PTH that are crucial for the signaling activity of PTH-(1-14). In addition, Carter and co-workers, carrying out Ala scan analysis and hydrophylic-to-hydrophobic substitutions in the 182-190 sequence of rPTH1-Rc, identified by homolog-scanning mutagenesis strategy to be a candidate for a ligand-binding site (Lee *et al.,* 1995a), suggest that F^{184} and L^{187} are important deterninants of functional interaction with residues 3-14 in PTH (Carter *et al.,* 1999b).

Homolog-scanning mutagenesis (Cunningham *et al.,* 1989) is a powerful technique. It generates chimeric receptors by systematically replacing segments of the PTH1-Rc with homologous segments of other class II GPCRs and has the potential to maximize surface expression and minimize perturbation of receptor conformation. Exploring the extracellular domains of the rPTH1-Rc by corresponding segments of the homologous rat secretin receptor reveals that the ectopic end of TM1 and the carboxyl-terminal of ECL1, ECL2, and ECL3 are involved in ligand binding (Lee *et al.,* 1995a). In ECL3, two specific residues, W^{437} and Q^{440}, were identified as major contributors to agonist binding. Interestingly, these two mutations did not affect the binding affinity of PTH(3-34), suggesting that these residues are involved in the interaction with the critical amino terminus of the hormone (Lee *et al.,* 1995a). Two chimeric receptors in which the entire amino-terminal domains of corticotropin-releasing receptor 1 and hPTH1-Rc were exchanged bound analogs of their cognate receptors with a specificity determined by the N-ECD (Assil *et al.,* 2001).

While calcitonin (CT) and PTH share little sequence homology, their functional domains have a similar organization. In both hormones the N-terminal portions function as activation domains, whereas the C-terminal portions contain the principal binding determinants. Although similar in structure, CTR and PTH1-Rc class II receptor glycoproteins have only 42% homology and are selectively activated only by their respective ligands. Bergwitz *et al.* (1996) created reciprocal CT-Rc/PTH1-Rc chimeras in which the N-ECD was exchanged between the two receptors. Similarly, chimeric ligands were synthesized in which the activation and binding domains of each ligand were exchanged to create CT/PTH hybrid peptides. Using a COS-7 mammalian expression system to assess ligand binding and cAMP accumulation, it was demonstrated that the reciprocal hybrid ligands [CT-(1-11)/PTH-(15-34) and PTH-(1-13)/CT-(12-32)] do not activate normal CT- or PTH1-Rc; they can, however, activate their respective N-ECD-PTH1-Rc/CT-Rc and N-ECD-CT-Rc/PTH1-Rc chimeras. This interaction was dependent on the receptor cognate N-ECD binding the hybrid with the cognate C-terminal portion. These chimeric receptors were then activated by the amino-terminal portion of the ligand interacting with the membrane-embedded domains of the receptor and the associated ECLs.

The discriminatory domains in PTH2-Rc, which allow response to PTH but not PTHrP, were studied using PTH1-Rc/PTH2-Rc chimeras with reciprocal exchanges among N-ECD, ECLs, and portions of the TMs (Bergwitz *et al.,* 1997; Turner *et al.,* 1998). The chimeric receptor N-ECD-PTH1-Rc/PTH2-Rc responded similarly to PTH and PTHrP (EC_{50} = 1.1 and 1.3 n*M,* respectively). However, this chimera had 100-fold higher apparent affinity for PTH than for PTHrP (Turner *et al.,* 1998). These findings suggest that in addition to the discriminatory role of the N-ECD, which predominantly affects binding, other domains of the PTH2-Rc may contain sites that restrict activation by PTHrP. These sites were located by generating PTH2-Rc mutants in which single or multiple nonconserved TM domain residues were mutated to the corresponding PTH1-Rc residues. Mutations within TM3 and 7 of PTH2-Rc (I244L in TM3 and C397Y, L399M and F400L in TM7) resulted in only partial recovery of affinity toward PTHrP. Turner and co-worker (1998) therefore concluded that the extracellular juxtamembrane portions of the TMs function as a selectivity filter or barrier that discriminates between Ile^5 and His^5 in PTH and PTHrP (Behar *et al.,* 1996a; Gardella *et al.,* 1996a), respectively, by accommodating the first and causing destabilizing interactions with the latter (Turner *et al.,* 1998).

Bergwitz *et al.* (1997) arrived at similar conclusions employing PTH/PTHrP point hybrids, [Trp^{23},Tyr^{36}]- and [Ile^5,Trp^{23},Tyr^{36}]PTHrP-(1-36)NH$_2$. While both analogs were equipotent and had similar affinities to the PTH1-Rc, only the former was an antagonist of PTH2-Rc (Gardella *et al.,* 1996a). Probing the pharmacological properties of these analogs with PTH1-Rc/PTH2-Rc domain, cassette, and point mutated chimeras revealed that I^{244} at the ectopic portion of TM3, as well as Y^{318} near the carboxyl end of ECL2, provide functional interactions with position 5 of the ligands that are involved in the PTH/PTHrP specificity switch (Bergwitz *et al.,* 1997).

Another series of experiments confirms the important role of PTH1-Rc TMs in ligand recognition and receptor structure. Mutation of a single amino acid (N192I) in the TM2 of the secretin receptor to the corresponding residue in the PTH receptor produced PTH binding and functional signaling by the secretin receptor (Turner *et al.,* 1996a). The reciprocal mutation in the PTH1-Rc (I234N) produced a PTH1-Rc that was responsive to secretin. Neither mutation significantly altered the response of the receptors to their own ligands. These results suggest a model of specificity wherein TM residues near the extracellular surface of the receptor function as a selectivity filter that blocks access of the wrong ligands to sites involved in receptor activation (Turner *et al.,* 1996a).

Clark *et al.* (1998) studied PTH1-Rc/PTH2-Rc chimeras in which the N-ECD and ECL3 of the two receptors were interchanged. They found that both domains in both receptors interact similarly with PTH and contribute to the differential interaction with PTHrP. Introduction of the ECL3 of PTH2-Rc into PTH1-Rc increased PTH- and PTHrP-stimulated AC

activity and maintained high binding affinity to PTH but eliminated high-affinity PTHrP binding. Similarly, exchanging ECL3 in PTH2-Rc for the one from PTH1-Rc preserved high-affinity binding but reduced the response to PTH. Interestingly, Q^{440} in the ECL3 of PTH1-Rc is important for PTH-(1-34) binding (Lee *et al.,* 1995a) and is predicted to participate in the binding pocket that accommodates Val^2 in the ligand (Rolz *et al.,* 1999). The corresponding residue in PTH2-Rc is R^{394}. Introduction of the Q440R mutation in the ECL3 derived from PTH1-Rc and the R394Q mutation in the ECL3 derived from PTH2-Rc restored the function of ECL3 chimeric receptors. Moreover, simultaneous interchange of N-ECDs and ECL3s eliminated agonist activation but not binding for both receptors. Simultaneous elimination of the E2-coded sequence (residues 62-106) from the N-ECD of PTH1-Rc and introducing the Q440/R394 mutation into the ECL3 of the PTH1-Rc restored function in the PTH2-Rc chimera. Taken together, these results suggest that interaction between N-ECD and ECL3 in PTH1-Rc is important for PTHrP recognition. To achieve high-affinity binding of PTHrP to the mutated PTH2-Rc, additional high-affinity interaction sites for PTHrP must be identified in PTH1-Rc and introduced to the PTH2-Rc (Clark *et al.,* 1998).

The reciprocal mutations of specific homologous domains and residues identify and delineate potential residues that are critical for local interactions with ligands of different pharmacological profiles and specificities and thus provide important insights into bimolecular ligand–receptor interactions. These studies have pointed to at least two distinct, independently functioning domains on the extracellular surface of the PTH1-Rc: (1) the N-ECD, which largely determines ligand binding specificity by interactions with the C terminus of PTH-(1-34), and (2) the TM5/ECL3/TM6 region of the receptor, which interacts with the N-terminal activation domain in PTH. Taken together, receptor chimera-based studies indicate that class II GPCRs share a similar overall structure with multiple functionally independent, ligand-specific domains. These domains are sufficiently different to permit synthetic hybrid ligands to bind and efficiently activate the complementary receptor chimeras.

Other Site-Directed Mutagenesis Studies

Mutagenesis has been instrumental in identifying polar residues within the hydrophobic TM domains of PTH1-Rc as important determinants of receptor function (Gardella *et al.,* 1996b; Turner *et al.,* 1996a). The polar residues R^{233} and Q^{451} located in TM2 and TM7, respectively, are highly conserved within class II GPCRs. Gardella and co-workers (1996b) found that mutating either R^{233} or Q^{451} resulted in reduced binding affinity and transmembrane signaling by the agonist but did not affect the binding of PTH-(3-34). These findings suggest that R^{233} and Q^{451} play important roles in receptor function by contributing to the interaction with the two critical N-terminal residues in PTH-(1-34) and thus affecting affinity and signaling. Combining both mutations, as in R233Q/Q451K, restored the binding affinity of the agonist almost to the wild-type receptor level but was devoid of activation of PTH-mediated cAMP or inositol phosphate signaling pathways. These results strongly suggest that residues in TM domains 2 and 7 are linked functionally, are proximal to each other, as in the bacteriorodopsin, and are involved in agonist-induced conformational changes affecting coupling to G protein (Gardella *et al.,* 1996). Moreover, mutation of three residues (S^{227}, R^{230}, and S^{233}) predicted to be aligned on the same face of TM2 resulted in blunted PTH-(1-34)-stimulated AC response and lower binding affinity for the agonist despite efficient cell surface expression (Turner *et al.,* 1996a). The same mutation at the corresponding sites in another member of the class II GPCRs, the secretin receptor, resulted in a similar reduction in AC activity. Taken together, these studies led Turner and co-workers (1996a) to propose that this ectopic region in TM2 participates in a signal transduction mechanism common to class II of GPCRs.

Characterization of Cysteins in the Ectopic Domain of PTH1-Rc

Cysteine to serine mutations of any of the six cysteines in the N-ECD severely impaired expression of the mutated receptor (Lee *et al.,* 1994a). However, mutations C281S or C351S, in the ECL1 and ECL2, respectively, resulted in a reduced level of cell surface expression and compromised binding affinity. Nevertheless, the double mutant C281S/C351S, however, had significant improvement in binding affinity, suggesting that these cysteins are involved in a disulfide bridge connecting the first and second ECLs as they are in rhodopsin and βAR (Lee *et al.,* 1994a).

Elucidation of the distinct pattern of the three disulfide bridges formed by the six cysteins in the extracellular N-terminal domain of PTH1-Rc is a major accomplishment (Grauschopf *et al.,* 2000). These six cysteins are highly conserved in class II GPCRs and therefore must contribute critically to receptor function. Bacterial expression of the N-ECD in inclusion bodies was followed by oxidative refolding, which generated stable, soluble, monomeric and functional protein (Grauschopf *et al.,* 2000). The N-ECD binds PTH-(1-34) with an apparent dissociation constant of $3-5\ \mu M$. Analysis of the disulfide bond pattern revealed the following pairwise arrangement: C^{131}-C^{170}, C^{108}-C^{148}, and C^{48}-C^{117} (Grauschopf *et al.,* 2000). This nonsequential pattern has the potential to contribute dramatically to the tertiary structure of the N-ECD and will therefore play an important role in future receptor-modeling studies.

Structure–activity studies of receptors using mutagenic techniques are inferential and cannot conclusively distinguish between direct bimolecular interactions and indirect effects resulting from the modification of local and global conformation. Understanding ligand–receptor interactions at the atomic level requires identifying the contact sites

between specific residues in the ligand and specific residues within the cognate receptor. Mapping this bimolecular interface will identify contact sites that contribute differentially to the bimolecular interaction. In addition to critical sites that contribute primarily either to binding affinity or to the conformational changes leading to signal transduction, there will be bimolecular interactions with less critical roles in both functions. Nevertheless, each additional bimolecular contact site contributes significantly to the identification and characterization of the bimolecular interface and therefore to the understanding of the ligand–receptor interaction.

Mutated Receptor-Based Genetic Disorders

JANSEN'S METAPHYSEAL CHONDRODYSPLASIA

Jansen's metaphyseal chondrodysplasia (JMC) is a rare form of short limb dwarfism associated with abnormalities in endochondral skeletal development, hypercalcemia, and hypophosphatemia, despite normal levels of PTH and PTHrP. Three missense mutations in the PTH1-Rc coding region, H223R, T410P, and I458R, have been discovered in patients with the disease (Schipani *et al.,* 1995, 1996, 1999). PTH1-Rc carrying any one of these mutations display constitutive, ligand-independent activation of the cAMP signaling pathway when tested *in vitro.*

The H223R, T410P, and I458R mutations are located at the cytoplasmic base of TM2, TM6, and TM7, respectively. In COS-7 cells transiently expressing the human I458R PTH1-Rc, basal cAMP accumulation was approximately eight times higher than in cells expressing the recombinant normal receptor. Furthermore, the I458R mutant showed higher activation by PTH than the wild-type receptor in assays measuring the activity of downstream effectors, AC and PLC. Like the H223R and T410P mutants, the I458R mutant does not constitutively activate basal inositol phosphate accumulation. Interestingly, these mutations all occur at TM regions near the intracellular loops of PTH1-Rc that are hypothesized to interact with and activate intracellular G proteins and the subsequent signaling cascade.

These same mutations in PTH1-Rc have also been utilized to identify PTH and PTHrP analogs with inverse agonist activity. Two peptides, [Leu^{11},D-Trp^{12}]hPTHrP-(7-34)NH_2 and [D-Trp^{12},Tyr^{34}]bPTH-(7-34)NH_2, which are highly potent antagonists for the wild-type PTH1-Rc, exhibited inverse agonist activity in COS-7 cells expressing either mutant receptor (H223R or T410P) and reduced cAMP accumulation by 30–50% with an EC_{50} of approximately 50 n*M* (Gardella *et al.,* 1996c). Such inverse agonist ligands may be useful tools for exploring the different conformational states of the receptor, as well as leading to new approaches for treating human diseases with an underlying etiology of receptor-activating mutations.

BLOMSTRAND'S LETAL CHONDRODYSPLASIA

Blomstrand's letal osteochondrodysplasia (BLC) is a rare lethal skeletal dysplasia characterized by accelerated endochondral and intramembranous ossification (Blomstrand *et al.,* 1985; Leroy *et al.,* 1996; Loshkajian *et al.,* 1997; Young *et al.,* 1993). The phenotype of BLC is strikingly similar to PTH1-Rc "knockout" mice, which display prominent pathology in the growth plate (Lanske *et al.,* 1999). In both the human disease and the PTH1-Rc-ablated mouse model, the growth plate is reduced in size because proliferating chondrocytes lack the normal columnar architecture as well as a greatly reduced zone of resting cartilage. This overall similarity of phenotype suggests that an inactivating mutation of PTH1-Rc is the underlying genetic defect in BLC. To date, two types of inactivating mutations have been documented in BLC patients (Karaplis *et al.,* 1998; Zhang *et al.,* 1998). The first is a single homozygous nucleotide exchange in exon E3 of the PTH1-Rc gene. This alteration introduces a P132L mutation in the N-ECD of the receptor (Zhang *et al.,* 1998). Proline 132 is conserved in all mammalian class II GPCRs. COS-7 cells expressing a GFP-tagged mutant receptor do not accumulate cAMP in response to PTH or PTHrP and do not bind radiolabeled ligand, despite being expressed at levels comparable to GFP-tagged wild-type PTH1-Rc. Thus, while full-length PTH1-Rc is being synthesized, it does not bind the ligand and it is functionally inactive.

Another mutation in PTH1-Rc detected in BLC patients results in the synthesis of truncated receptor fragments (Karperien *et al.,* 1999). Sequence analysis of all coding exons of the PTH1-Rc gene identified a homozygous point mutation in exon EL2 with one absent nucleotide (G at position 1122). This missense mutation produces a shift in the open reading frame, leading to a receptor truncated after amino acid 364 located in the ECL2. The mutant receptor, therefore, lacks TMs 5, 6, and 7.

Jobert and co-workers (1998) described a third point mutation in PTH1-Rc associated with BLC. This mutation (G→A at nucleotide 1176) leads to the deletion of 11 amino acids (residues 373-383) in the TM5 of the receptor (Jobert *et al.,* 1998). The mutated receptor is well expressed in COS-7 cells, but does not bind PTH or PTHrP and fails to elevate cAMP and inositol phosphate in response to these ligands.

All three mutations occur precisely at regions thought to be critical for (1) the interaction of PTH1-Rc with the activation domain at the extreme N terminus of PTH and PTHrP and (2) the activation of coupled G proteins at the cytoplasmic side of the receptor. Functional analysis of the mutant receptor in COS-7 cells and of dermal fibroblasts obtained from a BCL patient demonstrated that all of the BLC mutations described earlier are inactivating. Neither the transiently transfected COS-7 cells nor the dermal fibroblasts increased cAMP accumulation in response to PTH or PTHrP.

Integrated Studies of Ligand–Receptor Interactions

One of the most effective ways of characterizing any ligand–acceptor system is to study the intact bioactive bimolecular complex. Routinely, X-ray crystallography and

NMR spectroscopy are the tools of choice for analyzing the structure of bimolecular entities. These methods yield very detailed structural information that has been utilized in rational drug design and generated important leads for the development of novel therapeutic agents. Enzyme inhibitors, such as cathepsin K–inhibitor (Thompson *et al.,* 1997) and HIV protease–inhibitor (Miller *et al.,* 1989), and soluble protein acceptor–ligand systems, such as the human growth hormone (hGH)–extracellular domain of the hGH receptor (de Vos *et al.,* 1992), erythropoietin (EPO)–EPO-receptor (Livnah *et al.,* 1996), and ligand–FK506 binding protein (Shuker *et al.,* 1996), are just a few of a long list of successes demonstrating the power of studying the bimolecular complex and identifying intermolecular interfaces. It is imperative, however, to conduct structural studies under conditions that will not perturb biologically relevant conformations. Unfortunately, membrane-embedded proteins, such as GPCR, are not amenable to either NMR or X-ray analysis because of their large molecular weight, inability to form crystals, and great suceptibility to the manipulations required by these techniques.

Two traditional and indirect approaches, one "ligand centered" and the other "receptor centered," have been pursued to further understand the ligand–PTH Rc interaction and each has made important contributions (see preceding sections). The hormone-centered approach succeeded in mapping functional domains within the hormone that affects receptor binding and activation. In some cases, structural features responsible for biological properties have been identified down to the level of a single amino acid, which sometimes led to the development of important therapeutics. In the PTH/PTHrP field, the identification of the architecture of functional domains and the development of potent antagonists, partial agonists, and inverse agonists, signaling-selective analogs, and potent *in vivo* anabolic agents generate a very impressive list of accomplishments. However, this approach cannot be used to deduce the receptor domains that are in contact with the hormone across the interface. Furthermore, in some cases, modifying the primary structure of the hormone may result in altering the pattern of bimolecular interactions with the receptor. Although some structural modifications of the hormone may directly alter its interaction with an important complementary structural feature of the Rc, others may affect bioactivity through either local or global conformational changes within the hormone that prevent formation of an optimal "bioactive conformation." In essence, the hormone-centered approach is "blind" to the structure of the receptor.

The "receptor-centered" approach has also succeeded in providing valuable insights. Point-mutated and chimeric PTH-Rc — interspecies of PTH1-Rc, such as rat with opossum Rc, or interhormone Rc, such as PTH1-Rc with calcitonin or secretin Rc's — revealed the importance of specific Rc domains and single amino acids necessary for Rc function. However, analysis of the functional consequences that result from modifying the Rc structure alone cannot be used to identify unequivocally the interacting complementary structural elements in the hormone. Furthermore, one usually cannot distinguish Rc modifications that disrupt function as a result of local changes in an important "contact site" or a global conformational change. While local changes affect Rc interaction with a site in the hormone directly, global conformational changes lead to extensive modification of Rc topology, thereby altering interactions with the hormone indirectly. Hence, despite the attractiveness of both lines of investigation and the importance of their contributions, conclusions drawn from the hormone-centered and Rc-centered approaches have inherent limitations and are inferential at best.

In order to study the bimolecular interface of a dynamic system such as the ligand–receptor complex, we need to be able to freeze a bimolecular interaction and identify the interacting site. Photoaffinity labeling has emerged as an effective methodology for studying interactions of biological macromolecules with their ligands (Chowdhry and Westheimer, 1979; Dorman and Prestwich, 1994; Hazum, 1983; Hibert-Kotzyba *et al.,* 1995). The resultant photocross-linked conjugate can serve as a starting point for mapping "contact domains," and even "amino acid-to-amino acid contact points" between a biologically active compound and an interacting macromolecule (Bitan *et al.,* 1999; Blanton *et al.,* 1994; Boyd *et al.,* 1996; Girault *et al.,* 1996; Hadac *et al.,* 1999; Ji *et al.,* 1997; Kage *et al.,* 1996; Keutmann and Rubin, 1993; Kojr *et al.,* 1993; Li *et al.,* 1995; McNicoll *et al.,* 1996; Phalipou *et al.,* 1999; Williams and Shoelson, 1993).

A number of laboratories, including our own, have embarked on a challenging program to map the bimolecular interface between a large peptide hormone and a seven transmembrane-spanning G protein-coupled Rc. The approach, using photoaffinity scanning (PAS) to identify directly contact sites in the hPTH1-Rc responsible for hormone binding and signal transduction, has numerous stringent requirements, the fulfillment of which are crucial for success. Bioactive, specific cleavage-resistant, photoactivable, and radiolabled PTH analogs must be designed and synthesized. A rich and stable source of functional wild-type or mutant PTH-Rc, overexpressed preferentially in a homologous cellular background, must be developed and fully characterized pharmacologically. For purifying either the intact ligand–receptor conjugate or its ligand–receptor conjugated fragments, it will be advantageous to work with epitope-tagged hRc. Although optional, it may be very helpful to have antibodies to various hRc extracellular epitopes for use in purification and analysis. The PAS approach also requires devising an analytic strategy with sequential chemical and enzymatic cleavages; theoretical digestion maps to allow unambiguous identification of hormone-binding sites within the hPTH-Rc; readily available capacity to either synthesize or express receptor domains that contain the identified contact sites for conformational studies; and the capacity to generate site-directed mutated PTH1-Rc, express them stably or transiently, characterize them pharmacologically, and use them in the PAS technology to validate and/or delineate emerging results. Last but not least, this approach requires

access to tools that will allow to integrate cross-linking data with Rc mutagenesis data, and eventually with conformational analysis and molecular modeling data to generate a unified, experimentally based model of the hormone–Rc complex.

In summary, PAS technology is a multidisciplinary, integrated, iterative, and labor-intensive approach. Nevertheless, it is currently the only direct method that yields the best approximation of the actual ligand–receptor complex. Because of the nature of this approach, however it cannot yield molecular structures of the same resolution as those obtained by either X-ray crystallography or NMR analysis.

Photoreactive Analogs

Early efforts to generate a photoreactive, radiolabeled, and biologically active analog of PTH aimed to identify the receptor as a distinct molecular entity (Coltrera *et al.,* 1981; Draper *et al.,* 1982; Goldring *et al.,* 1984; Wright *et al.,* 1987). All of these studies used poorly characterized ligands containing nitroarylazide-based photophores and reported molecular masses ranging between 28 and 95 kDa for the hormone–receptor complex. Shigeno and co-workers (1988a,b) carried out a careful synthesis and characterization of the nitroarylazide-based photoligand and identified it as [$Nle^{8,18}$,Lys^{13}(N^{ε}-(4-N_3-2-NO_2-phenyl),Tyr^{34}]PTH-(1-34)NH_2, a fully active analog in ROS 17/2.8 cells. Using this photoaffinity ligand, they were able to identify in the same cells a plasma membrane glycoprotein corresponding to the PTH receptor that had the apparent molecular mass of 80 kDa (Shigeno *et al.,* 1988a,b).

Introduction of the arylketone-based photoaffinity scanning methodology (Adams *et al.,* 1995; Bisello *et al.,* 1999; Han *et al.,* 2000; Nakamoto *et al.,* 1995; Suva *et al.,* 1997) into the field of calciotrophic hormones and their corresponding receptors is the basis for the current approach to the characterization of the ligand–receptor bimolecular interface (Adams *et al.,* 1998; Behar *et al.,* 1999, 2000; Bisello *et al.,* 1998; Greenberg *et al.,* 2000; Suva *et al.,* 1997; Zhou *et al.,* 1997). Advantages of the benzophenone moiety as a photophore over the aryl azide moiety are numerous. A partial list includes the high efficiency of cross-linking—only a small amount is lost to hydrolysis and, as a result, very little nonspecific cross-linking is observed. Photoactivation is carried out at a wavelengths >330 nm, in which proteins are less susceptible to photodegradation. In addition, there is excellent compatibility with solid-phase peptide synthesis methodology. Furthermore, synthesis, purification, and biological evaluation can be conducted in the laboratory under normal ambient light conditions. This section summarizes major achievements in the design and development of benzophenone-containing PTH and PTHrP ligands and their contribution to the mapping of the bimolecular ligand–receptor interface.

Radioiodination was chosen as the tagging method of choice because of its high specific radioactivity translating into high sensitivity of detection of the radiolabeled conjugated ligand–receptor complex and the fragments derived from it. Therefore, successful PAS analysis requires maintaining the connectivity between the radiotag and the photophore throughout the controlled degradation of the conjugated ligand–receptor complex. Modifications in PTH-(1-34), which include $Met^{8 \text{ and } 18} \rightarrow Nle^{8 \text{ and } 18}$, $Lys^{13,26, \text{ and } 27} \rightarrow Arg^{13,26, \text{ and } 27}$, and $Trp^{23} \rightarrow$2-naphthylalanine^{23} (Nal), render the ligand resistant to the various chemical and enzymatic cleavage agents [i.e. CNBr, lysyl endopeptidase (Lys-C) and BNP-skatole, cleaving at the carboxyl side of Met, Lys, and Trp, respectively].

The fundamental requirement in any photoaffinity cross-linking study is that the photoreactive analogs have the same pharmacological profile as the parent peptide hormone. It will therefore be safe to assume that they share similar bioactive conformations and generate topochemically equivalent ligand–receptor complexes. The photoreactive benzophenone-containing analogs of PTH and PTHrP were designed specifically for PAS studies aimed at investigating the bimolecular interactions of the activation and binding domains of PTH and PTHrP with either PTH1-Rc or PTH2-Rc subtypes.

Identification of Contact Sites

Using PAS methodology, the positions for which contact sites have been identified are positions 1, 13, and 27 in PTH and positions 1, 2, and 23 in PTHrP (Adams *et al.,* 1995; Behar *et al.,* 2000; Bisello *et al.,* 1998; Carter *et al.,* 1999a; Greenberg *et al.,* 2000; Zhou *et al.,* 1997). Two different photophores were used in different studies: *p*-benzoylphenylalanine (Bpa) (Behar *et al.,* 2000; Bisello *et al.,* 1998; Carter *et al.,* 1999a) and the Lys(N^{ε}-*p*-benzoylbenzoyl) [Lys(N^{ε}-*p*Bz_2] (Adams *et al.,* 1995; Behar *et al.,* 2000; Greenberg *et al.,* 2000; Zhou *et al.,* 1997). The former has the benzophenone moiety attached to the peptide backbone through a β carbon, whereas the latter is presented on a relatively long side chain removed by six atoms from the backbone. These different modes of presentation of the benzophenone moiety may play a limited role in selecting cross-linking sites.

Position 1 in PTH Cross-Links to the Ectopic Site in TM6

Photocross-linking [Bpa^1,$Nle^{8,18}$,$Arg^{13,26,27}$,Nal^{23},Tyr^{34}] bPTH-(1-34)NH_2 (Bpa^1-PTH) to the human PTH1-Rc stably overexpressed (~400,000 Rc/cell) in human embryonic kidney cell line 293 (HEK293/C21) generates an 87-kDa photoconjugate (Bisello *et al.,* 1998). Chemical digestions by CNBr and BNPS-skatole, which cleave at the carboxyl end of Met and Trp, respectively, and enzymatic digestions by lysyl endopeptidase (Lys-C) and endoglycosidase F/N-glycosidase F (Endo-F), which cleave at the carboxyl end of Lys and deglycosylate aspargines at concensus glycosylated sites, respectively, generate an array of fragments. These radioiodinated fragments are characterized by SDS-PAGE, and the apparent molecular weights obtained are compared with the theoretic digestion restriction map of the

photoconjugated receptor. Although the resolving power of PAGE is limited, the combination of consecutive cleavages (e.g., Endo-F followed by Lys-C followed by CNBr) carried out in reversed order (e.g., Lys-C followed by BNPS-skatole and BNPS-skatole followed by Lys-C) is extremely powerful. It generates a reproducible pattern of fragments delimited by specific end residues and the presence or absence of glycosylation sites. Comparing the putative digestion map of the hPTH1-Rc with actual fragments identifies the sequence of the smallest radiolabeled ^{125}I-Bpa^{1}-PTH–PTH1-Rc-conjugated fragment (~4 kDa). This fragment consists of the ligand (4489 Da) modified by a very small moiety contributed by a Met residue belonging to the receptor (Bisello *et al.,* 1998). Two Met residues, 414 and 425, present at the mid region and the extracellular end of TM6, emerged as potential contact sites for position 1 in PTH. Contact between residue 1 in PTH and M^{414} requires the N terminus of PTH to protrude into the 7 helical and hydrophobic TMs bundle. In contrast, contact with M^{425} can be achieved while the N terminus is dipping only superficially into the TM bundle.

These biochemical methods are supplemented by molecular biology to provide additional resolving power to the PAS method. Transient expression of two point-mutated hPTH1-Rc, [M414L] and [M425L], generated fully active receptors in COS-7 cells (Bisello *et al.,* 1998). ^{125}I-Bpa^{1}-PTH lost its ability to photocross-link to [M425L] but not to [M414L], thus suggesting that M^{425} is the putative contact site for position 1 in PTH.

Behar and co-workers (2000) reported that radioiodinated PTHrP-based agonist [Bpa^{1},Ile^{5},Tyr^{36}]PTHrP-(1-36) NH_2 (^{125}I-Bpa^{1}-PTHrP), which carries a photophore at the same position as the photoreactive PTH analog ^{125}I-Bpa^{1}-PTH, forms a contact with M^{425} in hPTH1-Rc. This exciting finding confirms that the functional and conformational similarity between PTH and PTHrP extends to a common contact site for the N-terminal residue in the ligand. The location of this site at M^{425} in the ectopic portion of TM6 supports the prevailing view that these two hormones interact very similarly, if not identically, with the PTH1-Rc (Behar *et al.,* 2000).

Position 13 in PTH Cross-Links to a Juxtamembrane Location in N-ECD

Biochemical analysis of the photocross-linking product of radiolabled [Nle^{8,18},Lys^{13}(N$^{\varepsilon}$-*p*(3-I-Bz)Bz),Nal^{23}, Arg^{26,27},Tyr^{34}]bPTH-(1-34)NH_2 [Lys^{13}(*p*Bz$_2$)-PTH] with hPTH1-Rc expressed in HEK293/C-21 cells identifies a glycosylated radioactive band of ~6 kDa, which is delimited by Lys-C and CNBr cleavage sites at the N and C termini, respectively. The theoretical cleavage restriction map of hPTH1-Rc reveals that this minimal radiolabeled ^{125}I-Lys^{13} (*p*Bz$_2$)-PTH–hPTH1-Rc-conjugated fragment corresponds to hPTH1-Rc[173-189] and is located at the juxtamembranal end of the N-ECD (Zhou *et al.,* 1997).

Additional analysis of the 17 amino acid residues composing hPTH1-Rc[173-189] by a combination of site-directed mutagenesis followed by biochemical analysis further delineates the boundaries of the fragment containing the contact site for ^{125}I-Lys^{13}(*p*Bz$_2$)-PTH to obtain hPTH1-Rc [182-189], an 8 amino acid-sequence (Adams *et al.,* 1995). Several R-to-K single site-mutated receptors were generated. These include new Lys-C-susceptible cleavage sites that will allow further delineation of the contact site for position 13. The mutant [R181K]hPTH1-Rc was stably expressed in HEK293 cells (~200,000 Rcs/cell) and was fully functional. Compared to the wild-type receptor, Lys-C cleavage of the ^{125}I-Lys^{13}(*p*Bz$_2$)-PTH–[R181K] photoconjugate produces a smaller conjugated fragment (~18 vs ~9 kDa, respectively), corresponding to a cleavage site upstream to the N-glycosylated N^{176}. Interestingly, the only functional mutations that failed to cross-link to ^{125}I-Lys^{13}(*p*Bz$_2$)-PTH were the [R186K/A] mutants (Adams *et al.,* 1995). However, [R186K]hPTH1-Rc stably expressed in HEK293 cells cross-links effectively to ^{125}I-Bpa^{1}-PTH and displays wild-type receptor-like AC activity and binding affinity similar to that observed in HEK293/C-21 cells. These findings suggest that R^{186} participates in an interaction with the ligand that either provides a contact site for position 13 in the ligand or contributes an interaction that brings the ligand into the close spatial proximity required for cross-linking within the hPTH1-Rc[182-189] contact site (Adams *et al.,* 1995). This interaction does not appear to be essential for a productive ligand–receptor interaction, as [R186K] is fully functional and cross-links effectively with another photoreactive and bioactive analog, ^{125}I-Bpa^{1}-PTH.

Position 27 in PTH Cross-Links to ECL1

The two contact sites described earlier involve residues in the extended activation domain of PTH comprising residues 1-13. Greenberg and co-workers (2000) identified contact sites within the receptor that are involved in the interaction with the principal binding domain of the ligand (residues 24-34). This bimolecular interaction was probed by [Nle^{8,18},Arg^{13,26},L-2-Nal^{23},Lys^{27}(*N*$^{\varepsilon}$-*p*Bz$_2$),Tyr^{34}]bPTH (1-34)NH_2 [Lys^{27}(*p*Bz$_2$)-PTH], a potent agonist, modified by a benzophenone-containing photophore at position 27, and the study employed a combination of biochemical analysis of the photoconjugates and site-directed mutagenesis (Greenberg *et al.,* 2000). Analysis of the ^{125}I-Lys^{27} (*p*Bz$_2$)-PTH–PTH1-Rc photoconjugate by CNBr/Endo-F and BNPS-skatole/Endo-F degradation pathways produced an overlapping sequence of 67 amino acids corresponding to L^{232}-W^{298}. This contact domain includes part of TM2, ECL1, and the entire TM3. Secondary digestions of CNBr- and BNPS-skatole-derived fragments by endoproteinase Glu-C, which predominantly cleaves at the carboxyl side of Glu, converged on an overlapping 38 amino acid sequence corresponding to L^{261}-W^{298}, which includes part of ECL1 and the entire TM3 (Greenberg *et al.,* 2000).

This 38 amino acid contact site was further delineated by analyzing specific single point mutants transiently expressed in COS-7 cells. All three receptor mutants, [R262K], [L261M], and [L261A], were expressed and

displayed characteristic binding affinity and PTH-stimulated AC activity comparable to the wild-type receptor. [R262K] and [L261M] were designed to modify the Lys-C and CNBr cleavage patterns, respectively. The [L261A] was introduced to eliminate a favorable insertion site at position 261. Restriction digestion analysis of the ^{125}I-Lys^{27}(pBz$_2$)-PTH–[R262K] photoconjugate delineated the contact site as hPTH1-Rc[232-262]. Taken together, the minimal contact sites [261-298] and [232-262] obtained from the analysis of the wild-type and mutant [R262K] receptors, respectively, suggest either L^{261} or R^{262} as the contact site for Lys^{27}. Treatment of the ^{125}I-Lys^{27}(pBz$_2$)-PTH–[L261M]PTH1-Rc photoconjugate with CNBr generated a conjugated fragment similar in size to the ligand itself, thus confirming position 261 in the receptor as the contact site for position 27 in the ligand. This was further confirmed by the elimination of effective cross-linking of I^{125}-Lys^{27}(pBz$_2$)-PTH to the mutated receptor [L261A], in which a reactive insertion site, such as Leu, is replaced by Ala, a poor insertion site for the photoactivated benzophenone-derived biradical. Position 261, the contact site for position 27 in PTH, is located near the center of ECL1 (Greenberg *et al.,* 2000).

The identification of L^{261} in hPTH1-Rc as a contact site for Lys^{27} in PTH provides important information for mapping the PTH–PTH1-Rc interface. The ligand–receptor bimolecular interface includes three receptor domains: the juxtamembranal portion of N-ECD, the ectopic portion of TM6, and the ECL1. The remoteness of position 27 from positions 1 and 13 in PTH and that of L^{261} from R^{186} and Met^{425} in hPTH1-Rc generates an important additional structural constraint that can be used to refine the emerging experimentally based model of the PTH–PTH1-Rc complex.

Position 23 in PTHrPc Cross-Links to a Site Located at the Amino-Terminal End

Mannstadt and co-workers (1998) reported that the photoreactive analog of PTHrP, [Ile^5,Bpa^{23},Tyr^{36}]PTHrP-(1-36)NH$_2$ (Bpa^{23}-PTHrP), modified by a benzophenone moiety incorporated at position 23, cross-links to the contact site Y^{23}-L^{40} located at the very N-terminal end of rat PTH1-Rc. CNBr analysis of the ^{125}I-Bpa^{23}-PTHrP–rPTH1-Rc photoconjugate suggests that the contact site resides at the N terminus of the receptor, rPTH1-Rc[23-63]. A combination of CNBr cleavages and site-directed mutagenesis—single point mutation [M63I] and the double mutants [M63I,L40M] and [M63I,L41M])—further delineates the contact site to span the sequence 23-40. Earlier findings reported that the two mutant rPTH1-Rcs with deletions of residues 26-60 or 31-47 transiently expressed in COS-7 cells had little or no capacity to bind ^{125}I-PTH, thus suggesting that these regions are important for ligand binding (Lee *et al.,* 1994a). Further mutational analysis, which included cassette mutagenesis and Ala scan, found that mutants [T33A] and [Q37A] suffered the largest loss in binding affinity for ^{125}I-PTHrP and complete loss of binding affinity toward the antagonist [Leu^{11},D-Trp^{12}]PTHrP-(7-34)NH$_2$. These results led Mannstadt and co-workers (1998) to conclude that the first 18 amino acid residues of the PTH1-Rc include the contact site for position 23 in PTH and that T^{33} and Q^{37} are functionally involved in the binding of the 7-34 region in PTH rather than the 1-6 region.

The location of contact sites for two closely spaced residues in PTH/PTHrP (23 and 13) at both ends of the extracellular amino terminus of the receptor (within the sequence 23-40 and in proximity to R^{186}, respectively) is consistent with the current model of the ligand–receptor binding interface. The extensive length of the putative extracellular amino terminus of PTH1-Rc (~167 residues) may allow the formation of secondary and tertiary structures by the receptor within this domain so that it will be capable of simultaneously accommodating the bimolecular interactions mentioned earlier.

Cross-Linking of Position 1 of Antagonist vs the Same Position in Agonist

A detailed, atomic level understanding of the distinct ligand–receptor interactions that differentiate an agonist from an antagonist is of major importance. Behar and co-workers (2000) made a very interesting observation that directly distinguishes the nature of the bimolecular interactions of agonists and antagonists with PTH1-Rc. Radiolabeled [Bpa^2,Ile^5,Arg^{11,13},Tyr^{36}]PTHrP-(1-36)NH$_2$ (Bpa^2-PTHrP), a highly potent antagonist, was photoconjugated to hPTH1-Rc in HEK293/C-21 cells (Behar *et al.,* 2000). Unlike ^{125}I-Bpa^1-PTH and ^{125}I-Bpa^1-PTHrP, which are derived from potent agonists of PTH1-Rc and cross-link to it at M^{425}, ^{125}I-Bpa^2-PTHrP cross-links to both M^{425} and a proximal site within the receptor domain P415-M425 (Behar *et al.,* 2000). These results may reflect differences in binding modes of agonists and antagonists or in the interaction between the two consecutive positions in the PTHrP-(1-36) sequence and PTH1-Rc.

In an attempt to distinguish between these two possibilities, Behar and co-workers (2000) utilized the analog [Bpa^2,Nle^{8,18},Arg^{13,26,27},Nal^{23},Tyr^{34}]bPTH-(1-34)NH$_2$ (Bpa^2-PTH), which is a full agonist of PTH1-Rc and carries the same photoreactive moiety at the same position as the antagonist Bpa^2-PTHrP (Bisello *et al.,* 1998). Analysis of ^{125}I-Bpa^2-PTH photoconjugates with wild-type and [M414L] or [M425L] mutated hPTH1-Rc indicates that this ligand cross-links only to the ε-methyl of Met^{425}, which is similar to Bpa^1-PTHrP and Bpa^1-PTH cross-linking (Bisello *et al.,* 1998). These results, therefore, provide strong support for the hypothesis that the differences observed between the cross-linking of ^{125}I-Bpa^1- and ^{125}I-Bpa^2-PTHrP may reflect different interaction modes of agonists and antagonists with the PTH1-Rc.

Interestingly, two additional Bpa-containing PTHrP-(1-36) analogs, [Bpa^2,Ile^5,Trp^{23},Tyr^{36}]- and [Bpa^4,Ile^5,Trp^{23},Tyr^{36}]PTHrP-(1-36)NH$_2$, were reported to selectively antagonize and preferentially cross-link to hPTH1-Rc and

hPTH2-Rc stably expressed in LLC-PK_1 cells, respectively (Carter *et al.,* 1999a). However, in homologous systems composed of hPTH1- and hPTH2-Rc expressed in a human cellular background (HEK293/C-21 and HEK293/BP-16, respectively), Bpa^2-PTH is a full agonist and Bpa^4-PTH is a very weak agonist with a slightly better affinity for the hPTH2-Rc (Bisello *et al.,* 1998; Carter *et al.,* 1999a). Similar to [Bpa^4,Ile^5,Trp^{23},Tyr^{36}]PTHrP-(1-36)NH_2 (Carter *et al.,* 1999a), [Bpa^4,Ile^5,$Arg^{11,13}$,Tyr^{36}]PTHrP-(1-36)NH_2 displays poor binding affinity and negligible efficacy in HEK293/C-21 cells expressing the hPTH1-Rc (Behar *et al.,* 2000). Nevertheless, Carter and colleagues (1999a) reiterated the prevailing understanding that individual residues within the 1-5 sequence in PTHrP play distinct roles in modulating the interactions with PTH1- and PTH2-Rc.

Although PTH2-Rc may not be the physiological target for PTH or PTHrP, its structural resemblance to PTH1-Rc, its high-binding affinity, specific cross-linking, and effective coupling to the PTH-induced intracellular signaling pathways make it an attractive target for exploring structure–function relations in the PTH/PTHrP–PTH1-Rc system. Analysis of the photoconjugates obtained upon cross-linking ^{125}I-Bpa^1-PTH and ^{125}I-Lys^{13}($p$$Bz_2$)-PTH to hPTH2-Rc stably expressed in HEK293 cells (HEK293/BP-16, ~160,000 Rc/cell) revealed that both hPTH1-Rc and hPTH2-Rc use analogous sites for interaction with positions 1 and 13 (Behar *et al.,* 1999).

PAS methodology, although not perfect, offers the only readily available experimental approach to studying the bimolecular ligand–GPCR interface directly. To practice this methodology successfully requires introducing benzophenone moieties, radioiodine, and substitutions that provide resistance to specific chemical and enzymatic cleavages. The stringent requirements that a potential candidate molecule must meet to be employed in the PAS methodology result in a limited set of reagents. Photoreactive ligands thus represent only those molecules with the following characteristics. They tolerate the numerous modifications, maintain high-binding affinity, and efficaciously stimulate AC in a PTH-like manner, in the case of agonists, or effectively block agonist-stimulated AC, in the case of antagonists. The photoinsertion site of the benzophenone moiety is dictated by spatial proximity between the photophore and the potential insertion sites and their correct spatial disposition. However, it is also biased toward the more reactive insertion sites within its reactivity sphere. Finally, both the cleavages employed and the level of resolution allowed by SDS-PAGE limit photoconjugate analysis. In this respect, there is ample room for technological enhancements that will include epitope tagging, affinity purification, sequencing, and mass spectroscopic analysis. Validation of a putative contact site by site-directed mutagenesis is not necessarily benign. It generates some degree of perturbation, which we accept as long as the mutated receptor is expressed and functions similarly to the wild-type. Taken together, PAS is not the perfect method, but we believe it is the best available.

Experimentally Based Molecular Modeling

It is safe to assume that contact sites identified by the cross-linking studies described earlier are only a small fraction of the large ensemble that forms the bimolecular interface. It is also possible that not all contact sites revealed by PAS methodology will have the same functional significance. Nevertheless, all these contact sites will be part of the ligand–receptor interface and are therefore indispensable targets in mapping efforts. The ultimate objective of PAS studies is to generate a series of bimolecular structural constraints, which will be used in mapping the bimolecular interface. To this end, merging the information generated by PAS studies with information about the conformations of the ligand and receptor domains, as well as molecular modeling, is an integrated approach that results in an experimentally based ligand–receptor model. It contrasts with the more common approaches that predict conformation and molecular models solely on a theoretical basis.

The model for the PTH–PTH1-Rc complex is steadily evolving as new bimolecular contact sites are identified and additional conformational data on receptor domains are generated (Bisello *et al.,* 1998; Piserchio *et al.,* 2000a; Rolz *et al.,* 1999). Combining hydrophobicity profile analysis with a search of the Brookhaven Protein Data Bank (PDB) employing the Basic Logic Alignment Search Tool (BLAST) first identifies and then refines the location of the TM helices (Altschul *et al.,* 1990; Kyte and Doolittle, 1982). Identification of the TM domains of the PTH1-Rc is in good agreement with respect to those identified in peptides containing TM helical regions as determined by high-resolution NMR in micellar system (Mierke *et al.,* 1996; Pellegrini *et al.,* 1997b, 1998b). Arrangements of the TM heptahelical bundle in rhodopsin and bacteriorhodopsin (Grigorieff *et al.,* 1996; Henderson *et al.,* 1990; Pebay-Peyroula *et al.,* 1997; Schertler *et al.,* 1993; Schertler and Hargrave, 1995) were used as templates for the initial arrangement of the putative TM helical domains of the PTH-Rc and optimized to account for hydrophobic moment toward the membrane environment, helix–helix, helix–core, and helix–membrane interactions (Pellegrini *et al.,* 1997b).

Unlike the high structural similarity in the arrangement of the heptahelical TM domains bundle (Baldwin, 1993), the cytoplasmic and ectopic domains of GPCRs are extensively variable and no *a priori* structural model is available. The loops are constrained to some extent by the TM helical domains to which they are attached. Additional constraints are imposed by the three disulfide bridges at the extracellular N terminus (Grauschopf *et al.,* 2000) and the disulfide bridge connecting the first and second ECL. All of these cysteines are highly conserved in the class II GPCRs of which PTH1-Rc is a member. Therefore, constructing a good model for the ligand–ectopic/juxtamembrane bimolecular interface is a much more complicated endeavor.

A homology search with BLAST (Altschul *et al.,* 1990) has identified the conformational preferences of the C-terminal portion of the N-ECD proximal to TM1 of

PTH1- and PTH2-Rcs and ECL3 of PTH1-Rc (Bisello *et al.,* 1998; Rolz *et al.,* 1999). These homology searches suggest that the ECL3 adopts a helical conformations at T^{435}-Y^{443} (Rolz *et al.,* 1999) and that the juxtamembrane portion of N-ECD in PTH1-Rc and PTH2-Rc contains amphiphatic helices K^{172}-M^{189} and L^{129}-E^{139}, respectively (Bisello *et al.,* 1998; Rolz *et al.,* 1999). Unfortunately, such homology searches may not always result in the assignment of a distinct secondary structure to a specific receptor sequence.

Mierke and Pellegrini (1999) modeled the receptor and receptor–ligand complex in a H_2O/decane/H_2O (40 Å each) simulation cell that mimics the membrane milieu. The molecular simulation is carried out in multiple steps in which the heptahelical bundle and/or the cytoplasmic and extracellular domains are allowed to move freely. PTH, in its membrane-associated conformation, is then added to the receptor model, applying the ligand/receptor distance constraints derived from the cross-linking experiments, and additional simulations are carried out. At this stage, additional constraints obtained via site-directed mutagenesis and chimera receptor studies can be incorporated to enhance the modeling procedure.

The most direct way to identify conformational features of the cytoplasmic and ectopic domains of the GPCR is by generating these receptor fragments and examining them by NMR in a membrane mimetic system. Adding small portions of the corresponding TM(s) to the otherwise flexible receptor-derived termini or loops provides an anchor(s) that partially reproduces the native orientation of the receptor domain relative to the membrane-mimicking milieu. Another design element useful in restraining an excised loop sequence from assuming extended conformations is the covalent binding of both ends of the sequence by a linker of ~12 Å that approximate the distance between two consecutive TM domains (Schertler *et al.,* 1993; Schertler and Hargrave, 1995).

Mierke and co-workers have characterized the conformational features of the following PTH1-Rc domains: the third intracellular loop (ICL3), the C-terminal juxtamembrane portion of the N-ECD, and the ECL3 (Bisello *et al.,* 1998; Mierke *et al.,* 1996; Pellegrini *et al.,* 1997; Piserchio *et al.,* 2000a). These peptides were studied in a micellar system that mimics the cellular membrane and generates a micelle–water interface resembling the membrane–water interface. ICL3 was constructed as a 29 amino acid peptide with Cys residues in positions 1 and 28 (Pellegrini *et al.,* 1997b). Side chains of the two cysteines were bridged by an octamethylene linker to maintain the putative ~12-Å distance between two consecutive TM domains. Analysis of this constrained peptide revealed interesting conformational features that allow insight into ICL3–G protein interactions (Pellegrini *et al.,* 1997b).

More relevant to the ligand–receptor bimolecular interactions are analyses of the two ectopic domains that photocross-link to Lys^{13} and Lys^{27} in PTH-(1-34) (Adams *et al.,* 1998; Bisello *et al.,* 1998; Greenberg *et al.,* 2000). The sequence PTH1-Rc[172-189], which contains the 8 amino acid domain 182-189 identified as a contact site for position 13 in PTH (Adams *et al.,* 1998; Zhou *et al.,* 1997), was subjected to a combination of homology search and molecular dynamic calculations using a two-phase simulation cell consisting of H_2O and CCl_4 to mimic a membrane–water interface (Bisello *et al.,* 1998; Pellegrini *et al.,* 1998a). These analyses suggest that the segment R^{179}-E-R-E-V-F-D-R-L-G-M^{189} forms an amphipathic α helix whose axis is parallel to the membrane surface and points away from the heptahelical bundle (Fig. 7, see also color plate) (Bisello *et al.,* 1998; Pellegrini *et al.,* 1998a). ^{1}H-NMR analysis of the synthetic peptide hPTH1-Rc-[168-198] in the presence of micelles was carried out in combination

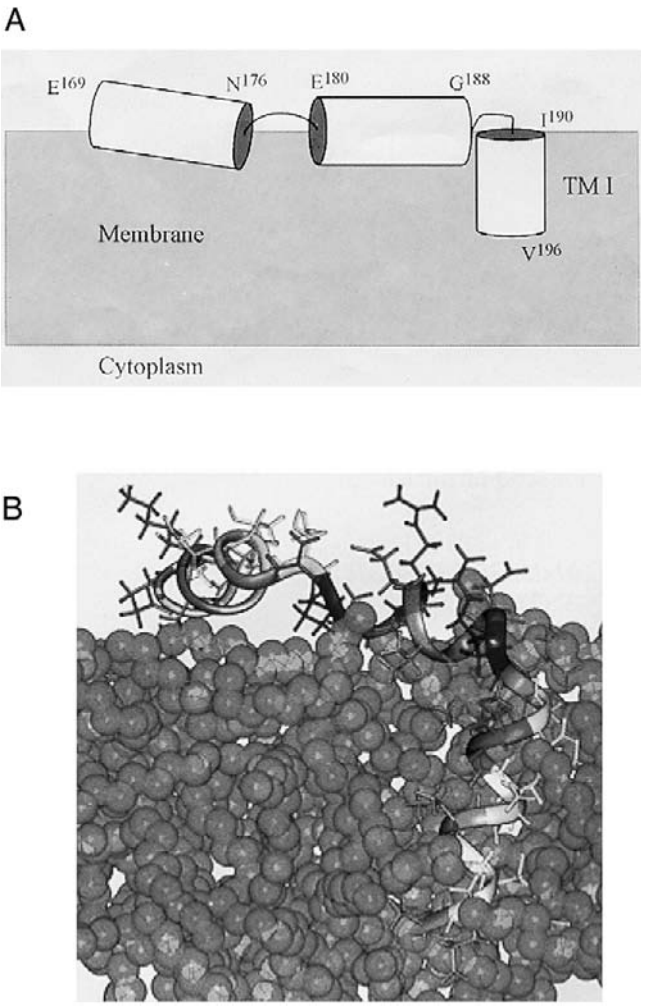

Figure 7 Structural features and topological orientation of PTH1-Rc[168-198], which consists of the juxtamembrane portion of N-ECD and the ectopic portion of TM1 (Pellegrini *et al.,* 1998a). (A) Schematic presentation of the experimentally determined conformation, which consists of three α helices, two of which have been determined to lie on the surface of the membrane; the third, at the top of TM1, is membrane embedded. (B) Molecular simulation of this peptide in a water/decane simulation cell used to refine the structure obtained in the presence of dodecylphosphocholine (DPC) micelles used in the NMR study. Decane molecules are shown in green as CPK space-filling spheres. The peptide molecule is colored according to hydrophobicity (blue, polar; red, hydrophobic). (See also color plate.)

with distance geometry and molecular dynamic simulation (Pellegrini *et al.,* 1998a). The analysis identifies three helical segments: 169-176, 180-188, and 190-196. The C-terminal helix, hPTH1-Rc[190-196], corresponding to the ectopic portion of the first TM helix, is hydrophobic and embeds perpendicularly into the micelle. The other two α helices, [180-188] and [169-176], lie on the membrane surface (Fig. 7). Polar residues in the linker, E^{177} and R^{179}, and in the middle helix, R^{181}, E^{182}, D^{185}, and R^{186}, are exposed to the solvent, whereas the hydrophobic residues, F^{173}, F^{184}, and L^{187}, project toward the hydrophobic membrane (Pellegrini *et al.,* 1998a). It is plausible that the positively charged Lys^{13} in PTH participates in stabilizing the Coulombic interaction with the negatively charged E^{182} and D^{185} located at the solvent-exposed hydrophilic phase of the receptor sequence. Nevertheless, this may not be an essential interaction, as analogs in which the ε amino on Lys^{13} is blocked by acylation maintain high affinity and efficacy. Point mutations replacing negatively charged amino acids with neutral or positively charged ones will help assess this putative interaction further.

Combining the putative TM bundle obtained in the modeling studies with the experimentally derived conformation of the synthetic hPTH1-Rc-[168-198] establishes a partial PTH1-Rc model that can be used to dock hPTH-(1-34) in its experimentally derived putative bioactive conformation (Pellegrini *et al.,* 1998b). This "on silicon" experiment results in the first generation of an experimentally based model of the PTH-hPTH1-Rc complex (Fig. 8, see also color plate) (Bisello *et al.,* 1998). Using cross-linking data as a docking cue places the C-terminal amphiphilic helix of the legand parallel to the membrane-aligned portion of the receptor-derived peptide. This positioning allows the formation of complementary Coulombic interactions between the polar residues in the C-terminal helix, comprising the principal binding domain of the ligand and the polar residues E^{177}, R^{179}, R^{181}, E^{182}, D^{185}, and R^{186} in the receptor-derived peptide. Interestingly, this docking procedure brings only M^{425}, and not M^{414}, into sufficient proximity to permit cross-linking to position 1 in PTH. Therefore, these observations are in complete agreement with the results obtained through cross-linking studies (Bisello *et al.,* 1998).

The contact site between position 27 in PTH and L^{261} in the ECL1 of PTH1-Rc (Greenberg *et al.,* 2000) offers another target for structural studies (Piserchio *et al.,* 2000a). The synthetic peptide hPTH1-Rc[241-285], composed of ECL1 and a few residues from the ectopic portions of TM2 and TM3 at the N and C termini of the loop, respectively, was subjected to detailed conformational analysis. These studies included high-resolution NMR in the presence of dodecylphosphocholine micelles followed by distance geometry calculations and molecular dynamic simulations. Piserchio and co-workers found that this receptor fragment contained three α-helical segments: [241-244], [256-264], and [275-284] (Fig. 9, see also color plate) (Piserchio *et al.,* 2000a). The first and last helices correspond to the ectopic portions of TM2 and TM3, respectively. Hydrophobic amino acids corresponding to the ectopic portion of the TMs are more strongly associated with the lipid micelle and may serve as membranal anchors. Moreover, all of the hydrophobic residues in the partially ordered central helical portion (terminated by the unique helix-breaking sequence P^{258}-P-P-P^{261}) project toward the lipid surface (Piserchio *et al.,* 2000a).

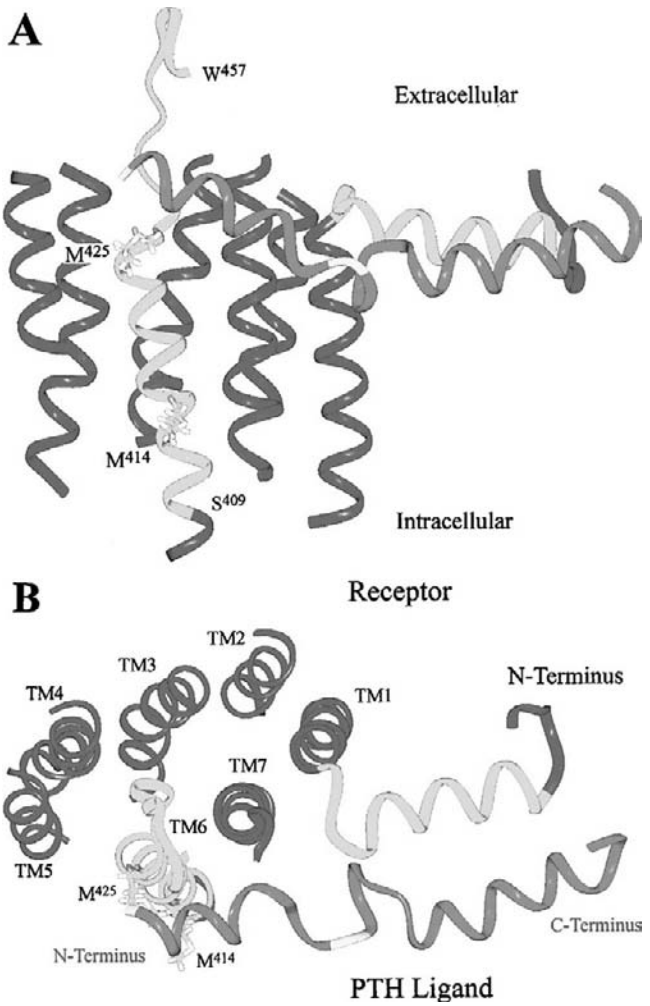

Figure 8 First generation of an experimentally based model of PTH–hPTH1-Rc complex. For clarity, only portions of the TM helices, N terminus, and the third extracellular loop are shown in *blue* (noncross-linked domains) and green (contact domains hPTH1-Rc[173-189] and hPTH1-Rc[409-437]) (A, side view; B, top view). The amphipathic α helix of the extracellular N terminus of the receptor is projecting to the *right*, lying on the surface of the membrane. The high-resolution, low-energy structure of hPTH-(1-34) determined by NMR in a micellar environment is presented in *pink*. Residues in cross-linking positions 1 and 13 of hPTH-(1-34) are denoted in *yellow*. The C-terminal amphipatic α helix of hPTH-(1-34) is aligned in antiparallel arrangement with the amphipatic α helix of the extracellular N terminus hPTH1-Rc[173-189]), contiguous with TM1 and encompassing the 17 amino acid contact domain (in *green*) to optimize hydrophilic interactions. Side chains of residue M^{414} and M^{425} within the "contact domain" comprised of TM6 and the third extracellular loop (hPTH1-Rc[S^{409}-W^{437}]) are shown (Bisello *et al.,* 1998). (See also color plate.)

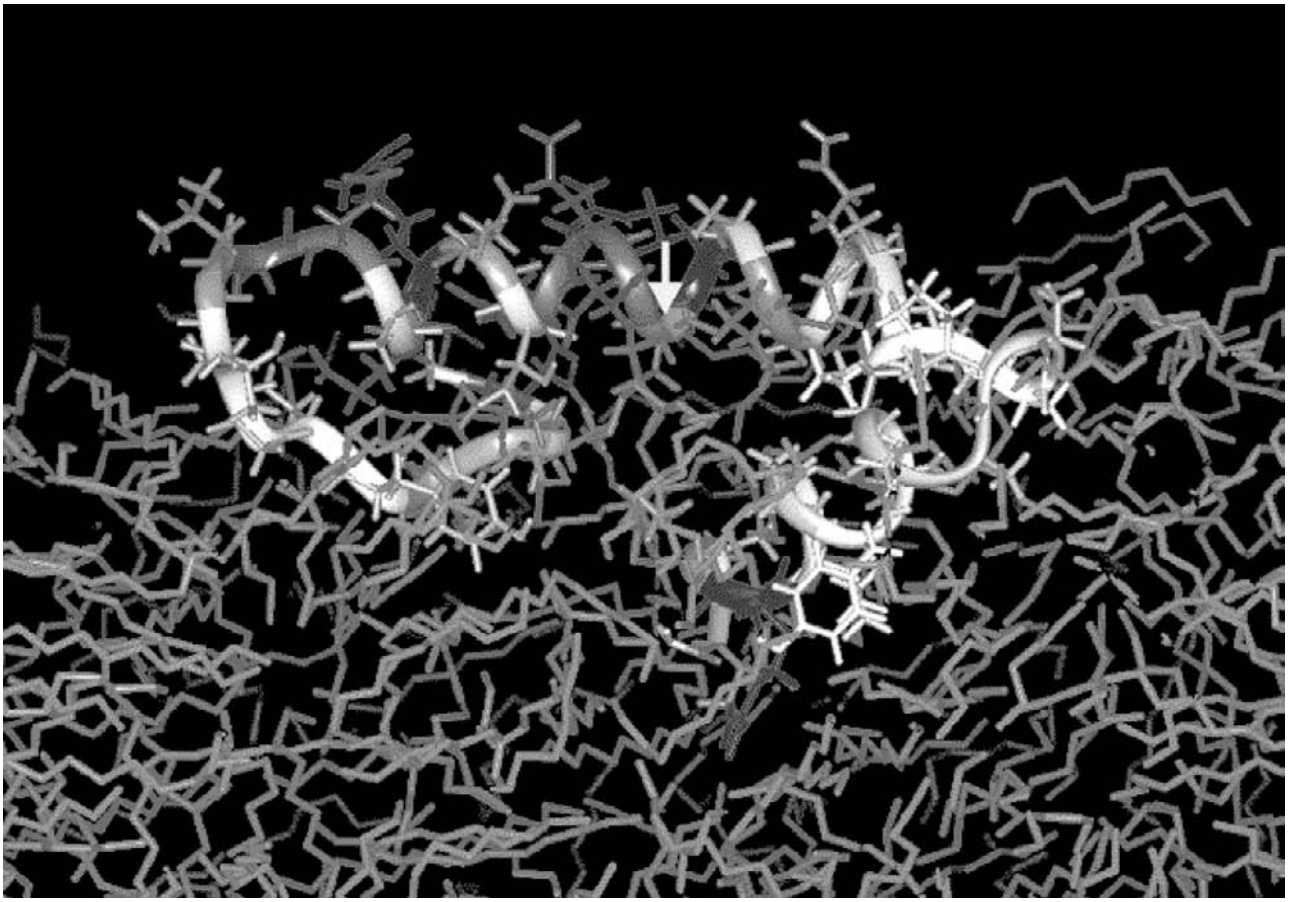

Figure 9 Structure of PTH1-Rc(241-285) comprising the ECL1 from the molecular dynamics simulation in a water/decane simulation cell. Peptide residues are colored according to side chain hydrophobicity (blue, polar; red, hydrophobic). Decane molecules are depicted in green; water molecules are not displayed for clarity. Residues belonging to TM2 and TM3 are embedded into the decane phase. The yellow arrow indicates the location of L^{261}, the cross-linking site to Lys^{27} in PTH-(1-34) (Piserchio *et al.,* 2000a). (See also color plate.)

The emerging structure of ECL1 is very helpful in understanding the bimolecular ligand–receptor interaction revealed by cross-linking studies. It suggests an antiparallel organization of the two amphiphilic helices: the C-terminal helix in PTH, which includes the photophore carrier, Lys^{27}, and the [256-264] helical portion in ECL1, containing the contact site L^{261} (Pellegrini *et al.,* 1998b). The two helices are oriented with their hydrophobic faces interfacing the membrane and their polar faces exposed to the solvent and are capable of forming numerous intermolecular interactions. Integrating these additional findings into the PTH–PTH1-Rc model results in the enhancement and refinement of the overall bimolecular topology. It defines more unambiguously the position of the C-terminal helix of PTH. This helix is now placed between the ECL1 and the C-terminal juxtamembrane helix of the N-ECD of hPTH1-Rc (Fig. 10). The topological organization of the receptor is consistent now not only with the individual bimolecular contact sites between position 1, 13, and 27 in PTH and the respective sites in PTH1-Rc (namely M^{425}, a site in the proximity of R^{186}, and L^{261}), but also accommodates the contact site between position 23 in PTHrP and Y^{23}-L^{40} in PTH1-Rc.

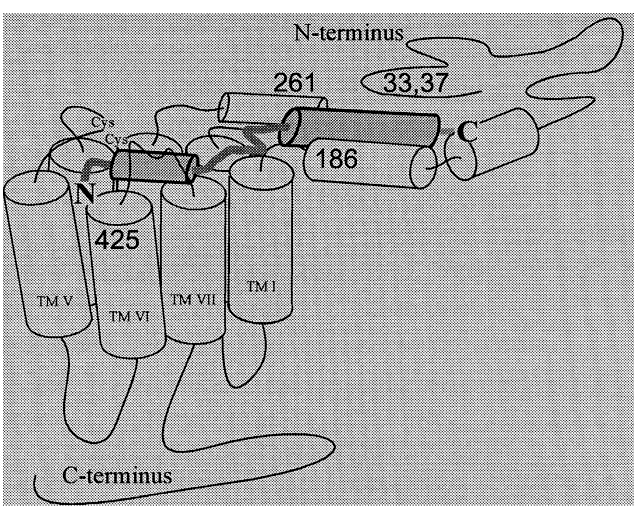

Figure 10 Topological organization of the PTH–PTH1-Rc complex. Schematics are the results of conformational analysis of ligand and receptor domains, contact sites as revealed by photoaffinity, cross-linking studies, and molecular modeling. The location of contact sites in PTH1-Rc derived from PAS studies is indicated (Ser^{1}–M425; Lys^{13}–R186; Trp^{23}–T33/Q37; Lys^{27}–L261). Helical domains in the receptor and ligand are presented as cylinders (Piserchio *et al.,* 2000a).

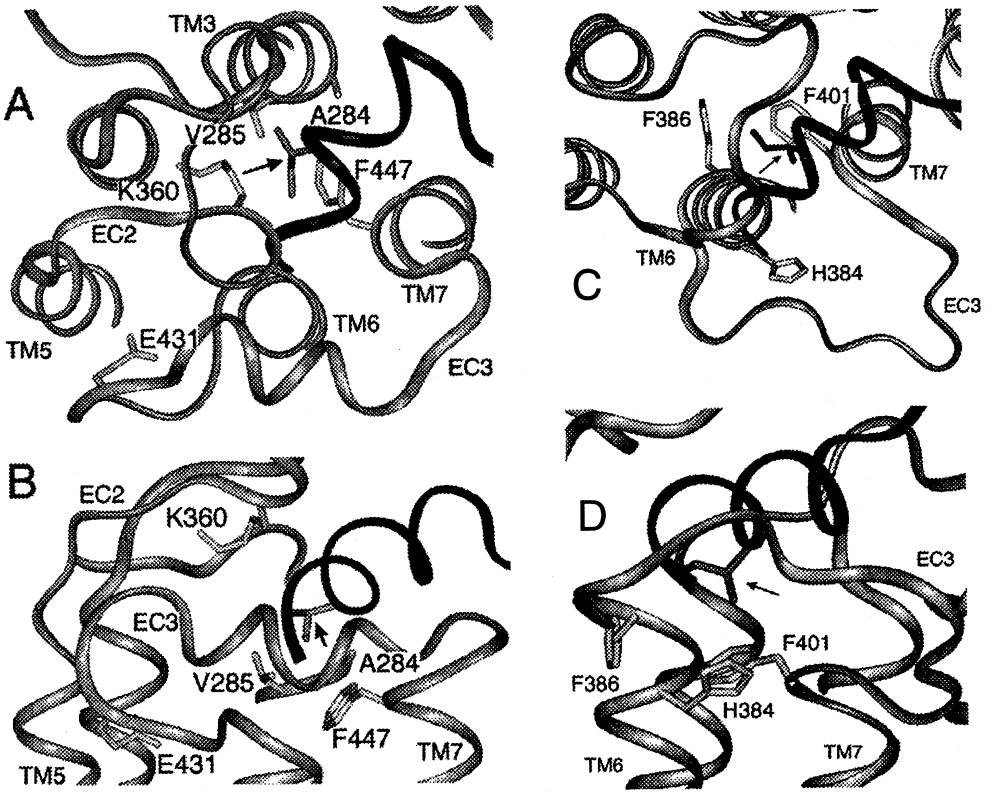

Figure 11 Illustration of the binding pocket for Ile^5 of PTH in PTH1- and PTH2-Rc (Rolz *et al.,* 1999). In PTH1-Rc Ile^5, the side chain (indicated by black arrow) is accommodated in a hydrophobic pocket made up of the ectopic portions of TM3 (A^{284} and V^{285}) and TM7 (F^{447}). Top (A) and side (B) views of this pocket in PTH1-Rc. The receptor and the ligand are depicted as ribbons in gray and black, respectively. In PTH2-Rc, the binding pocket for residue 5 in the ligand is more limited in size compared to the same pocket in PTH1-Rc due to the presence of F^{386} and F^{401} in the ectopic portions of TM6 and TM7, respectively. Top (C) and side (D) views of this pocket in PTH2-Rc. In addition, in PTH1-Rc the presence of E^{432} at the ectopic portion of TM6, in the entrance to the binding pocket of His^5, can attract His^5 by favorable Coulombic interaction. However, in PTH2-Rc the presence of H^{384}, at the bottom of the binding pocket for residue 5, will destabilize the interaction with an incoming side chain of His^5-containing ligand.

Rölz and co-workers studied in detail the emerging experimentally derived model complexes (Adams *et al.,* 1998; Bisello *et al.,* 1998; Zhou *et al.,* 1997) between PTH/PTHrP ligands and PTH1- and PTH2-Rc to identify interresidue contacts within the heptahelical TMs (Rolz *et al.,* 1999). They are able to offer insights that can explain ligand specificity (Behar *et al.,* 1996a; Gardella *et al.,* 1996a), the consequences of some site-directed mutations (Bergwitz *et al.,* 1997; Clark *et al.,* 1998; Gardella *et al.,* 1996b; Lee *et al.,* 1995a; Turner *et al.,* 1998), the constitutive activity of JCM-mutated receptors (Schipani *et al.,* 1995, 1997), the consequences of cross-linking studies (Bisello *et al.,* 1998), some structure–activity relations in the ligand (Cohen *et al.,* 1991; Rosenblatt *et al.,* 1976), and some aspects of signal transduction. For example, there is a loss of affinity for PTH-(1-34) following mutations W437A/L/E or Q440A/L in PTH1-Rc, an effect much reduced for PTH-(3-34) (Lee *et al.,* 1995a). The model positions both Q^{440} and W^{437} on the same face of ECL3, both projecting toward the center of the TM bundle (Rolz *et al.,* 1999). The side chains of these residues participate in forming the hydrophobic pocket that accommodates Val^2 in the ligand, providing stabilizing interactions by shielding it from the extracellular aqueous environment. Mutating Q^{440} and/or W^{437} to any smaller or a more polar residue will compromise the binding pocket for Val^2 by exposing it to water. The model also attempts to explain position 5 in PTH and PTHrP as a receptor-subtype specificity switch (Behar *et al.,* 1996a; Gardella *et al.,* 1996a). It suggests that in PTH1-Rc, the Ile^5 side chain is accommodated by a hydrophobic pocket. The bottom of this pocket is composed of hydrophobic residues at the ectopic end of TM3 (A^{284} and V^{285}) and TM7 (F^{447}), and it is large enough to accommodate either Ile^5 or His^5 (Fig. 11). In PTH2-Rc, due to the presence of F^{386} and F^{401} located at the ectopic ends of TMs 6 and 7, respectively, the binding pocket for residue 5 is reduced in size compared to the same pocket in PTH1-Rc. Therefore, the smaller pocket in PTH2-Rc cannot accommodate His^5-containing PTHrP or hybrid ligands and discriminates against them (Fig. 11) (Rolz *et al.,* 1999). These authors also encourage the use of their model as a tool for predicting the pharmacological consequences of

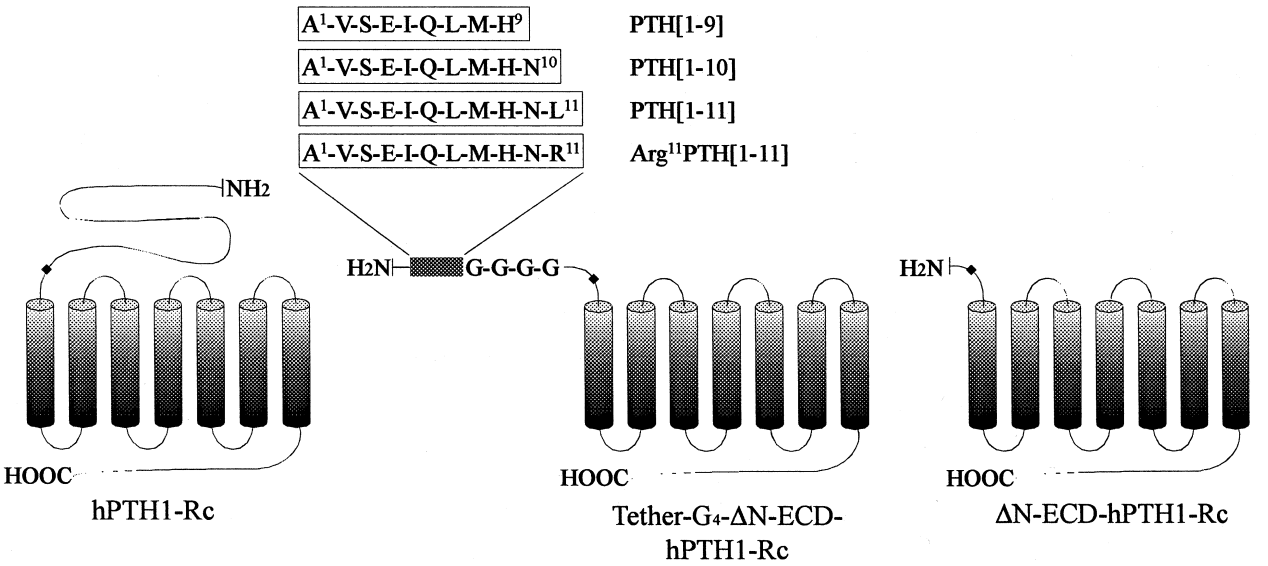

Figure 12 Design of ligand-tethered hPTH1-Rc. Schematics include the wild-type hPTH1-Rc on the left; N-ECD-truncated hPTH1-Rc (ΔN-ECD-hPTH1-Rc) on the right, and the tethered G_4-ΔN-ECD-hPTH1-Rc in the middle. Also listed are the different sequences derived from the N-terminal of PTH that are tethered to E^{182} (solid diamond) via a tetraglycine (G_4) spacer. All the recombinant receptors retain the 23 amino acid residues that form the signal sequence in the native receptor. Therefore, the putative N-terminal residue in all the receptors is Y^{23} generated upon cleavage by signal peptidase. The figure was modified from Shimizu and co-workers (2000a).

specific mutations such as receptor-subtype specificity-reversing mutations.

Ligand-Tethered hPTH1-Rc

Shimizu and coworkers (2000a) reported, what seems to be the absolute integration of ligand and receptor entities. Borrowing from the protease-activated thrombine receptor system (Kawabata and Kuroda, 2000), they generated constitutively active, ligand-tethered hPTH1-Rc (Shimizu *et al.,* 2000a). The elements they use in the construction of the ligand-tethered hPTH1-Rc are the following (Fig. 12): 1) a peptide as small as PTH-(1-14) that can stimulate weak cAMP formation with both wild-type and N-ECD-truncated rPTH1-Rc, rΔNt (Luck *et al.,* 1999); (2) residues 1-9 in PTH-(1-14), critical for interacting with the rΔNt (Luck *et al.,* 1999); (3) position 13 in PTH, which photocross-links to PTH1-Rc in the proximity of R186 (Adams *et al.,* 1995; Zhou *et al.,* 1997); and (4) the hydrophobic residues F^{184} and L^{187} in PTH1-Rc, which are functionally important for the interaction with the 3-14 portion of PTH-(1-34) (Carter *et al.,* 1999b). In this ligand-tethered hPTH1-Rc, N-ECD was truncated from E^{182}, which is juxtaposed to the TM1 (designated as ΔN-ECD-hPTH1-Rc). This truncated receptor was extended by a Gly_4 spacer (G_4-ΔN-ECD-hPTH1-Rc) and linked to N-terminal fragments of PTH. These fragments very in size from 9 to 11 residues (Shimizu *et al.,* 2000a).

Transient expression of the ligand-tethered receptor PTH-(1-9)-G_4-ΔN-ECD-hPTH1-Rc in COS-7 cells resulted in 10-fold higher basal cAMP levels compared to the wild-type hPTH1-Rc control. Tethering the extended and more potent [Arg^{11}]PTH-(1-11) resulted in 50-fold higher basal c-AMP levels than those seen with the wild-type hPTH1-Rc. Interestingly, like in PTH-(1-14) (Luck *et al.,* 1999), where Val^2, Ile^5, and Met^8 are the most critical residues for activation, these residues were also the most critical ones for the constitutive activity of the [Arg^{11}]PTH-(1-11)-G_4-ΔN-ECD-hPTH1-Rc (Shimizu *et al.,* 2000a).

The elegance of this study lies in devising a unique way to specifically "immobilize" the principal activation domain of the ligand in the proximity of the contact sites critical for receptor activation. Correspondence between the efficacy-enhancing substitutions in PTH-(1-11) and the tethered peptide supports the notion that both exercise the same interactions with the receptor that lead to receptor activation. The high effective molarity of the tethered ligand minimizes the role of binding affinity in bimolecular interactions compared to free ligand, thus allowing the identification of residues within the tethered ligand essential for induction of activity. However, the accessibility to the ligand tethered–receptor system requires the employment of molecular biology and therefore it is most applicable to tethered ligands composed of coded amino acids. In addition, stringent requirements for the efficient expression of tethered ligand–receptors in a relevant cellular background may turn out to be major obstacles in practicing and extending this approach in the future. It remains to be demonstrated whether the tethered ligand–receptor system is a source for identifying structural constraints that can contribute to the refinement of the experimentally based ligand–receptor model and to rational drug design. The elimination of most of the entropic component from the ligand–receptor interaction may generate contact interactions

and produce activation mechanisms that differ from those involved in the interaction with a diffusible ligand.

The quality of any model, namely its capacity to realistically represent ligand–receptor interactions and predict the nature of the interface, is based primarily on data and procedures used in its construction. A model can become highly speculative and thus only remotely relevant to biology if overloaded with data derived from indirect and circumstantial observations. It is important to avoid over interpretation of any model and remember the assumptions and approximations used in its construction. Finally, any extrapolation derived from any model must be tested in order to validate its predictive potential. In summary, the evaluation of any model of the ligand–receptor complex in the PTH/PTHrP system should follow the principles mentioned earlier.

Ligand–Receptor "Two-Site" Dynamic Model An Emerging Paradigm

The study of PTH/PTHrP–PTH1-Rc interactions has arrived to a very exciting stage in which several lines of evidence converged to allow understand ligand–receptor interaction at a much more detailed level. Moreover, the integrated and multidisciplinary approach to ligand–receptor studies in the PTH field turned to be synergistic in nature. A study by Hoare and co-workers (2001) brought together several key observations to suggest that ligand–PTH1-Rc interactions can be described by a "two-site" dynamic model (Fig. 13).

The foundation for this model was put down by numerous observations. Like several other class II GPCRs (Beyermann *et al.,* 2000; Holtmann *et al.,* 1995; Juarranz *et al.,* 1999; Stroop *et al.,* 1995), PTH1-RC can be devided into two functional domains: the large N-ECD (N domain) has been proposed to provide most of the principal binding interactions with the ligand (Bergwitz *et al.,* 1996; Juppner *et al.,* 1994) and the rest of the receptor, which includes the ECLs, TMs, and the ICLs designated as the juxtamembrane domain (J domain). Interactions of the ligand with this domain lead to activation and signal transduction (Bergwitz *et al.,* 1996; Gardella *et al.,* 1994; Juppner *et al.,* 1994; Turner *et al.,* 1996). A similar two functional domain architecture is also found in the ligands, PTH and PTHrP; the 15-34 sequence includes the principal binding domain (Caulfield *et al.,* 1990; Rosenblatt *et al.,* 1980), and the 1-14 sequence includes the activation domain for intracellular signaling through AC (Bergwitz *et al.,* 1996; Gardella *et al.,* 1991; Luck *et al.,* 1999; Shimizu *et al.,* 2000a,b; Takasu *et al.,* 1999a) and PKC (Takasu *et al.,* 1999a). Mutational analyses of the PTH1-Rc and analyses of PTH/PTHrP–PTH1-Rc photoconjugates revealed potential contact sites and spatial proximity between specific amino acid residues of the ligand and the receptor. Structural analyses of ligand and receptor domains, together with computer modeling, generated for the first time experimentally based models of the PTH–PTH1-Rc complex with atomic details.

In the "two-site" model of PTH/PTHrP–PTH1-Rc the C-terminal portion of PTH or PTHrP interacts with the N domain of the receptor and the N-terminal sequence of the ligand binds to the J-domain of the receptor (Bisello *et al.,* 1998; Juppner *et al.,* 1994; Luck *et al.,* 1999; Mannstadt *et al.,* 1999). Nevertheless, this model does not account for the dynamic nature of receptor conformation moving between G protein-coupled (GR) and -uncoupled (R) conformational states and its effect on the mechanism of ligand binding.

Hoare and co-workers (2001) reported that [$Ala^{3,10,12}$, Arg^{11}] rPTH-(1-14)NH_2 [PTH-(1-14)] and PTH-(3-34) bind

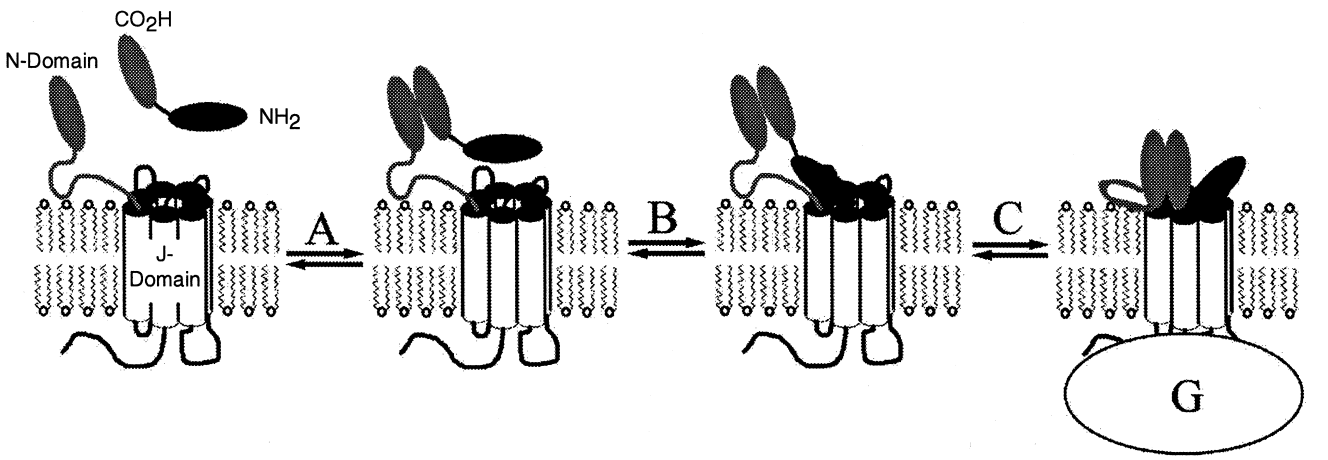

Figure 13 Model for modulation of PTH-(1-34) binding to the PTH1-Rc by the G protein. (A) The C-terminal portion of the ligand interacts with the N domain of the receptor. Subsequently, the N-terminal portion of the ligand binds to the J domain of the receptor (B). (C) Interaction of the receptor with the G protein forms RG, increasing the affinity to the J domain, possibly by producing a closure of receptor conformation. Reciprocally, interaction of the ligand with the J domain increases the affinity of receptor for the G protein, leading to its activation. Binding of the G protein to the other states of the receptor (the unoccupied and the one interacting only with the N-terminal portion of the ligand) has been omitted for clarity (Hoare *et al.,* 2001).

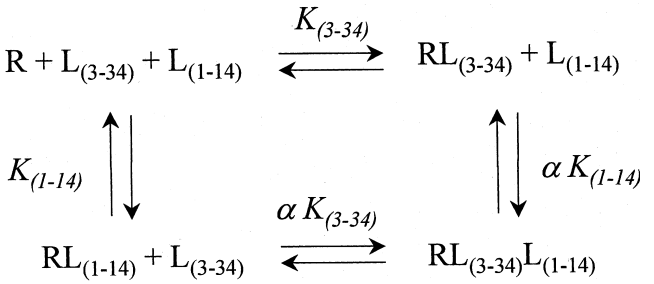

Figure 14 Model for simultaneous binding of ^{125}I-PTH-(3-34) and PTH-(1-14) to PTH1-Rc, where R is the receptor; $L_{(3\text{-}34)}$ is ^{125}I-PTH-(3-34); $L_{(1\text{-}14)}$ is PTH-(1-14); $K_{(3\text{-}34)}$ is the equilibrium association constant for ^{125}I-PTH-(3-34); $K_{(1\text{-}14)}$ is the equilibrium association constant for PTH-(1-14); and α is the cooperativity factor defining the effect of $L_{(1\text{-}14)}$ occupancy on the receptor-binding affinity of $L_{(3\text{-}34)}$ and reciprocally the effect of $L_{(3\text{-}34)}$ occupancy on the receptor-binding affinity of $L_{(1\text{-}14)}$ (Hoare *et al.,* 2001).

to two spatially distinct sites in PTH1-Rc (Fig. 14): the former binds selectively to RG, predominantly through the J domain, and only partially inhibits ^{125}I-PTH-(3-34) binding, and the latter binds predominantly to the N domain, is $GTP_{\gamma}S$ insensitive, and only partially inhibits PTH-(1-14)-stimulated AC activity. The higher binding affinity of agonist ligands to RG vs the R state of the receptor implies different conformations of the receptor in both states. At the uncoupled state, PTH-(1-14) and the PTH-(3-34) bind almost independently of each other. However, the negative cooperativity between the binding of ^{125}I-PTH-(3-34) and PTH-(1-14) is significantly greater in the RG state than in the uncoupled one. Moreover, agonist binding to the RG state is pseudo-irreversible. PTH-(3-34) inhibits PTH-(1-14)-stimulated cAMP accumulation, increases EC_{50} by 18-fold, and reduces E_{max} in a noncompetitive manner.

Taken together, these observations suggest that the receptor is in a "open" conformation in the uncoupled state, permitting simultaneous binding of both PTH-(3-34), the N domain interacting ligand, and PTH-(1-14), the J domain interacting ligand. At the coupled state, for which agonist has higher affinity than for the uncoupled state, the receptor is in a more "closed" conformation, preventing simultaneous access of both ligands to their preferred binding sites and trapping the ligand within the coupled receptor.

This study offers new and interesting insights on the mechanism of PTH–PTH1-Rc interaction. Although it does not offer structural details for the dynamic process, it does provide some new concepts that will stimulate additional studies to validate and refine the emerging model.

Future Directions

Our current understanding of bimolecular PTH/PTHrP–PTH1-Rc interactions at the atomic level and the events that follow receptor occupancy and receptor activation is still incomplete. Discovery of new PTH receptor subtypes and new endogenous ligands has provided new opportunities for understanding structure–function relations. When sufficiently advanced, the experimentally based models of the bimolecular ligand–receptor interface will allow us not only to understand the pharmacological profiles of PTH- and PTHrP-derived ligands better, but also serve as a molecular template for rational drug design to develop more selective and more efficacious hormone-derived and hormone mimetic drugs. This model will also provide the means for understanding aberrant mechanisms underlying pathological mutations of PTH1-Rc that lead to clinical disorders such as JMC and BLC.

The lack of a truly bone anabolic drug to complement the currently available arsenal of anti resorptive drugs for osteoporosis is highly noticeable. One area of research that has grown steadily in interest over the last decade is the potential utility of PTH- or PTHrP-derived agonists for the treatment of osteoporosis (Goltzman, 1999). It is very well established that low-dose, intermittent administration of several forms of PTH stimulates bone formation, leading to an overall anabolic effect on bone (Howard *et al.,* 1981; Tam *et al.,* 1982). Further observations have been made in animals and in human studies (Hock *et al.,* 1988; Hodsman and Fraher, 1990; Reeve *et al.,* 1990; Slovik *et al.,* 1986; Tada *et al.,* 1990; Tsai *et al.,* 1989; Wronski *et al.,* 1993). This beneficial effect on bone occurs despite the well-documented action of PTH in stimulating bone resorption via increased osteoclast number and activity. Nevertheless, instigating osteoblastic bone formation without concomitant activation of osteoclasts and resultant bone resorption remains an ultimate goal for the treatment of osteoporosis. To this end, future focus on the development of signaling-selective PTH or PTHrP analogs is one of the more promising directions for analog design.

The ongoing quest to identify the common molecular target for all bone anabolic agents and treatments may turn out to be either a very ambitious objective or a completely elusive one. However, based on current knowledge, dissection of PTH-induced signaling pathways to identify the anabolic one seems to be a more plausible and attainable goal. Nevertheless, discordance between binding affinity and signaling efficacy *in vitro* on the one hand and the predictive potential of these *in vitro* parameters for anabolic activity *in vivo* (Frolik *et al.,* 1999) on the other are puzzling. We anticipate that better understanding of the mechanism of PTH ligand–receptor interactions will provide the answers and guide the development of novel, potent, safe, and compliance-friendly bone anabolic drugs. Important subjects for future studies include characterizing the conformational changes in the PTH1-Rc induced by interaction with ligands of different pharmacological traits, elucidating the mechanisms involved in receptor coupling to the various G proteins, identifying and understanding the role of interactions of different adapter molecules participating in the signaling cascades, identifying and assigning function to the different PTH-induced signaling pathways and downstream effects that regulate gene expression and protein synthesis, and understanding the mechanism of receptor desensitization/

internalization and recycling, and their roles in PTH-induced activities *in vitro* and *in vivo.* Given the probability of substantial progress in many of the frontiers just listed, PTH or PTHrP agonists are likely to find clinical utility in the treatment of disorders of calcium and bone metabolism.

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CHAPTER 27

Actions of Parathyroid Hormone

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Parathyroid hormone is essential for the maintenance of calcium homeostasis through direct actions on its principal target organs, bone and kidney, and through indirect actions on the gastrointestinal tract. Parathyroid hormone acts directly on the skeleton to promote calcium release from bone and on the kidney to enhance calcium reabsorption. The indirect effects of parathyroid hormone on the gastrointestinal tract lead to greater calcium absorption through its actions to facilitate the conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D. Actions of parathyroid hormone at these three sites, the kidney, the skeleton, and the gastrointestinal tract, result in restoration of the extracellular calcium concentration. When the hypocalcemic signal for parathyroid hormone release returns to normal, calcium ion continues to regulate the release of parathyroid hormone. In the setting of a hypercalcemic signal, not due to abnormal secretion of parathyroid hormone (i.e., primary hyperparathyroidism), parathyroid hormone secretion is inhibited. The resulting physiological events associated with reduced concentrations of parathyroid hormone lead to reduced calcium mobilization from bone, a reduction in renal tubular reabsorption of calcium, and, by virtue of reducing the conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D, to reduced absorption of dietary calcium. Hence in hypercalcemic states, control of parathyroid hormone secretion by calcium reduces serum calcium levels.

Parathyroid hormone helps to regulate phosphorus metabolism. An increase in phosphorus leads to a reduction in the circulating calcium concentration. The resultant increase in parathyroid hormone leads to phosphaturia, a classical physiological effect of the hormone and restoration of the serum phosphorus concentration (see Chapter 20).

Cellular responsiveness to parathyroid hormone occurs via receptor-mediated activation of intracellular events and several major biochemical pathways. Details of the initial steps by which a change in extracellular calcium is sensed by the parathyroid cell and the mechanisms by which parathyroid hormone binds to its receptor, as well as the induction of several different messenger systems, are covered in Chapters 23, 26, and 28, respectively. This chapter focuses on the physiological and cellular effects of parathyroid hormone on the skeleton but also considers the kidney and the cardiovascular system, the latter being a recently recognized target of parathyroid hormone action.

Biologically Active Parathyroid Hormone and Its Peripheral Metabolism

The principal form of biologically active parathyroid hormone is the intact molecule, PTH(1-84). The half-life of PTH(1-84) in the circulation is less than 3 min. Clearance of parathyroid hormone occurs rapidly in the liver (60–70%), kidney (20–30%), and, to a much lesser extent, in other organs (Bringhurst *et al.,* 1988). Peripheral metabolism of parathyroid hormone is not altered by dietary calcium, hypercalcemia, or parathyroid disease (Bringhurst *et al.,* 1989; Fox *et al.,* 1983). Hepatic clearance is complex, involving high capacity, nonsaturable uptake by Kupffer cells

that cleave intact parathyroid hormone into discrete fragments and by hepatocytes that take up small amounts of hormone. Carboxy-terminal (C-terminal) fragments, resulting from cleavage between residues 33 and 34 and 36 and 37 of intact parathyroid hormone, are released by Kupffer cells into the circulation. The half-life of C-terminal fragments is 5–10 times longer than intact hormone. C-terminal fragments of parathyroid hormone are also metabolized and cleared by the kidney and hence do not reenter the circulation easily. The rapid metabolism of biologically active parathyroid hormone ensures that available parathyroid hormone for receptor binding is directed by the secretory rate of parathyroid hormone from the parathyroid glands in response to minute-to-minute fluctuations in the serum calcium concentration.

In normal settings, 70–95% of circulating parathyroid hormone is present as inactive C-terminal fragments; intact PTH(1-84) constitutes only 5–30% of the circulating forms of the molecule. The amino-terminal (N) fragment, PTH-(1-34), has biological activity comparable to PTH(1-84), and it may be generated during hepatic proteolysis of the intact molecule. Despite these points and the fact that many studies of PTH action have employed the N-terminal fragment, there is little evidence that it is present in appreciable quantities in the circulation. The liver-generated N-terminal product is degraded rapidly *in situ* (Bringhurst *et al.,* 1988). If the N-terminal fragment of parathyroid hormone serves biological functions *in vivo,* it would appear to be necessary to invoke local production at target sites themselves for which there is currently little convincing evidence.

Similar to C-terminal fragments, midregion fragments of parathyroid hormone are present in the circulation and are cleared through renal mechanisms. Under certain circumstances, studies have supported a biological role for these mid- and C- terminal fragments of parathyroid hormone. Murray and colleagues (1994), for example, have demonstrated that the C-terminal fragment, PTH(53-84), can stimulate alkaline phosphatase activity in osteoblast cells under conditions when the intact molecule is inhibitory. This and other C-terminal fragments have been considered as possible activators of osteoclast formation (Kaji *et al.,* 1994). The notion that cleavage products of polypeptide hormones may contain their own informational content gained further credence by studies with the sister molecule, parathyroid hormone-related protein (PTHRP). Both PTH and PTHRP contain dibasic sequences of amino acids that theoretically render them susceptible to intracellular processing enzymes. With PTHRP, several such cleavage products like PTHRP(67-86) and PTHRP(107-111) have been shown in special circumstances to stimulate placental calcium transport and to inhibit osteoclastic bone resorption, respectively (Care *et al.,* 1991; Fenton *et al.,* 1991, 1993). These observations suggest that PTH and PTHRP are polyhormones (or polyparacrine factors) that lead to the generation of a series of cell-specific, biologically active products. This concept is developed further in Chapters 28, 29, and 30. Studies suggesting the presence of unique receptor proteins for these nonclassical PTH products add strength to this hypothesis (Inomata *et al.,* 1995; Orloff and Stewart, 1995; Orloff *et al.,* 1995). The idea also becomes more interesting in view of the fact that these so-called breakdown products increase markedly in the presence of renal impairment.

Circulating heterogeneity of parathyroid hormone is due not only to peripheral metabolism but also to secretion of various fragments by the parathyroid glands themselves. Proteolytic degradation of parathyroid hormone within the parathyroid cell provides a calcium-sensitive mechanism to regulate intracellular parathyroid hormone content and the species of parathyroid hormone to be secreted. The absolute rate of hormone degradation is decreased under conditions of hypocalcemia, thus leading to increased secretion of intact parathyroid hormone. Intracellular hormone degradation is increased during states of hypercalcemia, leading to a reduction in secretion of intact parathyroid hormone (MacGregor *et al.,* 1979; Mayer *et al.,* 1979). *In vivo,* although secretion of intact parathyroid hormone persists at low levels when hypercalcemia is present, the ratio of mid- and C-terminal fragments to intact hormone is higher (D'Amour *et al.,* 1986; Kubler *et al.,* 1987). Thus, C-terminal fragments of parathyroid hormone are even more predominant in the circulation during hypercalcemic states, not due to hyperparathyroidism. Conversely, during hypocalcemia, intact parathyroid hormone becomes more evident in the circulation.

Most recently, a larger and heretofore not well-recognized molecule, PTH(7-84), has been found to circulate in appreciable quantities. Similar to other peptide fragments that do not contain the amino-terminal portion of the full-length molecule, this fragment is thought to be generated by peripheral metabolism or from the parathyroid gland when hypercalcemia is present. In renal failure, this fragment is likely to accumulate because its primary clearance route is the kidney. The recent development of an immunoradiometric assay that measures only the PTH(1-84) whole molecule may be of use in clinical situations where it is essential to measure only biologically intact hormone (see Chapter 75). It is also of interest that this large "inactive" fragment has been shown to harbor antagonist properties not only with respect to the calcemic actions of active parathyroid hormone, but also in nonparathyroid examples of hypercalcemia. The idea that PTH(7-84) may have its own actions, perhaps through a specific receptor system alluded to earlier, adds to the complexity of servomechanisms at which both parathyroid hormone-dependent and parathyroid hormone-independent hypercalcemia could be regulated, at least in part, by the parathyroid glands.

The proportion of N-terminal, C-terminal, large fragment, or intact PTH in the circulation has become a topic of great interest as our understanding of receptor–ligand interactions unfolds further (see Chapters 24 and 26). Cross-linking and mutagenesis studies suggest that interactions between the carboxyl-terminal portion of PTH(1-34) and the amino-terminal extracellular domain of the PTH receptor activate second messenger signaling (Erdman

et al., 1998; Luck *et al.,* 1999; Greenberg *et al.,* 2000; Grauschopf *et al.,* 2000). The carboxyl terminus of PTH induces receptor activation though conformational changes and/or G protein coupling and results in efficient receptor–ligand endocytosis (Huang *et al.,* 1999). In contrast, the N terminus of hPTH contains a critical activation domain for phospholipase C coupling (Takasu *et al.,* 1999). Investigations of crystalline hPTH(1-34) reveals an extended helical confirmation with a receptor-binding pocket in the N terminus and a hydrophobic interface at the C terminus (Jin *et al.,* 2000).

Cellular Actions of Parathyroid Hormone on Bone

There is a wealth of knowledge regarding the actions of PTH on bone cells *in vitro.* However, placing this information into the context of *in vivo* pharmacologic and physiologic actions of PTH is challenging. The cellular actions of PTH and the regulation of modeling and remodeling processes in bone were reviewed extensively by Parfitt in 1976 (Parfitt, 1976a,b,c,d). These concepts are revisited in this chapter but with references to new insights obtained from more recent pharmacologic studies.

PTH Regulation of Osteoblasts and Their Osteoprogenitors

Anabolic actions of parathyroid extract were first described in young rats, guinea pigs, kittens, and rabbits in the 1930s (Burrows, 1938; Pugsley and Selye, 1933; Jaffe, 1933; Parsons and Potts, 1972). At the time, the responses were thought to reproduce some of the pathologic processes of hyperparathyroidism in which an early destructive phase was followed by a reparative phase of bone formation (Heath, 1996). When hPTH(1-34) was first synthesized in the 1970s (Potts *et al.,* 1995), the anabolic effect of PTH on bone was revisited. Small clinical trials in the 1970s and early 1980s suggested that PTH could be used to restore bone in patients with osteoporosis (Cosman and Lindsay, 1998; Dempster *et al.,* 1993). The anabolic response of PTH has long been viewed with skepticism, as the literature was dominated by the dogmas that PTH was a hormone controlling calcium homeostasis, with its major effect manifest by stimulating bone resorption. Research of the past decade has shown reproducibly the osteoblast and its progenitor to be the primary *in vivo* target of PTH. The mechanisms of action are still not well understood, although we know that PTH regulates gene expression in the osteoblast, supporting synthesis of matrix proteins required for new bone formation (Parfitt, 1976a), proteins regulating osteoclast differentiation (Parfitt, 1976b), and proteins associated with matrix degradation and turnover (Parfitt, 1976c).

At the cellular level, several mechanisms of inhibition of proliferation by PTH occur and include inhibition of osteoprogenitor proliferation in young rats. Topoisomerase IIα, a marker of proliferation (Feister *et al.,* 2000), decreased expression of H4 (Onyia *et al.,* 1995) and caused a lack of stimulation of thymidine incorporation (Onyia *et al.,* 1995; Young, 1962, 1964; Onyia *et al.,* 1997). In young rats, in which proliferating cells are abundantly available adjacent to the growth plates and in cortical endosteum of the metaphyses and the periosteal diaphyseal surfaces (Kimmel and Jee, 1980; Kember, 1960), PTH targets cells in the G_1/S phase of the cell cycle (Onyia *et al.,* 1995; Young, 1962, 1964; Roberts, 1975; Hock *et al.,* 1994). The effects of PTH in young rats suggest possible roles in skeletal development, accretion of peak bone mass, and increased bone formation. In adult rats, there is no evidence that proliferating osteoprogenitor cells are targeted. Following exposure to [^{3}H-]thymidine in PTH-treated rats, there were no radiolabeled osteoblasts, and only unlabeled, nonproliferative bone surface cells are activated (Dobnig and Turner, 1995). An electron microscopy study supports the concept that PTH rapidly stimulates differentiation of nonproliferating osteoprogenitors lining quiescent bone surfaces (Roberts, 1975). *In vitro* studies have shown inconsistent effects of PTH on bone cell proliferation (Onishi *et al.,* 1997; Onishi and Hruska, 1997; Verheijen and Defize, 1995; Chaudhary and Avioli, 1998; Pfeilschifter *et al.,* 1993; Patridge *et al.,* 1985). In UMR-106 cells, PTH blocked entry of cells into S phase of the cell cycle, thereby increasing the number of cells in G_1, and cell proliferation was inhibited as a consequence of an increase in p27Kap1 (Onishi and Hruska, 1997). Histomorphometric studies have consistently shown early increases in bone-forming surfaces, consistent with the stimulation of differentiation of osteoprogenitors, rather than proliferation of osteoblasts (Cosma and Lindsay, 1998; Dempster *et al.,* 1993; Onyia *et al.,* 1997; Leaffer *et al.,* 1995; Hodsman and Steer, 1993; Meng, *et al.,* 1996).

When PTH binds to the G protein-coupled, seven-transmembrane receptor *in vitro,* cyclic AMP has been implicated as the key signaling pathway activated. The relative importance of cAMP *in vivo* is not well understood. Activation of calcium and protein kinase C (PKC) signal transduction pathways require higher doses of PTH *in vitro* than those required to activate the cAMP–protein kinase A (PKA) pathways. Thus, it has been proposed that cAMP is the key pathway by which the anabolic responses of PTH are effected (Potts *et al.,* 1995; Whitfield and Morley, 1998; Civitelli *et al.,* 1990). In fibroblasts, cAMP signaling activates PKA-controlled cell growth by abrogating signaling required for detachment of cells, inhibiting both progression through the cell cycle and apoptosis (Frisch, 2000; Howe and Juliano, 2000). If valid for osteoprogenitors, this could provide mechanisms by which PTH promotes differentiation but not proliferation of bone cells. In an additional control step, PTH has the capability to reverse the increase in cAMP through regulation of the inducible cAMP early repressor genes in cultured bone cell lines (Tetradis *et al.,* 1998; Bogdanovic *et al.,* 2000).

For a few years, it was thought that the use of certain analogs, such as hPTH(1-31), which induced anabolic

actions of PTH and activated only cAMP, validated the *in vitro* findings (Whitfield and Morley 1998). Subsequently, this analog was shown to activate cAMP *in vitro* under conditions in which there were high numbers of PTH1 receptors (Takasu and Bringhurst, 1998). In addition, an experiment in mice revealed that hPTH(1-31) was equivalent to hPTH(1-38) in stimulating cortical periosteal and trabeculear endosteal surfaces, but PTH(1-31) had a more attenuated effect on the cortical endosteum (Mohan *et al.,* 2000; Hock, 2000).

Stimulation of bone formation in endocortical surfaces is the hallmark of the anabolic effect of PTH irrespective of the species or experimental model (Cosman and Lindsay, 1998; Dempster *et al.,* 1993; Whitfield and Morley, 1998; Oxlund *et al.,* 1993). Thus, different mechanisms of action may be invoked in osteoblasts on differing bone surfaces within the same bone, a phenomenon that has not been considered in *in vitro* models. The magnitude of these differences is not enough to modify the outcome, as the increment of gain in bone mass and bone strength in PTH-treated mice remains equivalent between the two agonist ligands (Mohan *et al.,* 2000). The magnitude of changes in cyclic AMP and the PKA pathway cannot explain the paradoxal actions of PTH to inhibit and stimulate bone formation and to spare or stimulate osteoclast-mediated resorption. Application of new concepts on how waves of differing amplitude and frequency of cAMP and Ca^{+2} signaling traverse a cell and may be amplified in neighboring cells by activation of intracellular calcium-sensing receptors should be examined for the relevance to signal transection in bone cells in response to PTH (Bretschneider *et al.,* 1997; Dormann *et al.,* 1998; Hofer *et al.,* 2000; Thomas, 2000).

As one of the cAMP-dependent mechanisms, the upregulation of c-fos and the role of the immediate early gene comprising the AP-1 fos and jun family members have been widely studied. *In vivo,* PTH [as either hPTH(1-84) or hPTH(1-34)] increased c-fos, fra-2, junB, and c-jun significantly above baseline levels in the metaphyses of young mice at doses as low as 1 μg/kg (Stanislaus *et al.,* 2000; Liang *et al.,* 1999). Although other AP-1 members, δfosB (Sabatakos *et al.,* 2000) and fra-1 (Jochum, 2000), regulate bone formation, PTH did not regulate their expression in young rats (Stanislaus *et al.,* 2000). The regulation of c-fos in bone has the greatest magnitude of change of the PTH-regulated AP-1 family members and is time and cell dependent (Stanislaus *et al.,* 2000). *In situ* hybridization of young rat bone showed that bone-lining cells expressed an increased amount of c-fos within 15 to 60 min of exposure to hPTH(1-84) (0.2 mg/kg), followed by stromal cell and osteoclast expression after 1 to 2 hr (Lee *et al.,* 1994). Cells in which c-fos expression was delayed expressed few PTH1 receptors, consistent with an indirect effect of PTH on these cells (Lee *et al.,* 1994). *In vitro,* constant exposure to PTH(1-34) and PTHrP(1-34) induced c-fos gene expression rapidly in bone cell lines (Kano *et al.,* 1994; McCauley *et al.,* 1997; Clohisy *et al.,* 1992). Antisense olionucleotides complimentary to c-fos mRNA inhibited mRNA translation and antagonized the PTH- or PTHrP-induced inhibition of proliferation in UMR106 osteoblast-like cells, as well as induction of osteoclast differentiation in the presence of these cells (Kano *et al.,* 1994). The early inducible gene c-fos has also been implicated in the process of programmed cell death in other cells, and the PTH-induced increases in c-fos may be relevant to the inhibition of apoptosis in bone cells. Irrespective of whether anabolic or catabolic effects of PTH are induced, the increase in c-fos and interpretation of its significance is complex. It is not known if the increase in c-fos is due to activation of equivalent or different pathways in equivalent or different target cells.

The ability of PTH to promote commitment to osteoblast differentiation was demonstrated using a fibroblast osteoprogenitor colony-forming assay (CFU-f) with bone marrow stromal cells of neonatal or young rats (Ishizuya *et al.,* 1997; Nishida *et al.,* 1994). *In vitro,* PTH inhibits the expression and synthesis of matrix proteins, including collagen I, osteocalcin, and alkaline phosphatase, regardless of whether exposure is for a few hours or several days in differentiated osteoblasts (Tetradis *et al.,* 1998; Bogdanovic *et al.,* 2000; Clohisy *et al.,* 1992; Howard *et al.,* 1980, 1981; Dietrich *et al.,* 1976; Kream *et al.,* 1993; Raisz and Kream 1983a,b; Tetradis *et al.,* 1996,1997). *In vivo,* following an injection of PTH, collagen I mRNA was upregulated within 6 hr and bone matrix synthesis was increased within 24 hr in young growing rats (Onyia *et al.,* 1995, 2000; Hock *et al.,* 1994).

In vitro data may predict *in vivo* data associated with continuous exposure to PTH. Following continuous infusion of PTH in adult rats, osteoblasts are associated with fibrosis rather than new bone matrix (Dobnig and Turner, 1997; Kitazawa *et al.,* 1991), and the ratio of IGF I to IGF-BP3, detected in bone-lining cells by immunohistochemistry, is altered (Watson *et al.,* 1999). Prolonged exposure to PTH in cultured bone cells altered several nuclear matrix proteins (Bidwell *et al.,* 1998). Some of these appear to be architectural transcription factors and some, such as NMP4 (a nuclear matrix protein), and NP (a soluble nuclear protein), bind directly to the regulatory region of the rat type I collagen α(I) promoter in the presence of PTH (Bidwell *et al.,* 1998; Alvarez *et al.,* 1998). These experiments suggest that regulation of the nuclear matrix by PTH may modify the profile of transcribed genes under different exposures.

One of the most interesting recent developments in studies of the anabolic effects of PTH has been the consistent finding that PTH upregulates expression of both matrix-degrading proteins, such as matrix metalloproteinases and ADAMTS-1, and cytokines associated with regulating matrix degradation and turnover, such as interleukin (IL)-6 and IL-11 (Onyia *et al.,* 1995, 1997; Clohisy *et al.,* 1992; Greenfield *et al.,* 1993, 1995, 1996; Huang *et al.,* 1998; McClelland *et al.,* 1998; Winchester *et al.,* 1999; Elias, *et al.,* 1995). The role of the osteoblast in generating matrix metalloproteases was suggested by studies showing collagenase 3 to be a target of cbfa-1, a key transcription factor

linked to osteoblast differentiation (Jiminez *et al.,* 1999; Selvamurugan *et al.,* 2000a,b). Parathyroid hormone may induce retraction of osteoblasts from the bone surface through a calpain-dependent, proteolytic modification of the osteoblast cytoskeleton (Murray *et al.,* 1997). The consequence of cell retraction and detachment due to matrix degradation is apoptosis of cells that are unable to reattach. A transient increase in apoptosis in proliferating cells and osteocytes of young rat metaphyses was recorded during the initial response to PTH (Stanislaus *et al.,* 2000). A subset of osteoblasts may be highly susceptible to apoptosis. The existence of a susceptible osteoblast subset was suggested by data showing that cultured HEK293 cells transfected with high numbers of PTH1 receptor exhibited apoptosis when exposed to PTH (Turner *et al.,* 2000). Also, studies have suggested that local increases in phosphate concentrations may induce apoptosis in mineralizing cells (Meleti *et al.,* 2000; Boyan *et al.,* 2000). *In vitro,* PTH stimulated transport of inorganic phosphorus by a Na-dependent carrier in the rat osteosarcoma cell line UMR-106 in a dose-dependent manner, and P_i uptake was attenuated by PKC inhibitors or by downregulation of PKC by phorbol ester treatment (Arao *et al.,* 1994). Thus, the probability of apoptosis in susceptible cells may be enhanced by the ability of PTH to regulate phosphate homeostasis.

The transient increase in apoptosis of osteoblasts (Stanislaus *et al.,* 2000) and upregulation of matrix metalloproteinases (McClelland *et al.,* 1998; Zhao *et al.,* 1999) are consistent with mechanisms activating bone turnover. One consequence of matrix activation degrading enzymes may be that reconditioned bone surface can serve as an attractant for newly differentiating osteoblasts to increase bone-forming surfaces (anabolic action) or as an attractant for differentiating osteoclasts to continue resorption of old surfaces (catabolic action). Reattachment of detached osteoblasts may delay or inhibit their apoptosis. In mice and rats, continued intermittent PTH has been associated with inhibition of apoptosis (Stanislaus *et al.,* 2000; Jilka *et al.,* 1999). Differences in the profile of responses to intermittent exposure to PTH required to promote osteoblast differentiation and function and the profile of responses to continue with infusion of PTH that activate osteoclast differentiation and function have yet to be identified.

PTH Regulation of Osteoclasts and Their Osteoprogenitors

Barnicott (1948) first recognized the resorptive properties of PTH. In this study, remnants of parathyroid glands were laid on the inverted underside of calvaria and reimplanted under the skulls of mice. Significant bone resorption in the calvaria resulted. This work laid the foundation for *in vitro* assays utilizing rat and mouse bones in which resorption was induced routinely by PTH (Raisz, 1963, 1965). PTH indirectly enhances bone resorption and release of calcium from bone surfaces by activation of osteoclasts. This is despite evidence for direct binding of ^{125}I-bovine PTH(1-84) to avian osteoclats (Teti *et al.,* 1991) and for activation of resorption via a direct effect on the osteoclast (Mears, 1971; Miller and Kenney, 1985; Murrills *et al.,* 1990). The effect of PTH on osteoclasts is most likely indirect given recent data. *In vivo,* PTH induces a transient increase in apoptosis of osteoblasts (Stanislaus *et al.,* 2000) and upregulation of osteoblast matrix metaloproteinases (McClelland *et al.,* 1998; Zhao *et al.,* 1999). This is consistent with PTH-mediated mechanisms activating bone turnover in which protein synthesized by osteoblasts activates osteoclasts. In genetically modified mice in which collagenase is unable to cleave collagen I, daily PTH injections over the skull did not induce osteoclast-mediated resorption but did occur in control animals (Zhao *et al.,* 1999). *In vitro,* osteoclast-like cells in culture failed to respond to PTH unless cocultured with stromal or osteoblast-like cells (Kanzawa *et al.,* 2000; Suda *et al.,* 1999; Horwood *et al.,* 1998; McSheehy and Chambers, 1986; Fuller *et al.,* 1998a,b).

It is currently thought that stromal cells and osteoblast lineage cells regulate osteoclast differentiation through cell–cell contact by controlling the synthesis of osteoprotegerin (OPG/OCIS) and the ligand for the receptor activator of NF-κB (RANKL/ODF/TRANCE/OPGL) (Kanzawa *et al.,* 2000; Suda *et al.,* 1999; Horwood *et al.,* 1998). These two secreted proteins compete for binding to the osteoclast progenitor receptor activator NF-κB(RANK), a TNF receptor family member (Suda *et al.,* 1999). If RANKL binding to RANK predominates, as seen following PTH treatment of cultured osteoblast-like osteosarcoma cells transfected with the PTH1 receptor (Itoh *et al.,* 2000), osteoclast progenitors differentiate into osteoclasts in the presence of M-CSF (Suda *et al.,* 1999; Fuller *et al.,* 1998b). In addition, PTH downregulates OPG expression via a cAMP/PKA pathway in a variety of bone cell lines (Kanzawa, *et al.,* 2000). Reciprocal regulation of OPG and RANKL expression by PTH preceded its effects on osteoclast formation by 18–23 hr. These effects were more pronounced in primary bone marrow cells than in calvaria bone organ cultures or MC3T3.E1 cells (Lee and Lorenzo, 1999). *In vivo* studies of young rats treated once daily with hPTH(1-34) for 3 days showed an altered ratio of mRNA for osteoprotegerin and RANKL (Onyia *et al.,* 2000), but it is not known if the magnitude of these changes translates into relevant function.

Changes in resorption associated with once daily treatment of PTH are rarely detected in rat models *in vivo.* In humans and animals with osteonal bone skeletons, increased resorption in response to PTH in cortical bone occurs, but only after the increase in surface bone formation, which occurs during the first remodeling period (sigma) (Cosman and Lindsay, 1998; Hodsman and Steer, 1993; Mashiba *et al.,* 2000; Hirano *et al.,* 1999, 2000; Burr *et al.,* 2000). This increase in new bone-forming surfaces appears to offset the increase in resorption that precedes new osteonal formation in intracortical bone. As suggested by *in vitro* data (Kanzawa *et al.,* 2000; Suda *et al.,* 1999; Horwood *et al.,* 1998),

increased resorption plays a key role in the mechanisms activated when PTH is given by continuous infusion and is activated within the first 3 days of infusion in young rats (J. M. Hock, unpublished data). In humans and dogs, continuous infusion of PTH was associated with histomorphometric and biochemical data consistent with the early induction of resorption and increased bone turnover (Malluche *et al.,* 1982; Cosman *et al.,* 1991, 1998). In rats, resorption results in loss of bone mass despite a small increase in bone-forming surfaces (Watson *et al.,* 1999; Hock and Gera, 1992; Tam *et al.,* 1982).

It is not known if osteoclast activation following the continuous infusion of PTH is a direct effect due to shifts in the ratio of OPG and RANKL expression in osteoblasts and stromal cells or if the shift in matrix protein synthesis by osteoblasts to a more fibroblast-like profile (Dobnig and Turner, 1997) results in an extracellular matrix (ECM) feedback signal to activate increased bone turnover. One difficulty in developing experimental models of gene expression is to match the *in vivo* infusion dose of PTH with that of PTH given by once daily injections. PTH infusion induces hypercalcemia at doses above 80 μg/kg/day in rats, and infusion at 160 μg/kg/day was associated with increased mortality (Hock and Gera, 1992). When hPTH(1-34) was infused at 40 μg/kg/day in young rats and compared to a PTH injection of 40 μg/kg/day, there was upregulation of c-fos and IL-6 mRNA expression following injection and equivalent upregulation of c-fos but no alteration of IL-6 mRNA levels following infusion (Liang *et al.,* 1999). These responses were detected in the femur metaphysis, which is enriched for osteoblasts, but not in the cortical diaphysis, which contains predominantly hematopoietic and stromal cells. In primary cultures of bone marrow stromal cells from either metaphysis or diaphysis of rat femurs, PTH increased both c-fos and IL-6 expression (Tu *et al.,* 1997), suggesting that, *in vivo,* there are site-specific regulatory factors controlling the profile of genetic responses, in addition to those associated with different treatment regimens. Confounding their interpretation is a study in which serum IL-6 was increased in mice infused with PTH or rats in which PTH was locally injected over calvaria to mimic a resorption model. Blocking IL-6 release was associated with a decrease in resorption markers, with no change in biochemical markers of formation (Grey *et al.,* 1999; Pollock *et al.,* 1996). There were no measures of bone mass to ascertain if this altered profile was associated with a catabolic or anabolic effect, and no comparison was made to intermittent, once daily administration of PTH.

One frustrating limitation of these gene-profiling studies has been our lack of knowledge of how the balance of increased activation frequency may favor formation (anabolic effect of intermittent PTH) or resorption (catabolic effect of continuous PTH). A mathematical model that assumes a longer delay in osteoclast activation (due to a requirement for signals from the osteoblast to osteoclast progenitors) than the delay required for osteoblast differentiation argues that osteoblast function will predominate with intermittent PTH, whereas resorption will be greater with continuous PTH (Kroll, 2000). The importance of interval duration of PTH administration emphasized in this theoretical model has some support from preliminary data. In rats treated with PTH, six injections in 1 hr once daily or infusion for 1 hr once daily, induced an equivalent bone gain to that of one injection/day, whereas six injections over 8 hr or continuous infusion for longer than 1 hr/day resulted in bone loss (Dobnig and Turner, 1997; Frolik *et al.,* 1997; Turner *et al.,* 1998). While our knowledge of genetic regulation and signal transduction in bone cells has expanded, there is still limited understanding of the genes that differentially regulate modeling, growth processes, and remodeling *in vivo* to change the shape of bones or to regulate the spatial distribution of bone within a bone organ or within the skeleton as a whole.

The role of C-terminal fragments of parathyroid hormone in the process of bone resorption is controversial. It has been suggested that PTH(35-84), PTH(53-84), or PTH(69-84) may have a role in stimulating osteoclast-like formation (Takasu and Bringhurst, 1998; Kaji *et al.,* 1994). Mouse bone cell cultures, which contain both osteoclasts and progenitor cells, responded to the addition of these C-terminal fragments with an increase in bone resorption. No effect was noted, however, in the bone-resorbing activity of isolated rabbit osteoclasts, suggesting methodological or species differences. *In vivo,* no differences at the bone or molecular level have been shown between the responses to full-length hormone, hPTH(1-84), and hPTH(1-34) (Cosman and Lindsay, 1998; Stanislaus *et al.,* 2000; Ejersted *et al.,* 1993; Mosekilde *et al.,* 1991). An intact N terminus was required for PTH to increase bone mineral density in mature ovariectomized rats (Armento-Villareal *et al.,* 1997).

Candidate Cytokines and Hormones as Mediators of PTH Actions

Several candidate agents have been implicated as mediators in regulation of the osteoblast axis by PTH. Growth hormone (GH) has been evaluated either as a direct regulator of bone cell biology or as a stimulator of IGF-1, which was known to stimulate osteoblast proliferation and differentiation *in vitro* (Canalis *et al.,* 1994; Schmidt *et al.,* 1994). A study of young male hypophysectomized rats showed that GH was required for PTH to increase bone mass equivalent to the increment seen in treated, intact controls (Hock and Fonseca, 1990). As the effects of GH on bone may be mediated by IGF-1, it was inferred that IGF-1 might be required for the anabolic effect of PTH. It is possible that this IGF-1 mechanism may only be activated during skeletal development when growth plates are still active. The addition of PTH to older, mature hypophysectomized female rats resulted in increased osteoblast number and trabecular bone volume, even in the absence of GH (Schmidt *et al.,* 1995). Other studies of PTH in combination with either GH alone or combined with IGF-1 in intact

old female rats showed no minor additional effect of either GH or IGF-1 (Gunness and Hock, 1995; Mosekilde *et al.,* 2000). It has been suggested that GH in older animals may enhance the effect of PTH by stimulating periosteal formation to change the geometry of the bones, making them more resistant to fracture in biomechanical tests (Mosekilde *et al.,* 2000); data to prove this additive effect of GH are limited. Collectively, these data suggest that GH is required for the anabolic effect of PTH during the "adolescent" phase of skeletal growth in rats, but is not necessary after skeletal maturation in rats.

The role of IGF-1 and its binding proteins, especially IGF-BP5, which is anabolic in cultured bone cells (Gabbitas and Canalis, 1998; Canalis, 1997), remains ambiguous. One elegant study showed that a neutralizing IGF-1 antibody blocked the effects of PTH on collagen synthesis in cultured fetal rat calvaria and implicated IGF-1 as a key mediator of PTH effects on osteoblasts (Canalis *et al.,* 1989). In calvarial bone cells isolated from 2-day rats and treated with hPTH(1-34) for 6 hr, osteoblast commitment to differentiation was blocked by the IGF-1 antibody (McCauley *et al.,* 1997). These data suggest that IGF-1 may be one of the mediators of PTH effects on skeletal growth and maturation. Bone organ culture or cells isolated from fetal or neonatal animals may not respond to hormonal stimulation under the same constraints as those from postnatal and mature rats or mice (Canalis *et al.,* 1994). Alternatively, as IGF-1 inhibits collagenase (Canalis *et al.,* 1995), IGF-1 may mediate a different aspect of the anabolic mechanism, namely regulating the process by which osteoblasts condition the bone surface as a prerequisite to attract osteoclast progenitors to bone. As skeletal cells secrete the six known IGF-binding proteins (IGFBPs) and two of the four known IGFBP-related proteins (IGFBP-rP), there may be additional levels of regulation if IGF-1 mediates the action of PTH *in vivo* (Schmid *et al.,* 1989; LaTour *et al.,* 1990; McCarthy *et al.,* 1991; Moriwake *et al.,* 1992; Shimasaki *et al.,* 1991). IGFBPs may prolong the half-life of IGF-1 and compete with its receptors for binding. IGF-BP4 may inhibit osteoblast function *in vitro,* whereas IGF-BP5 enhances the stimulatory effects of IGF-1 (Pereira and Canalis, 1999; Qin *et al.,* 1998). PTH induces IGFBP-rP-1 and IGFBP-rP-2 in osteoblasts by transcriptional control (Pereira and Canalis, 1999; Pereira *et al.,* 2000).

In cultured mouse cells and bone organ culture, hPTH-(1-34) increased expression of FGF-2 and FGF receptors 1 and 2 within 30 min (Hurley *et al.,* 1999). In cultured rat osteoblasts, TGFβ may downregulate the PTH1 receptor to decrease receptor binding and signal transduction by cAMP (Jongen *et al.,* 1995). This may be a reciprocal action to the ability of PTH to increase TGFβ1 by the PKC pathway and TGFβ2 via the PKA pathway in cultured human and rat osteoblasts (Wu and Kumar, 2000). However, *in vivo* studies have not shown gene expression of growth factors to be significantly regulated by PTH until after 4 weeks of treatment (Watson *et al.,* 1995; Pfeilshifter *et al.,* 1995); the effects are dose dependent and occur at higher doses than those needed to induce an increase in bone mass (Stanislaus *et al.,* 2000; Pfeilshifter *et al.,* 1995; Sato *et al.,* 1997). This delay in upregulation suggests that an increase in growth factors may be more an indication of the highly significant increase in osteoblast numbers and function rather than a primary mediator of the anabolic actions of PTH. Withdrawal of PTH treatment in rats results in downregulation of the osteoblast response in 24–48 hr (Gunness-Hey and Hock, 1989), but the molecular and cellular consequences have not studied to determine the effects on the multiple cytokines regulating local bone cell balance.

Cellular Actions of Parathyroid Hormone on Kidney Phosphate Transport

The classic action of parathyroid hormone on the kidney is to cause phosphaturia. Approximately 85% of the phosphorus reabsorption occurs in the proximal tubule. A low phosphorus intake stimulates the tubular phosphorus absorption and a high phosphorus intake inhibits phosphorus reabsorption. The alterations occur independent of changes in PTH, serum calcium levels, or extracellular fluid volume. Phosphorus transport in the proximal tubule occurs against an electrochemical radiant. Reabsorption is transcellular, absorptive, and dependent on the low concentration of intracellular calcium maintained on the basolateral side by Na,K-ATPase. In the proximal tubule, phosphorus is transported into the cell through the actions of a membrane-bound, sodium–phosphate cotransporter, Npt2(NaPi2).

The actions of parathyroid hormone place a further constraint on the already limited capacity of the kidney to reclaim filtered phosphate. Whether this active transport process is expressed simply as a maximal rate ($TmPO_4$) or in relationship to the glomerular filtration rate ($TmPO_4$/GFR), the parathyroid hormone is inhibitory when present and permissive when absent. Inhibition of phosphate transport occurs primarily in the proximal convoluted tubule and the pars recta (Agus *et al.,* 1981). Phosphate reabsorption may also be inhibited by PTH in the distal tubule. This effect is accompanied by the inhibition of sodium and fluid reabsorption. Renal tubular reabsorption of phosphate is an active process and, when corrected for GFR, is an accurate measure of renal responsiveness to parathyroid hormone, one of the earliest physiological observations to be made for parathyroid hormone (Ellsworth and Howard, 1934).

The cellular basis for phosphate transport in the kidney has been explored extensively. Lederer and McLeish (1995) demonstrated that activation of purinergic P2 receptors attenuated the inhibitory effect of parathyroid hormone on Na^+-dependent phosphate transport by a G-protein-dependent mechanism. In LLC-PK1 kidney cells that were stably transfected with the PTH/PTHRP receptor, PLC activation and parathyroid hormone-stimulated phosphate transport were dependent on receptor density. This finding is in contrast to the intracellular cyclic AMP response to parathyroid

hormone. Thus, receptor-dependent parathyroid hormone effects on phosphate uptake are linked more closely to PKC activity than to cyclic AMP (Guo *et al.,* 1995). Other investigators have shown the importance of PTH1 receptor density in intracellular calcium and cAMP pathways (Jobest *et al.,* 1997).

In cultured opposum kidney cells, parathyroid hormone-induced phosphaturia is modulated by luminal cAMP, which, in turn, is metabolized to adenosine by brush-border membrane ectoenzymes such as ecto-nucleotidase (5′-NU). Parathyroid hormone stimulates 5′-NU in a time-, concentration-, and protein synthesis-dependent manner (Siegfied *et al.,* 1995). In this system, downregulation or inhibition of protein kinase C attenuated the effect of parathyroid hormone, supporting a role for PKC activation in parathyroid hormone-induced renal phosphate loss.

The coupling of PTH to its receptors has been best characterized in the proximal tubules of the kidney. These ligand–receptor interactions mediate the regulation of TmP/GFR in the proximal tubule, whereas those in the distal nephron regulate calcium reabsorption. Both of these mechanisms are coupled with different intracellular signal transduction systems. Utilizing prolonged exposure of parathyroid hormone and a protein PKC activator (mezerein) in an opossum kidney cell line (OK/E), assessments were made on cAMP production, PKC activity, and Na-dependent phosphate transport. Short-term exposure (5 min) to PTH stimulated cAMP production, whereas a longer incubation (6 hr) reduced cAMP production. Na-dependent phosphate transport was maximally inhibited under desensitizing conditions and was not affected by reintroduction of PTH. Addition of a PKC activator, mezerein, for 6 hr enhanced PTH-, cholera toxin-, and forskolin-stimulated cAMP production, suggesting enhancement of $G_s\alpha$ receptor coupling, and increased adenylate cyclase activity. However, PKA- and PKC-dependent regulation of the sodium–phosphate transporter was blocked in mezerein-treated cells. Thus, PTH-induced decreases in cAMP production were associated with a reduction in membrane-associated PKA activity, whereas the PKC activator, mezerein, increased cAMP production and decreased membrane and cytosolic PKA activity. This differential modulation of cAMP production in the regulation of sodium-dependent phosphate transport suggests that agonist specific activation and downregulation of PKC isoenzymes may be involved in the changes in cAMP production and sodium-dependent phosphate transport (Cole, 1997).

To determine differences between PKA and PKC intracellular signaling pathways, the effect of PTH on renal proximal tubule sodium-dependent phosphate transport was tested in opossum kidney cells. PKC-dependent phosphorylation of phospholipase A_2 stimulates arachadonic acid release, a potent inhibitor of P_i transport. Arachadonic acid is metabolized to 20-hydroxyeicosatetraenoic acid (20-HETE) in the proximal tubule. The addition of 20-HETE specifically inhibited sodium-dependent phosphate transport in OK cells and is thought to be one of the mediators of PTH action (Silverstein *et al.,* 1998). Further studies attempted to determine the effects of protein kinase A and protein kinase C activation on membrane expression of NaPi-4, the type II sodium–phosphate cotransporter in opossum kidney (OK cells). Treatment of OK cells with PTH decreased the expression of NaPi-4 as early as 2 hr, and the effect was sustained for over 24 hr. A nonhydrolyzable cAMP analog, 8-bromo-cAMP, inhibited NaPi-4 expression by over 90% over a 24-hr period. Phorbol ester inhibited NaPi-4 expression less than 10%. PTH(3-34), a fragment that stimulates PKC only, inhibited phosphate transport but had no effect on NaPi-4 expression. These studies suggest that PKA inhibits sodium–phosphate uptake in OK cells by downregulation of NaPi-4 expression and does not provide a role for PKC (Lederer *et al.,* 1998). These results were confirmed and extended with the use of unique PTH analogs that signal specifically through the PKA or PKC pathway. In isolated murine proximal tubules, PTH(1-34), an activator of PKC, and PKA was effective when added to either the apical or the basolateral perfusate. PTH(3-34), which signals PKC, acted only via the luminal perfusate. Activation of PKA with 8-bromo-cAMP or PKC with a phorbol ester mimicked the effects. *In vivo,* the specific analogs downregulated the sodium–phosphate cotransporter (designated NaPi-IIa in this study) in the brush border membrane. These studies indicate that functional PTH receptors are located on both membrane domains and that apical PTH receptors may preferentially initiate stimulation of phosphate reuptake through a PKC-dependent mechanism (Traebert *et al.,* 2000).

The proteolytic pathway in the regulation of Na-Pi cotransporter II by PTH is also an important regulatory pathway. In opossum kidney cells, inhibition of lysosomal degradation prevented the PTH-mediated degradation of the transporter. Inhibition of the proteosomal pathway, however, did not have the same effect. Lysosomal inhibitors prevented the PTH-mediated degradation of the sodium–phosphate transporter, but they did not inhibit the PTH-mediated inhibition of the Na-Pi cotransporter. The Na-Pi cotransporter was constitutively transported to and degraded within endosomes/lysosomes, and degradation was enhanced in the presence of PTH (Pfister *et al.,* 1998).

In vivo studies have confirmed many of the effects on the Na-Pi cotransporter. Both dietary phosphorus and PTH are important physiological regulators of phosphate reabsorption in the renal proximal tubule. In thyroparathyroidectomized (TPTX) rats, Na-Pi-II protein and mRNA were increased in the kidney transport compared to sham-operated animals. Administration of PTH to TPTX rats caused a decrease in the amount of Na-Pi-II protein but did not change the levels of Na-Pi-II mRNA. Dietary phosphorus deprivation in TPTX rats did not alter the amount of Na-Pi-II mRNA or protein. Switching TPTX animals from a low phosphorus diet to a high phosphorus diet decreased Na-Pi-II immunoreactivity from superficial nephrons but not from juxtamedullary nephrons. These studies suggest that dietary phosphorus can regulate the amount of Na-Pi-II protein in superficial nephrons in a PTH-independent

manner (Takahashi *et al.,* 1998). The type II Na-Pi cotransporter gene (designated Npt-2) is in the renal brush border membrane and undergoes endocytosis in the presence of PTH. In mice homozygous for the disrupted Npt-2 gene, decreased renal phosphorus reabsorption occurs in response to PTH. PTH has no effect on serum phosphorus concentration, on fractional excretion of P_i, or Na-dependent P_i transport in renal brush border in the transgenic mice. In contrast, PTH elicits a fall in serum phosphorus concentration, an increase in urinary phosphorus concentration, and a decrease in brush border membrane Na-Pi cotransport with a corresponding reduction in Npt-2 protein in wild-type mice. Both Npt-2 −/− and Npt-2 +/+ mice exhibit a significant rise in the urinary cAMP/creatinine ratio in response to PTH, proving that there is not a generalized resistance to PTH in Npt-2 −/− mice. Phosphorus deficiency per se also does not account for the PTH resistance in Npt-2 −/− mice, as phosphate-depleted normal mice respond to PTH. These transgenic animals have shown that Npt-2 gene expression is critical for PTH effects on renal phosphorus handling (Zhao and Tenenhouse, 2000). In distal tubules, PTH inhibits phosphate reabsorption, but the transporters that are involved in this process have not yet been characterized.

Cation/Anion Transport

Parathyroid hormone inhibits proximal tubule reabsorption of sodium, bicarbonate, calcium, and phosphate (Agus *et al.,* 1981; Pollock *et al.,* 1986). Extracellular calcium homeostasis is highly dependent on the multiple roles that the kidneys play in the regulation of calcium reabsorption. The mechanisms of calcium reabsorption occur throughout the entire length of the nephron but vary significantly among segments. There is extensive recovery of calcium by the proximal tubules that is under regulation by a paracellular pathway and not known to be regulated by hormones. In the thick ascending limbs, calcium absorption occurs through cellular and paracellular routes. Parathyroid hormone and calcitonin regulate the cellular components while the paracellular component is modulated by sodium reabsorption. The least understood area of calcium reabsorption is in the distal tubule. It is thought here that calcium absorption is transcellular, although a definitive transport system has not been well characterized. PTH, 1,25-dihydroxyvitamin D, and calcitonin all play regulatory roles in the distal nephron.

The amount of calcium filtered per day by a 70-kg man is greater than 10 g and four times more than the calcium content of the entire extracellular fluid compartment. To maintain neutral calcium balance, approximately 98% of filtered calcium is reabsorbed along the renal tubule. Of this filtrate, 70% of filtered calcium is reabsorbed in the proximal tubule. Twenty percent of filtered calcium is reabsorbed in the loop of Henle, and the distal convoluted tubule is responsible for approximately 8% of calcium reabsorption and is the major site of urine calcium excretion. The basolateral membrane of the thick ascending limb of the loop of Henle now has receptors identified that are stimulated by increased levels of serum calcium, resulting in a decrease activity of the Na-K-Cl_2 pump (Brown *et al.,* 1998). Cellular calcium absorption across the polarized epithelial cells involves entry across an apical (mucosal, luminal) membrane, followed by extrusion across the basolateral membrane into the interstitial fluid. Apical calcium entry is mediated by calcium channels, and basolateral efflux involves energy-dependent extrusion accompanied by plasma membrane Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger.

Oral or intravenous phosphate administration will cause an increase in distal calcium reabsorption in kidney and reduced calcium excretion. Extrarenal mechanisms may contribute to the hypocalciuria associated with phosphate administration. Calcium and phosphate may form a complex in the intestine, decreasing the amount of calcium available for absorption. In addition, phosphate can complex with calcium and bone in soft tissues, which will further reduce the filtered load of calcium. In a phosphate-replete state, hypercalciuria occurs. Excess phosphate will stimulate PTH secretion, resulting in reduced ionized calcium, which further enhances PTH release, which, in turn, enhances calcium reabsorption. It has been proposed that a defect in proximal tubular calcium absorption may be present, as well as a direct effect of phosphate to decrease calcium reabsorption in the distal nephron.

PTH is the principal regulator of renal tubule calcium reabsorption. Increased levels of PTH increase renal tubule calcium reabsorption and reduce glomerular filtration. At the same time, PTH reduces glomerular filtration and thus the filtered load of calcium by reducing the glomerular ultrafiltration coefficient. Patients with hyperparathyroidism, however, are hypercalciuric. The PTH-induced increase in tubule calcium reabsorption produces hypercalcemia and an increased filtered load of calcium. Overall, this produces an increase urinary calcium excretion. PTH increases calcium reabsorption in the distal convoluted tubule through the facilitation of opening of calcium channels. Genetic disorders of calcium excretion have been described and are often due to inactivating mutations of renal ion channels and transporters (Scheinman, 1999). In addition, PTH stimulates the conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D in the renal proximal tubule, which will further enhance intestinal calcium absorption and bone resorption, leading to an increase in the filtered load of calcium. Chronic hypercalcemia will decrease renal reabsorption of phosphorus, whereas chronic hypocalcemia has the opposite effect.

In human studies, the increase in urinary calcium excretion in response to a calcium load occurs with inverse changes in PTH levels. It is often thought that the effect of changes in serum calcium on its own excretion is poorly understood in human subjects. Using a PTH clamp protocol, calcium-regulated renal calcium and magnesium handling were explored in eight male normal volunteers. Graded calcium infusions were given, and PTH was maintained in the

normal range while the subjects were placed on a high and then a low sodium diet. The curve describing urinary sodium as a function of serum calcium was sigmoidal on both high and low sodium diets. This study, as well as previous studies, shows that there is a PTH-independent calcium-dependent change in renal calcium, magnesium, and sodium handling, which is mediated, in part, by the calcium-sensing receptor in the loop of Henle (Brown and Hebert, 1997) .

The role of PTH in the pathogenesis of postmenopausal osteoporosis was difficult to decipher. Although all postmenopausal women are estrogen deficient, a subset has higher rates of bone resorption and greater bone loss. One proposed defect to explain these findings was an impairment in renal calcium conservation. At baseline, there were no differences in PTH concentrations or renal-filtered load of calcium in 19 osteoporotic postmenopausal women compared to 19 elderly normal women. Before PTH infusion, osteoporotic women had lower values for tubular reabsorption of calcium and higher urinary calcium excretion when corrected for glomerular filtrate. After infusion of hPTH-(1-34), tubular reabsorption of calcium increased and calcium excretion decreased in both groups. This study suggests that postmenopausal women with osteoporosis had a PTH-independent defect in renal calcium conservation that may have impacted negative calcium balance (Heshmati *et al.,* 1998). Decreased sensitivity to PTH, resulting in lower bone turnover, better renal calcium conservation, and less osteoporosis, has been noted in Black women compared to White women. Even at baseline, Black women have superior renal calcium conservation, and in response to administration of 1,25-dihydroxyvitamin D, Black women have a greater decrement in PTH. These data suggest that Black women conserve calcium more efficiently under both static and dynamic conditions (Cosman *et al.,* 2000).

PTH is the major regulator of renal 1α-hydroxylase. When serum levels of calcium decrease, parathyroid glands release more calcium due to sensing by the calcium-sensing receptor. The increase in PTH restores calcium levels to normal by its direct effects on bone and kidney. In addition, indirect effects include stimulation of renal 1α-hydroxylase, which accelerates the conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D (calcitriol). Enhancement of intestinal calcium absorption by 1,25-dihydroxyvitamin D aids in the restoration of calcium levels to normal. Vitamin D receptors are present on the parathyroid glands and act as sensors to detect adequate levels of 1,25-dihydroxyvitamin D, thus helping in the regulation of PTH synthesis and release.

Cellular effects of PTH on other cations and ions are less well described. For example, in the distal tubule, little data have been gathered regarding the effects of PTH on sodium excretion. Expansion of the extracellular fluid volume with sodium chloride decreases tubular magnesium reabsorption and increases urinary magnesium excretion. The decrease in proximal magnesium resorption parallels the decline in sodium reabsorption. In a cell culture model of distal tubular epithelium, Rodriquez-Commes and colleagues (1995) used equivalent short circuit current as an estimate of net sodium transport. Parathyroid hormone increased the current in A6 cells in a dose-dependent manner, and the effect appeared to be mediated by both cyclic AMP and intracellular calcium. PTH is responsible for both water and electrolyte transport along the nephron. Using the TPTX rat model, clearance and microperfusion studies were performed to assess the role of PTH. Normal levels of PTH were necessary to maintain concentrating ability in the animal model. In isolated medullary-collecting tubules, the lack of PTH did not alter antidiuretic hormone-induced changes in osmotic permeability, suggesting that PTH-mediated effects occurred at other locations in the nephron (Zaladek Gil *et al.,* 1999).

Numerous chloride channels have been isolated from kidney tubules, and several of them have been implicated in the maintenance of calcium homeostasis. Mutation in the chloride channel designated ClC-5 can result in the formation of kidney stones. In the thyroparathyroidectomized rat model, in the presence of vitamin D deficiency, lower serum and higher urinary calcium concentrations occur compared to control animals. ClC-5 mRNA and protein levels were decreased compared to control and vitamin D-deficient animals. Replacement of PTH reversed these findings, suggesting that PTH modulates the expression of ClC-5 in the kidney cortex, but PTH did not regulate ClC-5 gene expression in the medulla (Silva, 2000).

At the cellular level, parathyroid hormone inhibits activity of the apical membrane Na^+/H^+ exchanger. PTH(1-34) and PTHRP(1-34) stimulate adenylyl cyclase and PKC activities in renal proximal tubule OK cells. PTH(3-34), PTH(28-42), and PTH(28-49) enhance PKC activity only. All fragments inhibited the Na^+/H^+ exchanger, suggesting that signal transduction from the parathyroid hormone receptor to the Na^+/H^+ exchanger could be mediated by PKC or PKA. Inhibition of PCK attenuated the PTH(28-42)-induced inhibition of the Na^+/H^+ exchanger activity (Azarani *et al.,* 1995).

Other Renal Actions of PTH

Alternative roles for parathyroid hormone in the regulation of kidney metabolism were evaluated by Nissim and co-workers (1995). The action of parathyroid hormone on the regulation of renal glutamine and ammonia metabolism was shown to be stimulatory. Using OK kidney cells as a model, parathyroid hormone and acute acidosis stimulated glutamine metabolism by both phosphate-dependent glutaminase and glutamate dehydrogenase pathways. It was proposed that this mechanism may be mediated by decreased activity of the Na^+/H^+ exchanger. The relationship between parathyroid hormone and acidosis is of particular interest because of the well-known effect of parathyroid hormone to inhibit bicarbonate transport in the parts recta (Tam *et al.,* 1982).

Other effects of parathyroid hormone on the kidney have been tested in animal models. In a model of renal insufficiency in rats, osteopontin mRNA increased and alkaline

phosphatase decreased in renal tissue exposed to parathyroid hormone (Liang and Barnes, 1995).

Megalin is a multifunctional receptor expressed at the apical surface of proximal tubules and belonging to the low-density lipoprotein receptor gene family. This receptor is important for the endocytosis of macromolecules filtered at the glomerulus (Farquahr, 1995). It is known that proximal tubules are important in the clearance of PTH, and it has been shown that megalin mediates the endocytosis of PTH (Hilpert, 1999). Megalin-mediated PTH endocytosis was specific and purified megalin specifically recognized full-length PTH(1-84) and synthetic amino-terminal peptide fragments. In megalin-deficient mice, excretion of amino-terminal, but not of carboxyl-terminal, PTH fragments increased substantially (Willnow *et al.,* 1999).

Renal Expression and Action of PTH/PTHRP Receptors

The type I receptor (PTH1r) for PTH and PTH-related peptide is a G-protein-coupled receptor with seven transmembrane domains highly expressed in bone and kidney. The PTH1r has been implicated in specific genetic diseases affecting calcium homeostasis. Thus, intense investigations of its receptor and peptide ligands have been of great interest. The discovery of a PTH2 receptor subtype that responds to PTH but not PTHrP has further complicated our understanding of PTH action. The use of site-directed mutagenesis and photoaffinity cross-linking methods has provided specialized and specific exploration of receptor–ligand interactions (Mannstadt *et al.,* 1999).

The gene encoding the PTH/PTHRP (PTH1r) receptor has been cloned and several promoter regions have been identified. The first promoter was cloned from human kidney (Adams *et al.,* 1995; Juppner, 1994; Orloff *et al.,* 1994). A second promoter region located >3 kb upstream of this original promoter was amplified from mouse kidney RNA and corresponds to two previously unidentified exons composed of an untranslated sequence (McCuaig *et al.,* 1995; McCuaig *et al.,* 1994). These cDNAs are highly homologous to the 5′ end of a cDNA isolated from human kidney. This promoter, which is not (G+C)-rich and lacks a consensus TATA element, is highly tissue specific. It is strongly active in the kidney.

A third human promoter, P3, has also been identified and is widely expressed more so in the kidney than in other tissues. A third human promoter, P3, is widely expressed and accounts for approximately 80% of renal PTHr transcripts in the adult. In contrast, P1 activity in the kidney is weak. P2 is active at midgestation in many human tissues, including bone. P2-specific transcripts are spliced differentially in a number of human cell lines in adult tissues (Bettoun *et al.,* 1998).

Function of all three promoters is inhibited by CpG methylation *in vitro.* The first promoter (P1) is selectively hypomethylated in the adult kidney *in vivo,* suggesting that demethylation is required for renal P1 function. This occurs early and is established by 11.75 weeks of fetal age, several weeks prior and to and consistent with the onset of P1 activity. P3 is unmethylated at midgestation and is inactive. This promoter exhibits characteristics of both tissue-specific and ubiquitously active promoters. The adult methylation patterns of P1 and P3 indicate that their function requires factors expressed late in development (Bettoun *et al.* 2000).

Additional studies have revealed that the PTH/PTHRP receptor in the kidney is controlled by physiological perturbations. A model of secondary hyperparathyroidism caused by a vitamin D-deficient diet in rats is associated with a twofold reduction in PTH/PTHRP receptor mRNA. When the secondary hyperparathyroid state was corrected by vitamin D, the complement of PTH/PTHRP mRNA was restored (Turner *et al.,* 1995). In another model of secondary hyperparathyroidism, namely chronic renal failure, it could also be shown that PTH/PTHRP receptor mRNA was downregulated. Control of the secondary hyperparathyroidism with parathyroidectomy restored PTH/PTHRP mRNA levels. Of interest, verapamil was also able to restore PTH/PTHRP mRNA levels in this model (Tian *et al.,* 1994). Finally, dexamethasone had a potentiating effect on the PTH/PTHRP receptor system in OK cells by altering binding to and immunoreactivity of the PTH/PTHRP receptor (Abou-Samra *et al.,* 1994).

Actions of Parathyroid Hormone on the Vasculature and Cardiovascular System

Although not originally conceived as a vasoactive substance, it is now generally appreciated that both thyroid hormone and PTHRP have important effects to regulate vascular tone. Parathyroid hormone-induced relaxation of vascular smooth muscle (Mok *et al.,* 1989) is a mechanism for a classical hypotensive property that was first demonstrated by Collip and Clark (1925). The actions of parathyroid hormone and PTHRP as vascular smooth muscle relaxants raise questions about the physiological significance of both in this regard. PTHRP has been studied intensely because of the attractiveness of a mechanism that would depend on local production of this paracrine factor. Studies by Hongo *et al.* (1991) and Rian *et al.* (1994) have demonstrated that PTHRP is produced locally by vascular smooth muscle and endothelial cells. Sensitivity to forces of stretch, as shown in smooth muscle of the bladder (Yamamoto *et al.,* 1992) and in the oviduct (Thiede *et al.,* 1991), argues for very sensitive and regional signaling mechanisms. To help discern the intracellular signaling mechanisms that may be associated with changes in blood pressure, specific parathyroid hormone fragments were injected via the femoral vein into anesthetized rats. Human PTH(1-30)NH_2 and hPTH(1-31)NH_2 had potent hypotensive effects when injected intravenously and specifically stimulated adenyl cyclase activation. Infusion of hPTH(7-84), which stimulates phospholipase C, did not cause hypotension in this model. These data suggest that

the hypotensive effects of PTH are mediated through an intracellular cAMP mechanism (Whitfield *et al.,* 1997).

Several studies have addressed the clinical relevance of the association between parathyroid hormone levels and blood pressure measurements. The prevalence of hypertension has been noted to be higher in patients with primary hyperparathyroidism than in the general population. In a population health survey, serum parathyroid hormone levels were measured in a large number of subjects aged 39–79. In a follow-up study in 1998, 72 subjects had elevated levels of PTH and 100 subjects had normal serum PTH levels. After excluding patients with hyperparathyroidism, measurements of serum calcium, serum vitamin D, bone mineral density, and systolic and diastolic blood pressures were obtained. Subjects with elevated PTH levels had significantly lower serum calcium levels and intakes of dietary calcium than those with normal PTH levels. Differences in vitamin D intake or serum levels did not differ between the two groups. Subjects with elevated levels of parathyroid hormone had significantly lower bone mineral density in the lumbar spine than those with normal PTH levels. Females with elevated serum PTH levels had significantly higher systolic and diastolic blood pressures, but this was not true for male subjects (Jorde *et al.,* 2000). A second study looked at the association between serum parathyroid hormone levels in normotensive elderly subjects undergoing 24-hr ambulatory blood pressure monitoring. In this group of 123 subjects aged 63–88 years, serum PTH levels correlated to nocturnal systolic blood pressure, nocturnal diastolic blood pressure, daytime systolic blood pressure, and mean 24-hr systolic blood pressure on univariate and multivariate analysis. Nocturnal, daytime, and mean 24-hr blood pressures were not correlated to serum calcium, 25-hydroxyvitamin D, age, body mass index, or alcohol consumption. In this study, gender differences were also determined. However, men had higher levels of diastolic blood pressure than women. The authors concluded that serum PTH levels were strongly related to blood pressure, particularly nocturnal blood pressure in elderly subjects (Morfis *et al.,* 1997).

To further explore the relationship between blood pressure and levels of parathyroid hormone, interventional studies have been performed in human subjects. The acute administration of parathyroid hormone was utilized to mimic the role of secondary hyperparathyroidism in the pathogenesis of hypertension in patients with renal failure. Because uremia is characterized by insulin resistance and hyperinsulinemia, administration of physiologic doses of hPTH(1-34) was performed under conditions of a euglycemic clamp technique in 10 healthy male subjects. The study design was a double-blind, crossover using a sham infusion or 200 units of hPTH(1-34). The infusion of hPTH(1-34) resulted in a significant increase in mean arterial pressure compared to sham infusions. Intra-individual changes in intracellular calcium concentration determined in platelets were significantly correlated with changes in mean arterial pressure, but insulin sensitivity was not affected by PTH infusion. Thus, subacute administration of physiologic doses of parathyroid hormone under hyperinsulinemic conditions alters intracellular calcium and blood pressure in healthy subjects (Fliser *et al.,* 1997).

The effects of parathyroid hormone on the vasculature extends to potential actions on vascular reactivity as well as the blood pressure per se. Nilsson *et al.* (1999) have shown that in primary hyperparathyroidism, there is an abnormal vasodilatory response to the local infusion of metacholine and nitroprusside. Parathyroid hormone has also been shown to modulate the secretion of endothelin-1, whereas endothelin, in turn, may influence parathyroid hormone secretion. Endothelin levels have been shown to be elevated in the plasma of patients with primary and secondary hyperparathyroidism (Lakatos *et al.,* 1996). More recently, Smith *et al.* (2000) have shown that in mild primary hyperparathyroidism, certain indices of vascular stiffness were higher in primary hyperparathyroidism than in control subjects. Commentary by Silverberg (2000) places this observation in a clinical context.

The most clinically relevant question is whether improvement of blood pressure will occur in patients with elevated levels of parathyroid hormone. Several studies have evaluated blood pressure measurements before and after parathyroidectomy (Schleiffer, 1992). In most cases, hypertension occurs after extirpation of the abnormal parathyroid gland(s) and restoration of parathyroid hormone to normal levels (Sancho *et al.,* 1992). In one study of hypertensive patients on maintenance dialysis, 19 patients were evaluated 1 month before total parathyriodectomy, the first month after surgery, and also after 16 months. There was neither a clinical nor a statistically significant change in either systolic or diastolic blood pressure over time (Ifudu *et al.,* 1998).

In addition to vascular dilatory properties, parathyroid hormone and PTHRP have major effects in influencing cardiac function (Dipette *et al.,* 1992, Schluter and Piper, 1998). These actions include increases in heart rate, coronary blood flow, and contractility. PTH and PTHRP are active in the absence of changes in autonomic reflexes (Bogin *et al.,* 1981; Chui *et al.,* 1991, Dipette *et al.,* 1992; Roca-Cusachs *et al.,* 1991; Tenner *et al.,* 1983). In isolated perfused hearts, parathyroid hormone and PTHRP are positive inotropic agents (Nickols *et al.,* 1989). These studies were not able to determine whether parathyroid hormone and PTHRP are directly inotropic agents because both agents have concomitant effects on heart rate and coronary blood flow, each perturbation having the potential to influence inotropy.

To determine whether parathyroid hormone and PTHRP can directly stimulate cardiac contractility, the isolated, perfused rat heart was studied under conditions where the individual contributions of heart rate, coronary blood flow, and contractility could be assessed independently (Ogino *et al.,* 1995,). In this model, both parathyroid hormone and PTHRP stimulated heart rate, coronary blood flow, and contractility in a dose-dependent manner. When heart rate was fixed by pacing, the effect on coronary blood flow and contractility was still appreciated. However, when heart rate and coronary blood flow were rendered constant by pacing and by maximal dilatation (nitroprusside), respectively, neither parathy-

roid hormone nor PTHRP could directly increase inotropy. These studies provide evidence that the cardiac actions of parathyroid hormone and PTHRP are mediated by effects on heart rate and coronary blood flow as compared with direct actions on contractility per se. More recent studies have called attention to the actions of parathyroid hormone and PTHRP on heart rate as due to increases in the pacemaker current, I_f, of the sinoatrial node (Hara *et al.,* 1995, Hara *et al.,* 1998). These observations provide an electrophysiological basis (Shimoyama *et al.* 1998a) and, more recently, a biochemical basis (Shimoyama *et al.,* 1998b) for the actions of parathyroid hormone and PTHRP to directly alter automaticity of the heart. The demonstration that PTHRP is expressed in the heart (Moniz *et al.,* 1990, Moseley *et al.,* 1991), coupled with the known sensitivity of PTHRP to mechanical stretch forces (Pirola *et al.,* 1994; Thiede *et al.,* 1990; Yamamoto *et al.,* 1992), provides an attractive hypothesis by which PTHRP could be induced to improve cardiac function when the heart is challenged by increases in end diastolic volume.

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CHAPTER 28

Renal and Skeletal Actions of Parathyroid Hormone (PTH) and PTH-Related Protein

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The kidney is the focal point for the physiological regulation of mineral ion homeostasis by circulating parathyroid hormone (PTH). By directly controlling renal tubular reabsorption of calcium and phosphate and the synthesis of $1,25(OH)_2D$, PTH exerts control over both intestinal absorption and urinary excretion of these key mineral ions. Renal tubular responses to PTH deficiency, PTH or PTHRP excess, or defects in function of the type-1 PTH/PTHrP receptor (PTHR) lead to alterations in blood calcium, phosphate, or $1,25(OH)_2D$ that are the hallmarks of numerous clinical disorders, described later in this volume. This chapter reviews current understanding of the mechanisms whereby PTH (and PTHrP) control renal tubular epithelial function. The discussion will focus principally on the known actions of PTH, as relatively little is known of the possible physiologic actions of PTHrP in the kidney. Because the PTHR recognizes the active amino termini of both ligands equivalently, however, it is likely that the effects described for PTH would pertain to PTHrP as well. Expression and action of PTHrP in the kidney are discussed in the last section of the chapter. Whereas species of PTH or PTHrP receptors distinct from the PTHR have been discovered recently (see Chapter 24), the role(s) of these, if any, in normal renal physiology currently is unknown. While not unequivocally proven in each case, it is likely that the effects of PTH and PTHrP described here are mediated by the PTHR.

PTHR Expression, Signaling, and Regulation in the Kidney

The PTHR is widely expressed within the kidney among cells with dramatically different physiologic roles. The response to PTHR activation observed in individual renal cells is a complex function of the number and location of expressed PTHRs on the cell surface; the cell-specific expression of effectors capable of coupling to the PTHR; the cell-specific repertoire of PTHR-regulated genes; enzymes, channels, and transporters; the local concentrations of PTH or PTHrP ligand; exposure to other agents that regulate PTHR function heterologously; and the pattern of recent exposure to PTHR ligand(s).

PTHR Expression within the Kidney

The PTHR is widely but not universally expressed by the various cell types that collectively comprise the mammalian nephron. Early work, based on measurements of regional cAMP responses (Ardaillou *et al.,* 1983; Chabardes *et al.,* 1980; Dousa *et al.,* 1977; Morel, 1981; Sraer *et al.,* 1978) and PTH radioligand binding *in vivo* (Rouleau *et al.,* 1986), indicated that PTHRs are expressed in glomeruli, proximal convoluted (PCT), and straight tubules (PST), the cortical

thick ascending loop of Henle (cTAL), and portions of the distal nephron, including the distal convoluted tubules (DCT), connecting tubules (CNT), and early portions of the cortical collecting ducts (CCD). More recently, these functional observations have been confirmed by *in situ* hybridization of tissue sections or by reverse-transcriptase polymerase chain reaction (RT-PCR) of microdissected nephron segments, using probes derived from cloned PTHR cDNA (Lee *et al.,* 1996; Riccardi *et al.,* 1996; Yang *et al.,* 1997). Minor disparities regarding PTHR expression in Henle's loop and CCD arising from use of these sensitive molecular techniques likely reflect methodologic issues (Lee *et al.,* 1996; Riccardi *et al.,* 1996; Yang *et al.,* 1997).

Given that circulating PTH peptides may be filtered at the glomerulus and appear in the tubular urine, it is of interest that PTHRs are expressed on the apical (luminal) as well as the basolateral membranes of proximal tubular cells (Amizuka *et al.,* 1997; Kaufmann *et al.,* 1994; Shlatz *et al.,* 1975). However, these apical-membrane receptors appear not to be coupled tightly, if at all, to adenylyl cyclase (Kaufmann *et al.,* 1994; Shlatz *et al.,* 1975). Moroever, a high-capacity apical peptide-uptake mechanism, mediated by the multifunctional endocytic clearance receptor megalin (Hilpert *et al.,* 1999), likely would limit the access of filtered bioactive PTH peptides to these receptors. PTHRs are also expressed within the vasculature of the kidney, including peritubular (but not glomerular) endothelial cells and vascular smooth muscle cells (Amizuka *et al.,* 1997). As discussed further later, such receptors may mediate local or systemic vascular effects of PTHRP and PTH, respectively.

As described in more detail in Chapter 24, the PTHR gene incorporates multiple promoters and 5′-untranslated exons and therefore can generate multiple transcripts via alternative promoter usage and different patterns of RNA splicing (Amizuka *et al.,* 1997; Bettoun *et al.,* 1998; Jobert *et al.,* 1996; Joun *et al.,* 1997; McCuaig *et al.,* 1995). It is of interest that certain promoters (i.e., P1 in mouse and P3 in human) seem to be used exclusively in kidney cells, whereas a different promoter (P2) is employed to generate those PTHR mRNAs that are widely expressed in other tissues and organs (Amizuka *et al.,* 1997; Bettoun *et al.,* 1998; Joun *et al.,* 1997). Whether these differences simply reflect opportunities for tissue-specific gene regulation or lead to expression of structurally different forms of the PTHR (Jobert *et al.,* 1996; Joun *et al.,* 1997) remains to be established.

PTHR Signal Transduction in Renal Cells

The PTHR is known to couple to multiple intracellular signal transducers and effectors, including but perhaps not limited to G_s and the G_q/G_{11} family of heterotrimeric G proteins (Abou-Samra *et al.,* 1992) (see also Chapters 24 and 26). Administration of PTH *in vivo* leads to the rapid generation of nephrogenous cAMP (McElduff *et al.,* 1987; Tomlinson *et al.,* 1976) and to activation of protein kinase C (PKC) in basolateral renal cortical membranes (Bellorin-Font *et al.,* 1995). This signaling plurality via the PTHR has been abundantly confirmed and further characterized in extensive studies *in vitro,* which have involved isolated renal tubules or slices, primary renal cortical cell cultures, a widely employed established opossum kidney cell line with characteristics of PCTs (OK cells), immortalized immunoselected distal tubular cells, and various established epithelial cell lines of renal origin (i.e., COS-7, HEK293, LLC-PK1), devoid of endogenously expressed PTHRs, which have been transfected with cDNA encoding the cloned PTHR (Abou-Samra *et al.,* 1992; Azarani *et al.,* 1995, 1996; Bringhurst *et al.,* 1993; Coleman and Bilezikian, 1990; Friedman *et al.,* 1996, 1999; Goligorsky *et al.,* 1986a; Guo *et al.,* 1995; Henry *et al.,* 1983; Hruska *et al.,* 1986, 1987; Janulis *et al.,* 1992; Jobert *et al.,* 1997; Martin *et al.,* 1994; Meltzer *et al.,* 1982; Nemani *et al.,* 1991; Offermanns *et al.,* 1996; Pines *et al.,* 1996; Quamme *et al.,* 1989a; Schneider *et al.,* 1994; Siegfried *et al.,* 1995; Smith *et al.,* 1996; Tamura *et al.,* 1989; Teitelbaum and Strewler, 1984). Collectively, these studies indicate that PTH can activate adenylyl cyclase, protein kinase A (PKA), phospholipase C (PLC), PKC, and cytosolic-free calcium (Ca_i^{2+}) transients, as well as phospholipase A_2 (PLA_2) (Derrickson and Mandel, 1997; Ribeiro *et al.,* 1994; Ribeiro and Mandel, 1992) and phospholipase D (PLD) (Friedman *et al.,* 1996, 1999). Other signaling mechanisms may be recruited by PTHRs in renal cells as well. For example, PTH-induced activation of mitogen-activated protein kinases in renal epithelial cells may proceed via activation of nonreceptor tyrosine kinases, phosphorylation of EGF receptors, and subsequent assembly of active Ras/Raf-1/MEK complexes (Cole, 1999; Lederer *et al.,* 2000).

The repertoire of PTHR signaling appears to differ depending on the region of the nephron in which it is expressed. For example, cells of proximal tubular origin manifest an acute spiking Ca_i^{2+} response that likely is triggered by inositol trisphosphate released via PLC activation. Cells of distal tubular origin, in contrast, exhibit a very delayed and sustained Ca_i^{2+} response (probably due to apical Ca^{2+} entry; see later) and show PKC activation in the absence of PLC stimulation (Friedman *et al.,* 1996). The PKC response to PTH in these DCT cells may be mediated by PLD (Friedman *et al.,* 1999). The coupling of specific PTHR-generated signals to the various physiologic responses to PTH or PTHRP that occur in different renal epithelial cells has not yet been fully clarified and will be discussed further later.

Regulation of PTHR Signaling in Renal Cells

As in other PTH/PTHRP target cells, the responsiveness of renal epithelial cells to PTH or PTHRP may be regulated, both by previous or chronic exposure to the homologous ligand and by other agonists that do not interact directly with the PTHR. Desensitization of renal cellular responsiveness during continuous exposure to high concentrations of PTH or

PTHRP has been well documented and studied extensively. Chronic hyperparathyroidism, either primary or secondary to calcium or vitamin D deficiency, as well as acute infusion of PTH, leads to PTH resistance in humans or animals, manifested by impaired cAMP and phosphaturic responses (Bellorin-Font *et al.,* 1995; Carnes *et al.,* 1980; Forte *et al.,* 1976; Tomlinson *et al.,* 1976; Tucci *et al.,* 1979). In humans, the cAMP response may be desensitized more readily than the phosphaturic response at low doses of hormone (Law and Heath, 1983). Similar desensitization is observed in cultured renal epithelial cells (Fujimori *et al.,* 1993; Guo *et al.,* 1997; Henry *et al.,* 1983; Pernalete *et al.,* 1990; Urena *et al.,* 1994a).

Several factors may contribute to this renal resistance to PTHR activation, including a reduced number of cell surface PTHRs, persistent occupancy of PTHRs by ligand, and defective coupling between available PTHRs and the G proteins that mediate activation of effectors such as adenylyl cyclase or PLC (i.e., a "postreceptor" defect). The relative roles of these factors in causing PTHR desensitization appear to vary according to the specific situation and experimental system (Carnes *et al.,* 1978; Forte *et al.,* 1976; Mahoney and Nissenson, 1983; Mitchell *et al.,* 1988; Tamayo *et al.,* 1982; Turner *et al.,* 1995). As reviewed in more detail in Chapters 24 and 26, it is clear that PTHRs are internalized rapidly following ligand occupancy and activation, a response that lowers cell surface receptor expression and that is due to PTHR phosphorylation by both PTHR-dependent activation of "signal kinases" (PKA, PKC) and the action(s) of generic G protein-coupled receptor kinases (Blind *et al.,* 1996; Dicker *et al.,* 1999; Qian *et al.,* 1998). The particular PTHR-generated signals that mediate PTHR desensitization in renal epithelial cells may be cell type specific. For example, in OK proximal tubular cells, homologous desensitization of the PTHR cAMP response is PKC dependent (Pernalete *et al.,* 1990), whereas in PTHR-transfected LLC-PK1 cells, desensitization is pathway specific, i.e., adenylyl cyclase is fully desensitized by cAMP-dependent signaling only, whereas desensitization of the PLC response is linked to prior PLC activation (Guo *et al.,* 1997). Control of receptor expression may be an important mechanism for modulating the relative, as well as the absolute, intensities of signaling along the various transduction pathways coupled to the PTHR. Thus, as shown in a series of PTHR-transfected LLC-PK1 renal epithelial cell subclones that comprised a broad range of receptor expression, the magnitude of the PLC response was influenced much more strongly by changes in cell surface PTHR density than the adenylyl cyclase response (Guo *et al.,* 1995). This was interpreted as evidence that the coupling between G_s and the PTHR in these cells is more efficient than that between the PTHR and the G_q that presumably mediates PLC activation. In any event, it is clear that changes in PTHR expression may allow differential modulation of PTHR signaling responses in a given renal cell.

Expression of PTHRs on the surface of kidney cells is also controlled by the rate of PTHR gene transcription, although current understanding of this process is incomplete. Hypoparathyroidism, induced by either parathyroidectomy or dietary phosphate depletion, strongly upregulates PTHR mRNA levels in rat renal cortex (Kilav *et al.,* 1995). Curiously, the opposite effect, i.e., suppression of PTHR mRNA by exposure to high concentrations of PTH, has not been observed either *in vivo* or *in vitro* (Kilav *et al.,* 1995; Urena *et al.,* 1994a). Renal PTHR mRNA expression is reduced in rats with renal failure, but this apparently is due to some aspect of uremia or renal disease other than secondary hyperparathyroidism *per se*, as it is not prevented by parathyroidectomy (Largo *et al.,* 1999; Urena *et al.,* 1994b, 1995). In rats with secondary hyperparathyroidism due to vitamin D deficiency, renal cortical PTHR mRNA levels actually were found to be twice as high as normal, a change that could not be corrected by normalizing serum calcium (Turner *et al.,* 1995). This experiment has been interpreted as evidence of a suppressive action of vitamin D on PTHR gene transcription in the proximal tubule, although this may not be true of all renal epithelial cells. For example, PTHR expression is upregulated severalfold by $1,25(OH)_2D_3$ in immortalized DCT cells (Sneddon *et al.,* 1998). In cultured OK cells, TGFβ1 was shown to diminish PTHR mRNA expression, but the possible physiologic significance of this effect *in vivo* has not been clarified (Law *et al.,* 1994). PTHR mRNA expression was not affected by the mild secondary hypoparathyroidism induced by ovariectomy in rats nor by subsequent estrogen treatment (Cros *et al.,* 1998).

Calcium and Magnesium Excretion

The action of PTH to maintain blood calcium was among the first to be described, and early observations in animals or patients with hypoparathyroidism or hyperparathyroidism clearly implicated abnormalities in renal calcium handling (see Chapters 56 and 62). Alterations in serum magnesium concentrations frequently are encountered also in patients with parathyroid disorders, which led to the understanding that PTH participates in magnesium homeostasis as well (see Chapter 21). The mechanisms whereby Ca^{2+} and Mg^{2+} are reabsorbed are similar and interrelated in some regions of the nephron but different in others.

Sites and Mechanisms of Calcium and Magnesium Reabsorption

Calcium and Mg^{2+} are reabsorbed at many sites along the nephron (de Roufignac and Quamme, 1994; Friedman, 1998). Approximately 60% of filtered Ca^{2+}, but only 20% of filtered Mg^{2+}, is reabsorbed by the proximal tubule. Reabsorption here is almost entirely passive, driven by both the ambient lumen-positive voltage and the progressive concentration of these ions within the tubular urine as Na^+ and water are reabsorbed along the proximal segments (de Roufignac and

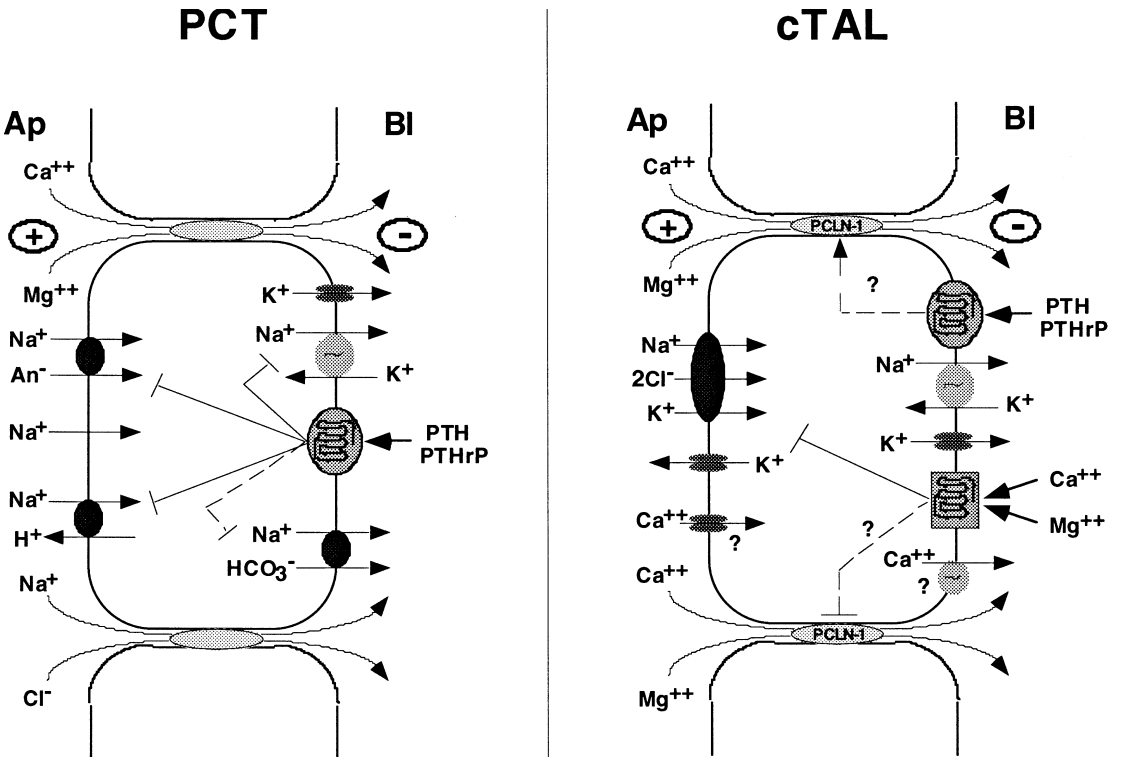

Figure 1 Calcium and magnesium reabsorption in PCT and cTAL. In the PCT (left), Ca^{2+} and Mg^{2+} are reabsorbed passively via paracellular routes at rates driven by the lumen-positive transepithelial voltage and limited by the conductance of the intercellular junctions for these cations. Transpeithelial voltage, depicted as positive at the apical ("Ap") relative to the basolateral ("Bl") side of the epithelium, is generated by paracellular diffusion of Cl^- ions, which, like Ca^{2+} and Mg^{2+} ions, are concentrated progressively along the lumen by active transcellular Na^+ reabsorption. Major mechanisms of Na^+ reabsorption shown include Na^+/H^+ exchange, Na^+-dependent cotransport of anions (phosphate, amino acids, sulfate, etc.), and a small apical Na^+ conductance, all driven by the low intracellular Na^+ concentration established by the Na^+-K^+-ATPase, which pumps 3 Na^+ ions out for each 2 K^+ ions that enter the cell. The stoichiometry of the basolateral electrogenic Na^+/HCO_3^- cotransporter (1 Na^+ per 3 HCO_3^- ions) allows for active basolateral extrusion of some Na^+ because of the negative intracellular potential (not shown) and the favorable HCO_3^- concentration gradient that drive HCO_3^- exit. PTHRs expressed in PCT inhibit Na^+ transport by multiple mechanisms and thereby moderately impair Ca^{2+} and Mg^{2+} reabsorption (dashed lines indicate responses about which some uncertainty exists). In cTAL (right), Ca^{2+} and Mg^{2+} reabsorption again occurs mainly via voltage-dependent paracellular transport, although transcellular Ca^{2+} transport, presumably mediated by apical Ca^{2+} channels and basolateral Ca^{2+}-ATPases, has also been described ("?"). Apical NKCC2 cotransporters and ROMK K^+ channels maintain the lumen-positive transepithelial voltage necessary for cation transport, which is inhibited by the Ca^{2+}/Mg^{2+}-dependent activation of the CaSR and by the loop diuretic furosemide. Chloride exits across the basolateral membrane via one or more Cl^- channels, including ClC-Kb (not shown). The channel protein parcellin-1 appears to be critical for paracellular cation transport in the cTAL and could be a target for CaSRs and PTHRs, which, respectively, reduce and augment cation transport in this nephron segment.

Quamme, 1994; Frick *et al.,* 1965; Friedman, 1998) (Fig. 1). In the proximal tubule, the route of reabsorption for both Ca^{2+} and Mg^{2+} is almost entirely paracellular, and differences in permeability of the intercellular tight junctions for the two cations presumably account for the preferential reabsorption of Ca^{2+} here.

Both Ca^{2+} and Mg^{2+} are also passively reabsorbed in the cTAL of Henle's loop, although here the permeability for Mg^{2+} may be greater than that for Ca^{2+}, as 60% of Mg^{2+} but only 20% of Ca^{2+} is reabsorbed in this segment. The lumen-positive transepithelial voltage gradient that drives Ca^{2+} and Mg^{2+} transport in the cTAL is maintained by, and proportional to, the rate of Na^+-K^+-$Cl^-{}_2$ transport, which is dependent, in turn, on the activities of the NKCC2, ClC-Kb, and ROMK transporters (Hebert *et al.,* 1984). The calcium-sensing receptor (CaSR) is also especially strongly expressed in Henle's loop, and activation of this receptor by high peritubular Ca^{2+} or Mg^{2+} concentrations inhibits Ca^{2+} and Mg^{2+} reabsorption in the cTAL, presumably by reducing the transepithelial voltage gradient (Brown *et al.,* 1998) (see Chapter 23). It is also possible that the CaSR may mediate inhibition by Ca^{2+} and Mg^{2+} of the cAMP response to PTH (Bapty *et al.,* 1998; Slatopolsky *et al.,* 1976; Takaichi and Kurokawa, 1986). Paracellin-1, a novel member of the claudin family of tight-junction proteins that is expressed only in Henle's loop and the DCT, has been identified as the cause of an autosomal recessive renal magnesium and Ca^{2+}-wasting disorder (Simon *et al.,* 1999). While not yet demonstrated directly, it seems likely that the expression of paracellin-1 may control the passive permeability of the cTAL for both Ca^{2+} and Mg^{2+}. There is also some evidence for active, transcellular transport of Ca^{2+} by

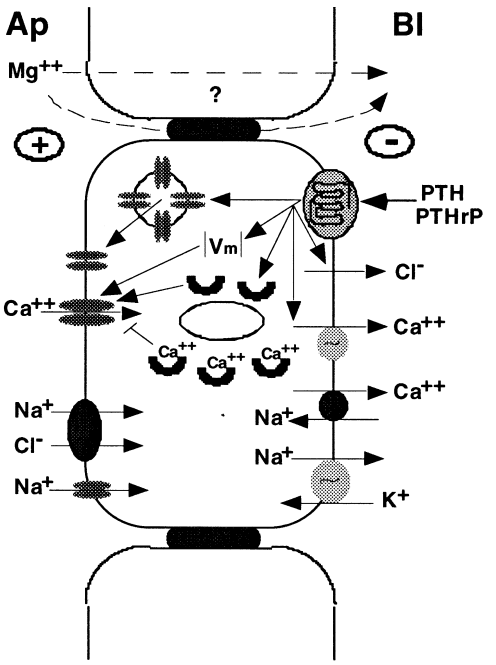

Figure 2 PTH regulation of distal tubular calcium reabsorption. In DCT, Ca^{2+} reabsorption involves apical Ca^{2+} entry via voltage-sensitive Ca^{2+} channels and subsequent basolateral extrusion by Ca^{2+}-ATPases and, uniquely, Na^+/Ca^{2+} exchangers driven by the Na^+-K^+-ATPase. Multiple Ca^{2+} channels may be expressed here, including CaT2 and the ECaC channel that is activated by hyperpolarizing voltages (increased $|V_m|$). Inhibition of the thiazide-sensitive NaCl transporter, with continued basolateral Cl^- exit, hyperpolarizes the cell toward the K^+ equilibrium potential, which then increases Ca^{2+} entry by CaT2/ECaC and possibly other channels activated by hyperpolarizing potentials. Calbindin D28K binds and shuttles Ca^{2+} from the apical membrane to the basolateral sites of active Ca^{2+} extrusion, thereby buffering the cytoplasm from high concentrations of transported Ca^{2+}. Calbindin D28K is induced by $1,25(OH)_2D_3$ and may directly activate apical Ca^{2+} channels, which otherwise are inhibited by intracellular Ca^{2+} ions. PTHR activation leads to insertion of additional apical Ca^{2+} channels, hyperpolarization of the cell (? via enhancing basolateral Cl^- exit), and thus activation of Ca^{2+} channels, increased calbindin D28K expression, and stimulation of the basolateral Ca^{2+}-ATPase. The routes and mechanisms of Mg^{2+} reabsorption in DCT are unknown.

the cTAL (Friedman and Gesek, 1995). Calcium-sensitive cation channels have been found in cTAL apical membranes (Chraibi *et al.,* 1994), as have Ca^{2+}-ATPases that would be necessary for extrusion across the steep basolateral electrochemical gradient (Caride *et al.,* 1998).

Finally, small but critical fractions of filtered Ca^{2+} and Mg^{2+} — approximately 5–10% each — are reabsorbed in the distal nephron (i.e., the DCT, CNT, and early CCD). The mechanism of Mg^{2+} reabsorption by the distal nephron is obscure, but it seems to be closely related to that of NaCl, in that both pharmacologic (thiazide diuretics) and genetic (Gitelman's syndrome) inhibition of the thiazide-sensitive NaCl cotransporter (TSC) impairs Mg^{2+} reabsorption. In contrast, Ca^{2+} reabsorption in the distal nephron, which involves transcellular active transport against an unfavorable electrochemical gradient (Costanzo and Windhager, 1978; Lau and Bourdeau, 1995; Shareghi and Stoner, 1978), is promoted by TSC inhibition, which hyperpolarizes the apical cell membrane. Cells of the distal nephron express several proteins that are required for effective transcellular active Ca^{2+} transport (Friedman, 1998) (see Fig. 2). Calcium enters the apical membrane via multiple Ca^{2+} channels (Barry *et al.,* 1998; Matsunaga *et al.,* 1994), one of which, ECaC, has been cloned from rabbit and shown to be expressed in the distal tubule, to be activated by hyperpolarizing voltages, and to be inactivated by intracellular Ca^{2+} (Hoenderop *et al.,* 1999a,b;). Another Ca^{2+} transporter, CaT2, with properties similar but not identical to those of ECaC, has been cloned from rat and also shown to be expressed in distal tubular cells (Peng *et al.,* 2000). These cells also express the vitamin D-dependent calbindin-D28K calcium-binding protein, which can transport Ca^{2+} across the cytoplasm while buffering the submicromolar cytosolic-free Ca^{2+} concentration against the high mass flux of transported Ca^{2+} (Bronner and Stein, 1988; Liu *et al.,* 1996; Van Baal *et al.,* 1996). Calbindin-D28K may also directly activate apical membrane Ca^{2+} channels (Bouhtiauy *et al.,* 1994). Extrusion of transported Ca^{2+} across the basolateral membrane can occur via both a direct Ca^{2+}-ATPase and a high-capacity Na^+/Ca^{2+} exchanger driven by the transmembrane Na^+ gradient.

PTH Regulation of Renal Calcium and Magnesium Excretion

Administration of PTH *in vivo* increases the net renal reabsorption of both Ca^{2+} and Mg^{2+} (Bailly *et al.,* 1984; Burnatowska *et al.,* 1977; de Roufignac and Quamme, 1994; Everhart-Caye *et al.,* 1996; Massry and Coburn, 1973; Peacock *et al.,* 1969). PTH augments Mg^{2+} reabsorption in the cTAL (De Rouffignac *et al.,* 1991; Di Stefano *et al.,* 1990; Shareghi and Agus, 1982) and possibly in the distal nephron as well (Bailly *et al.,* 1985), but the mechanism(s) involved is obscure. PTH may increase the transepithelial voltage that drives paracellular Mg^{2+} (and Ca^{2+}) transport in the cTAL, but this is controversial (Di Stefano *et al.,* 1990; Shareghi and Agus, 1982) and, in any event, is unlikely to explain the magnitude of the PTH effect (de Roufignac and Quamme, 1994). Other experiments indicate that the PTH response probably is mediated by an increase in paracellular Mg^{2+} permeability (Wittner *et al.,* 1993). In this regard, it will be of interest to learn if PTH upregulates paracellin-1 expression or permeability.

Although PTH increases net renal Ca^{2+} reabsorption overall, it inhibits Ca^{2+} reabsorption somewhat in the PCT (Agus *et al.,* 1971, 1973; Amiel *et al.,* 1970). As will be discussed further later, this results from the action of PTH to reduce Na^+ reabsorption (via inhibition of both NaP_i cotransport and Na^+/H^+ exchange) and Na^+-K^+-ATPase activity, processes that otherwise support net solute and water reabsorption and thereby establish the elevated intraluminal concentrations of Ca^{2+} and Cl^- required for the effective paracellular movement of Ca^{2+} in the PCT. In contrast, PTH augments Ca^{2+} reabsorption in the cTAL and in the distal nephron, especially

in the CNT (Agus *et al.,* 1973; Bourdeau and Burg, 1980; Imai, 1981; Shareghi and Stoner, 1978; Shimizu *et al.,* 1990a), and it is these actions that account for the overall positive effect of PTH on renal Ca^{2+} reabsorption. The mechanism of the PTH effect in the cTAL has not been studied intensively but likely proceeds via an increase in transepithelial voltage and enhanced paracellular Ca^{2+} transport (Di Stefano *et al.,* 1990), although some evidence suggests a component of transcellular transport as well (Friedman, 1988).

The distal nephron clearly is the major site at which PTH regulates Ca^{2+} transport. PTH exerts several specific actions in these cells that independently contribute to increased Ca^{2+} reabsorption. PTH increases Ca^{2+} uptake across apical membranes of distal tubular cells, an effect that can be observed in apical membrane vesicles isolated following PTH administration *in vivo* or to isolated tubules *in vitro* (Bourdeau and Lau, 1989; Lajeunesse *et al.,* 1994). In cultured cells obtained from mouse cTAL and DCT, PTH induced a delayed (10 min) and sustained increase in cytosolic Ca^{2+} that was of extracellular origin, was blocked by dihydropyridine Ca^{2+} channel antagonists, and appeared to result from the exocytosis of membranes harboring preformed but functionally latent intracellular Ca^{2+} channels (Bacskai and Friedman, 1990). These channels were of low conductance and were activated by hyperpolarizing voltages (Matsunaga *et al.,* 1994), features also reported for the subsequently cloned ECaC channel (Hoenderop *et al.,* 1999b). It is not yet clear, however, if either ECaC or CaT2 serves as a major route of regulated Ca^{2+} entry in distal tubular cells, nor is it established that these channels are regulated by PTH. Importantly, PTH acutely hyperpolarizes distal tubular cells, at least in part by increasing basolateral Cl^- conductance (Gesek and Friedman, 1992). This action could activate ECaC or CaT2 channels (Hoenderop *et al.,* 1999b; Peng *et al.,* 2000) and also increase both the driving force for apical membrane Ca^{2+} entry and the rate of Na^+/Ca^{2+} exchange at the basolateral membrane (White *et al.,* 1996). Increased Na^+/Ca^{2+} exchange has been demonstrated following PTH administration *in vivo* and *in vitro* (Hanai *et al.,* 1986; Scoble *et al.,* 1985). Moreover, activation of Na^+/Ca^{2+} exchange is critical for the action of PTH to increase Ca^{2+} reabsorption, as this can be blocked completely in rabbit CNTs and DCT cells either by disrupting the Na^+ gradient that drives the Na^+/Ca^{2+} exchanger with ouabain or monensin or by removing extracellular Na^+ from the basolateral compartment (Shimizu *et al.,* 1990b; White *et al.,* 1996). The fact that this exchanger is expressed only in the distal, and not the proximal, nephron may explain, at least in part, why distal and not proximal tubular cells can conduct transcellular Ca^{2+} transport (Bouhtiauy *et al.,* 1991; Lajeunesse *et al.,* 1994; White *et al.,* 1996, 1997). PTH may also increase Ca^{2+} extrusion by activating basolateral Ca^{2+}-ATPase (Levy *et al.,* 1986), although this is not observed in all systems (Bouhtiauy *et al.,* 1991). Finally, expression of the calbindin-D28K protein in renal cortex has been shown to decrease following parathyroidectomy and to increase following PTH infusion into intact rats (Hemmingsen *et al.,* 1996). The powerful inductive effect of $1,25(OH)_2D_3$ on calbindin-D28K expression in the distal nephron (Christakos *et al.,* 1981; Rhoten *et al.,* 1985) may be involved in mediating this action of PTH, given that PTH augments $1,25(OH)_2D_3$ synthesis (see later) and that $1,25(OH)_2D_3$ directly accelerates the distal tubular calcium reabsorptive response to PTH *in vitro* (Friedman and Gesek, 1993). Other evidence indicates that PTH can increase calbindin-D28K independently of $1,25(OH)_2D_3$ or serum calcium, however (Hemmingsen *et al.,* 1996).

PTHR Signal Transduction in Regulation of Calcium and Magnesium Excretion

The particular PTHR-generated signals responsible for these various effects of PTH on components of the distal tubular Ca^{2+}-reabsorptive response are not fully clarified. The initial entry of Ca^{2+} across the apical membrane seems to require activation of both PKA and PKC in immortalized murine DCT cells (Friedman *et al.,* 1996, 1999). In many experimental systems, the PTH effect on distal Ca^{2+} transport can be mimicked by cAMP analogs or phosphodiesterase inhibitors (Bourdeau and Lau, 1989; Lau and Bourdeau, 1989; Shimizu *et al.,* 1990b), although in isolated rabbit CNT/CCD tubules, in which this cAMP mimicry also pertains, the effect of PTH was prevented by chelerythrine, a PKC inhibitor, but not by dideoxyadenosine, an adenylyl cyclase inhibitor that did block PTH-dependent cAMP accumulation (Hoenderop *et al.,* 1999a). Further evidence implicated a Ca^{2+}-independent ("atypical") PKC as a mediator of this PTH effect (Hoenderop *et al.,* 1999a). In murine DCT cells, PTH activates the mitogen-activated protein kinase ERK2 via a PKC-dependent mechanism, and inhibition of the ERK2 kinase (MEK) blocks PTH-induced increases in cytosolic Ca^{2+} (Sneddon *et al.,* 2000). Similarly, the ability of dibutyryl cAMP to promote Ca^{2+} transport in rabbit distal tubules was potentiated greatly by phorbol esters, which exerted no effect alone, and PKC inhibitors did block the effect of the combination of phorbol and cAMP analog as well as that of the cAMP analog alone (Hilal *et al.,* 1997). PTH stimulation of Na^+/Ca^{2+} exchange, transepithelial hyperpolarization, and, in canine cells, Ca^{2+}-ATPase is also reproduced by cAMP analogs (Bouhtiauy *et al.,* 1991; Hanai *et al.,* 1986; Levy *et al.,* 1986; Scoble *et al.,* 1985; Shimizu *et al.,* 1990a), although, as just noted, such evidence clearly does not exclude a role for other PTHR messengers in these processes as well. Considering that PTH may have to orchestrate a series of independent "elemental responses" to achieve effective distal tubular Ca^{2+} reabsorption, including membrane hyperpolarization, increased exocytosis of latent Ca^{2+} channels, increased calbindin-D28K expression, increased Na^+/Ca^{2+} exchange [this possibly secondary entirely to the hyperpolarization and increased cytosolic-free Ca^{2+} (Friedman, 1998)] and increased Ca^{2+}-ATPase activity, and that these responses may not all occur in the same cells, it is perhaps not surprising that some ambiguity persists regarding the roles of PKA vs PKC (or other PTHR-activated effectors)

in controlling overall distal tubular Ca^{2+} transport. Apparent requirements for multiple effectors may reflect a convergence of several signals on a single mechanism, independent actions of different effectors on one or more of the elemental cellular responses that contribute to the overall Ca^{2+}-reabsorptive response, or both.

Phosphate Excretion

Phosphaturia was one of the earliest recognized actions of PTH (Albright *et al.,* 1929; Collip, 1925; Ellsworth and Howard, 1934; Hiatt and Thompson, 1956). With the advent of micropuncture analysis, it became clear that the effect of PTH to inhibit phosphate reabsorption occurs almost entirely in the proximal tubules, especially in the late portion of the PCT (Agus *et al.,* 1971, 1973; Amiel *et al.,* 1970; Brunette *et al.,* 1973; LeGrimellec *et al.,* 1974; Strickler *et al.,* 1964; Wen, 1974). Some evidence points to a small component of PTH-inhibitable phosphate reabsorption in the distal nephron as well (Amiel *et al.,* 1970; Bengele *et al.,* 1979; Pastoriza-Munoz *et al.,* 1978; Pastoriza-Munoz *et al.,* 1983; Wen, 1974).

Mechanisms of Proximal Tubular Phosphate Reabsorption

Extensive experimentation with isolated perfused tubules, renal membranes, and membrane vesicles since the mid-1970s, reviewed exhaustively by Murer and colleagues (Gmaj and Murer, 1986; Murer *et al.,* 1991; Murer, 1992), has provided a clear picture of the mechanisms of proximal tubular phosphate reabsorption. Phosphate (P_i) must be moved across the apical membrane of the cell against a steep electrochemical gradient imposed by the strongly negative intracellular potential. This is accomplished by Na^+/P_i cotransporters energized by the high transmembrane Na^+ gradient. Early biochemical analyses had indicated that multiple such Na^+/P_i cotransporters, with distinct kinetic, allosteric, and physical properties, are located within the renal cortex (Levi, 1990; Walker *et al.,* 1987). Some of these may be so-called "housekeeping" cotransporters, presumed to reside on the basolateral membranes, that are expressed ubiquitously by all cells and involved in maintaining intracellular P_i concentrations, whereas others are epithelial specific and devoted to the specialized function of transepithelial phosphate transport (Murer, 1992) (Fig. 3).

Three major classes (types I, II, and III) of Na^+/P_i ("NaP_i") cotransporters, products of different genes, have been cloned and shown to be expressed in PCT cells (Custer *et al.,* 1993, 1994; Murer and Biber, 1997; Tenenhouse, 1997). Type I and type IIa NaP_i cotransporters are localized to the apical brush border membrane of PCT cells (Murer *et al.,* 1996). [Type IIb transporters, closely related to type IIa, are expressed in intestine but not in kidney (Hilfiker *et al.,* 1998),] Type III NaP_i cotransporters, originally identified as cell surface virus

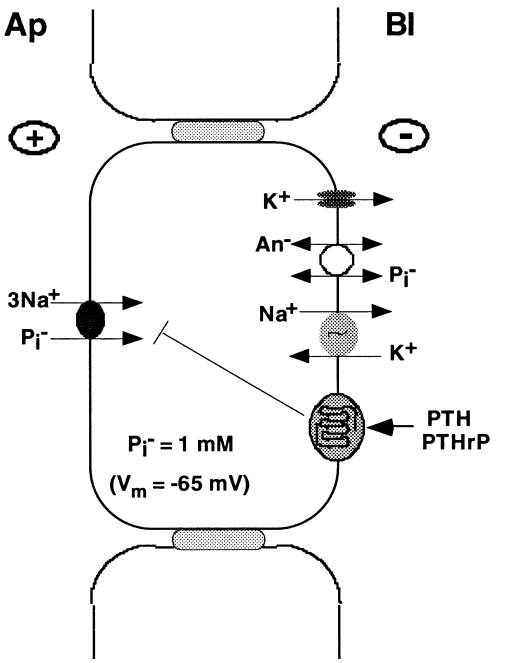

Figure 3 Phosphate reabsorption in the proximal tubule. Phosphate (P_i) must be transported actively across the apical membrane of the PCT cell because of the strongly interior-negative potential and the fact that the cytosolic P_i concentration (1 m*M*) is roughly 100-fold above equilibrium. This transport is accomplished by an electrogenic type IIa NaP_i cotransporter [stoichiometry = 3 Na^+ ions per Pi (mono- or dibasic) ion] that is energized by the steep transmembrane Na^+ gradient established by the basolateral Na^+-K^+-ATPase. Activity of this cotransporter is reduced by PTHR activation. Mechanisms of basolateral P_i exit are not well understood, but an anion exchanger could allow P_i to leave the cell passively.

receptors (Glvr-1 and Ram-1), are widely expressed, both within and outside the kidney (Kavanaugh and Kabat, 1996). Type III cotransporters, which are regulated by extracellular P_i deprivation and, via a PKC-dependent mechanism, by PTHRs, are also expressed by DCT cells and thus might be involved in phosphate reabsorption in both proximal and distal nephrons (Fernandes *et al.,* 2001; Tenenhouse *et al.,* 1998). As shown by targeted gene disruption in mice, type IIa cotransporters account for 70% of renal P_i reabsorption (Beck *et al.,* 1998). They are 80- to 90-kDa glycoproteins that are predicted to span the membrane eight times, with both their amino and carboxyl termini oriented into the cytosol (Murer and Biber, 1997). Type IIa cotransporters are electrogenic and transport Na^+ and $H_2PO_4^-$ in a molar ratio of 3:1 (Murer and Biber, 1997). Expression and activity of type IIa NaP_i cotransporters ("NaP_i-2", in rat; "NaP_i-3" or "NPT2" in human) are strongly regulated by both parathyroid status and dietary phosphate (Keusch *et al.,* 1998; Lotscher *et al.,* 1999; Murer *et al.,* 1996; Pfister *et al.,* 1997; Ritthaler *et al.,* 1999; Takahashi *et al.,* 1998), and PTH regulation of serum P_i and tubular NaP_i reabsorption is lost in mice lacking type IIa cotransporters (Zhao and Tenenhouse, 2000). Thus, regulation of NaP_i-2 activity is the principal mechanism whereby PTH controls phosphate reabsorption in the PCT.

PTH Regulation of Proximal Tubular Phosphate Reabsorption

Early work had demonstrated that PTH rapidly lowers the maximal rate of NaP_i cotransport in brush border membrane vesicles and that recovery from this effect requires new protein synthesis, suggesting that PTH causes degradation of NaP_i cotransporters (Dousa *et al.,* 1976; Gmaj and Murer, 1986; Hammerman, 1986; Malmstrom and Murer, 1987). Functional and immunohistochemical analyses of NaP_i-2 protein expression in rat kidney and in cultured OK cells have confirmed that PTH induces a rapid (15 min) movement of NaP_i-2 protein away from the apical membrane, into the subapical endocytic apparatus, followed by a microtubule-dependent delivery to lysosomes and proteolytic degradation (Kempson *et al.,* 1995; Keusch *et al.,* 1998; Lotscher *et al.,* 1999; Pfister *et al.,* 1997; Zhang *et al.,* 1999) (see Fig. 4). This PTH-induced retrieval of NaP_i-2 from apical membranes requires the presence of a specific dibasic amino acid motif in the most carboxyl-terminal of the putative cytosolic loops of NaP_i-2 and apparently is not mediated by the direct action of kinases upon the NaP_i-2 protein (Karim-Jimenez *et al.,* 2000). PTH does not acutely reduce NaP_i-2 mRNA expression, although parathyroidectomy does lead to increases of severalfold in levels of both apical NaP_i-2 protein and mRNA (Kilav *et al.,* 1995; Pfister *et al.,* 1998; Saxena *et al.,* 1995; Takahashi *et al.,* 1998).

PTHR Signal Transduction in the Regulation of Phosphate Excretion

Early experiments *in vivo* or with isolated renal membranes indicated a role for cAMP-dependent actions of PTH in regulating phosphate excretion, based mainly on mimicry of the PTH effect by cAMP analogs or cAMP phosphodiesterase inhibitors (Agus *et al.,* 1973; Gmaj and Murer, 1986; Hammerman, 1986). Many of these studies were conducted before the cAMP-independent signaling features of the PTHR were recognized, however (Dunlay and Hruska, 1990).

The involvement of specific PTHR-generated signals in NaP_i regulation has been pursued extensively *in vitro* using the OK opossum kidney cell line, which expresses both type IIa NaP_i cotransporters and PTHRs (Juppner *et al.,* 1991), exhibits other features of PCT cells, and manifests PTH-dependent inhibition of NaP_i cotransport (Caverzasio *et al.,* 1986; Cole *et al.,* 1988; Lederer *et al.,* 1998; Martin *et al.,* 1994; Murer, 1992; Pfister *et al.,* 1999; Segal and Pollock, 1990). There is general agreement that direct pharmacologic activation of either PKA or PKC can inhibit NaP_i activity in OK cells. The importance of the cAMP response of PTHR was highlighted by experiments in which expression of a dominant-negative inhibitor of PKA (mutant PKA regulatory subunit gene) in OK cells completely blocked NaP_i downregulation by PTH (Segal and Pollock, 1990) and by the demonstration that NaP_i regulation by PTH was unaffected when PLC/PKC activation was completely

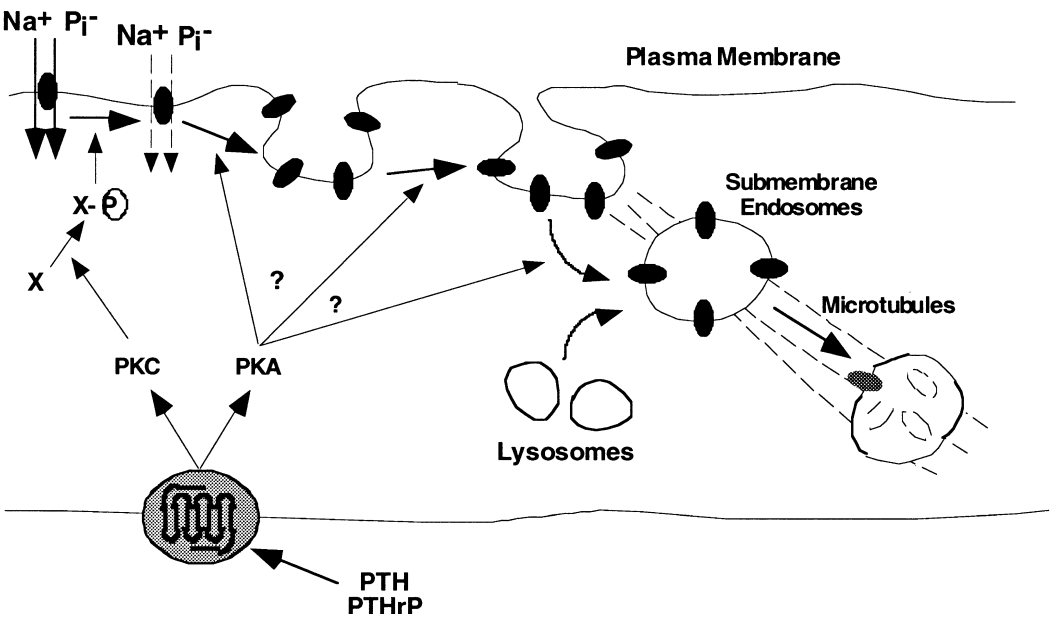

Figure 4 Regulation of NaP_i cotransport by PTH. Activation of PTHRs on the basolateral membrane of PCT cells stimulates PKA and PKC. PKC induces a rapid decrease in the activity of NaP_i-2 transporters expressed on the apical surface, an effect that is mimicked by PTH(3-34). This may involve phosphorylation of one or more intermediary proteins ("X"), as consensus PKC phosphorylation sites within the NaP_i-2 protein can be eliminated without affecting this regulatory effect of PKC. Activation of PKA also impairs NaP_i cotransport, but this effect is more delayed and involves retrieval of surface NaP_i-2 cotransporters by a microtubule-dependent process of endocytosis, lysosomal fusion, and degradation. The responsible PKA substrates and details of their actions currently are unknown ("?").

inhibited by the drug U73122 (Martin *et al.,* 1994). However, a role for PKC was suggested by findings that NaP_i activity could be at least partially regulated by PTH analogs, such as PTH(3-34), at concentrations that do not activate adenylyl cyclase or PKA but which do stimulate PKC (Cole *et al.,* 1988, 1989; Pfister *et al.,* 1999; Reshkin *et al.,* 1991). Moreover, NaP_i regulation in OK cells by PTH(1-34) can be blocked by pharmacologic inhibition of PKC and may be observed at concentrations that activate PKC but are too low to measurably stimulate PKA (Cole *et al.,* 1988; Malmstrom *et al.,* 1988; Quamme *et al.,* 1989a,b). The NaP_i-2 protein can be phosphorylated and contains several consensus sites for PKC, but mutation of these sites does not interrupt PKC- or PTH-dependent downregulation of NaP_i-2 activity (Hayes *et al.,* 1995; Karim-Jimenez *et al.,* 2000; Murer *et al.,* 1996). Thus, PKC-dependent phosphorylation of other proteins, which then act to regulate NaPi-2, may mediate this effect (Hayes *et al.,* 1995; Murer *et al.,* 1996).

Studies involving selective application of PTH(1-34) or PTH(3-34) to the apical vs basolateral surfaces of perfused murine proximal tubules indicate that PTHRs present in both membrane domains can induce rapid retrieval of apical NaP_i proteins but that this occurs via an exclusively PKC-dependent mechanism via activation of apical PTHRs, whereas basolateral PTHRs apparently accomplish this through a pathway more dependent on PKA (Traebert *et al.,* 2000). *In vivo,* apical PTHRs presumably may be activated by PTH peptides that are filtered through the glomerulus and then appear in the luminal fluid. Other experiments suggest that PKA and PKC activation may lead to temporally and qualitatively distinct changes in NaP_i-2 protein expression and activity (Lederer *et al.,* 1998; Pfister *et al.,* 1999) (Fig. 4). For example, PTH(3-34) initially inhibited NaP_i activity comparably to PTH(1-34), but did so with no, or much less, induced clearance of the protein from the cell surface, which suggested that the main effect of PKC was to reduce the activity of the cotransporter, whereas that of PKA may relate more directly to the physical removal of the protein from the apical membrane via endocytosis (Lederer *et al.,* 1998; Pfister *et al.,* 1999). Thus, while a coherent view has yet to fully emerge, it seems reasonable to conclude at present that activation of PKA and PKC via the PTHR each can separately downregulate NaP_i activity, that stimulation of both kinases may be necessary for a full response to the hormone, and that PTHRs located on different surfaces of PCT cells may be coupled to different distal effectors of NaP_i regulation.

Sodium and Hydrogen Excretion

Studies *in vivo* and with isolated renal tubules *in vitro* have established that PTH produces an acute natriuresis and diuresis and rapidly inhibits proximal tubular acid secretion (HCO_3^- reabsorption) (Agus *et al.,* 1971; Bank and Aynedian, 1976; McKinney and Myers, 1980; Puschett *et al.,* 1976; Schneider, 1975). As illustrated in Fig. 1, Na^+ reabsorption in the PCT proceeds via both the active, transcellular route and the passive, paracellular pathway. These mechanisms account for roughly 60 and 40%, respectively, of Na^+ reabsorption (Rector, 1983). Much of the transcellular Na^+ reabsorption in PCTs involves Na^+-dependent cotransport of anions such as phosphate, sulfate, and amino acids or the operation of apical Na^+/H^+ exchangers.

PTH Regulation of Proximal Tubular Sodium and Hydrogen Excretion

Effective reabsorption of Na^+ and HCO_3^- in the proximal tubule requires the concerted activities of apical Na^+/H^+ exchangers (type 3 NHEs or NHE3s), basolateral Na^+-K^+-ATPases (to maintain the transmembrane Na^+ gradient), and electrogenic basolateral Na^+-$3HCO_3^-$ cotransporters, among others (Alpern, 1990). PTH exerts at least three or four independent actions that conspire to powerfully inhibit Na^+ and HCO_3^- reabsorption. These include inhibition of apical Na^+/H^+ exchange, apical Na^+/P_i^- cotransport, basolateral Na^+-K^+-ATPase activity, and, possibly, basolateral Na^+-HCO_3^- cotransport (see Fig. 1).

PTH strikingly inhibits the activity of the amiloride-sensitive NHE3 in proximal tubular apical brush border membranes and in OK cells (Helmle-Kolb *et al.,* 1990; Hensley *et al.,* 1989; Kahn *et al.,* 1985; Pollock *et al.,* 1986), directly impairing both Na^+ reabsorption and H^+ excretion. Conversely, parathyroidectomy increases NHE3 exchanger activity (Cohn *et al.,* 1983). In rats, NHE3 protein and mRNA expression are increased and decreased, respectively, during sustained hyper-and hypoparathyroidism (Girardi *et al.,* 2000). The possibility that PTH may inhibit basolateral base exit via regulation of Na^+-$3HCO_3^-$ cotransporters is unsettled, as this has been observed in proximal tubules of rat (Pastoriza-Munoz *et al.,* 1992) but not of rabbit (Sasaki and Marumo, 1991). However, *in vivo* or *in vitro* administration of PTH greatly reduces the activity of the basolateral Na^+-K^+-ATPase in rat proximal tubules (Derrickson and Mandel, 1997; Ominato *et al.,* 1996; Ribeiro *et al.,* 1994; Ribeiro and Mandel, 1992).

PTHR Signal Transduction in the Regulation of Sodium and Hydrogen Excretion

The mechanisms whereby PTHRs regulate these various effectors of proximal tubular Na^+ and H^+ excretion are both different and complex. Within 30–60 min of PTH exposure *in vivo,* NHE3 is phosphorylated and inactivated, after which it is sequestered (but not destroyed) via a more delayed internalization to a high-density intracellular membrane fraction (Fan *et al.,* 1999; Hensley *et al.,* 1989; Zhang *et al.,* 1999). Experiments in OK cells also indicate that PTH reduces the sensitivity of the exchanger to the intracellular H^+ concentration (Miller and Pollock, 1987). Functional analysis of expressed recombinant NHE3 exchangers (Zhao *et al.,* 1999a) supports previous evidence (Agus *et al.,* 1973; Kahn *et al.,* 1985; Sasaki and Marumo, 1991; Weinman *et al.,* 1988) that NHE3 is a direct substrate for PKA. Involvement

of the cAMP/PKA signaling cascade in PTH regulation of NHE3 was suggested by the demonstration that PTH(1-34), but not a PTH(3-34) analog devoid of PKA activity, induced NHE3 internalization in rat proximal tubules (Zhang *et al.,* 1999). Also, PTHrP(1-34) inhibited NHE3 activity in an OK cell subclone in which this peptide could increase cAMP but not cytosolic Ca_i^{2+}, PLC, or PKC (Maeda *et al.,* 1998). Careful temporal analysis of events in cultured OK cells indicates that PTH induces a rapid, cAMP/PKA-dependent phosphorylation of multiple serine residues within the cytoplasmic "tail" of NHE3 that is maximal within 5 min and is associated with a reduction in exchanger activity but no change in NHE3 surface expression (Collazo *et al.,* 2000). This initial inactivation of surface exchangers, due either to phosphorylation *per se* or association with regulatory proteins, is followed by a more delayed, dynamin-dependent endocytosis of NHE3 proteins that becomes evident by 30 min.

However, NHE3 activity can be regulated by PKC via processes independent of NHE3 phosphorylation, and evidence developed using both kinase inhibitors and signal-selective PTH analogs in OK cells strongly supports the involvement of PKC- and PKA-dependent pathways in NHE3 regulation by PTHRs (Azarani *et al.,* 1995, 1996; Helmle-Kolb *et al.,* 1990; Kahn *et al.,* 1985). By analogy with mechanisms of PTH-regulated PCT phosphate and DCT calcium excretion, then, it is likely that these two PTHR signal kinases exert cooperative but distinct effects in controlling NHE3 expression and activity.

In the case of basolateral Na^+-K^+-ATPase, analysis of PTH regulation has disclosed a novel pathway of PTHR signaling. Administration of PTH(1-34) *in vivo* causes a rapid inactivation of proximal tubular basolateral Na^+-K^+-ATPase activity without inducing destruction or sequestration of the pump proteins (Zhang *et al.,* 1999). In this case, PTH(3-34) does mimic the action of PTH(1-34) by activating PKC (not PKA) (Ominato *et al.,* 1996; Ribeiro *et al.,* 1994). This occurs via PTHR coupling to a Gq/G11 family member and leads to a series of further responses, which include activation of PLA_2, generation of arachidonic acid, and metabolism of arachidonate via the P450 monooxygenase pathway to produce active eicosanoids, notably 20-hydroxyeicosatetraenoic acid ("20-HETE") (Derrickson and Mandel, 1997; Ominato *et al.,* 1996; Ribeiro *et al.,* 1994; Ribeiro and Mandel, 1992). In a manner as yet unknown, 20-HETE then leads to inhibition of Na^+-K^+-ATPase activity (Derrickson and Mandel, 1997; Ominato *et al.,* 1996). This monooxygenase-dependent pathway accounts for most of the PTH regulation of Na^+-K^+-ATPase activity, although a portion of the response is attributable to cAMP/PKA activation (Ribeiro and Mandel, 1992).

PTH Regulation of Sodium and Hydrogen Excretion beyond the Proximal Tubule

While it is true that PTH strongly inhibits proximal tubular HCO_3^- reabsorption, this is compensated to some extent by its effect to increase HCO_3^- reabsorption in Henle's loop and H^+ secretion in the CD (Bank and Aynediian, 1976; Bichara *et al.,* 1986; Paillard and Bichara, 1989). Moreover, phosphaturia induced by PTH also contributes to net acid secretion (Mercier *et al.,* 1986), and PTH actually can increase net renal acid secretion during metabolic acidosis (Bichara *et al.,* 1990). Similarly, in perfused mouse CTAL, PTH may exert an antinatriuretic effect, manifested as augmented paracellular transport driven by an increased transepithelial voltage (Wittner and Di Stefano, 1990). Thus, the overall effect of PTH on renal acid and sodium excretion may vary markedly depending on the particular physiologic state of the organism.

Vitamin D Metabolism

Synthesis of $1,25(OH)_2D_3$ is increased by PTH and reduced by parathyroidectomy (Fraser and Kodicek, 1973). This results from regulated expression, in proximal tubular cells, of the $25(OH)D_3$ 1α-hydroxylase gene, the promoter for which is induced rapidly by PTH *in vitro* (Garabedian *et al.,* 1972; Kong *et al.,* 1999a; Murayama *et al.,* 1999). This effect of PTH can be overridden *in vivo* by the direct suppressive action of hypercalcemia on 1α-hydroxylase expression (Weisinger *et al.,* 1989). It is variably impaired in older animals or humans, even though indices of PTHR signaling *per se* remain normal (Friedlander *et al.,* 1994; Halloran *et al.,* 1996). PTH induction of 1α-hydroxylase mRNA is transcriptional, additive to that of calcitonin, occurs in the genetic absence of the vitamin D receptor, and is antagonized by coadministration of $1,25(OH)_2D_3$, which directly inhibits expression when given alone (Murayama *et al.,* 1999).

The signaling pathways employed by the PTHR to increase $1,25(OH)_2D_3$ synthesis have been examined extensively *in vivo* and *in vitro*. Involvement of cAMP is suggested by the fact that the PTH effect can be mimicked by cAMP analogs, forskolin, or phosphodiesterase inhibitors (Armbrecht *et al.,* 1984; Henry, 1985; Horiuchi *et al.,* 1977; Korkor *et al.,* 1987; Larkins *et al.,* 1974; Rost *et al.,* 1981; Shigematsu *et al.,* 1986). Moreover, in a transformed murine proximal tubular cell line, transciptional induction of the 1α-hydroxylase occurred with either PTH or forskolin, and the effects of both were blocked by the PKA-selective inhibitor H89 (Murayama *et al.,* 1999). However, careful studies of the effects of added PTH in isolated perfused rat proximal tubules have correlated rapid (30–60 min) increases in $1,25(OH)_2D_3$ synthesis with PKC activation on the basis of (a) concentration dependence [PKC and $1,25(OH)_2D_3$ synthesis were increased at PTH concentrations 100- to 1000-fold lower than required for PKA activation], (b) selective inhibition by PKC inhibitors, and (c) activation by truncated PTH analogs [i.e., PTH(3-34), PTH(13-34)] that can trigger PKC but not PKA in this system (Janulis *et al.,* 1992, 1993). More information clearly is needed, but available data seem most consistent with both a predominant effect of cAMP/PKA on transcriptional regulation of 1α-hydroxylase gene expression and a more rapid, posttransciptional effect of PKC on 1α-hydroxylase enzyme activity.

The 25(OH)D 24-hydroxylase also is regulated by PTH. In kidney homogenates, cultured proximal tubular cells, and certain proximal tubular cell lines, PTH inhibits 24-hydroxylase activity by mechanisms that may involve cAMP (Henry, 1985; Matsumoto *et al.,* 1985; Shigematsu *et al.,* 1986; Shinki *et al.,* 1992; Tanaka and DeLuca, 1984). It also antagonizes the inductive effect of $1,25(OH)_2D_3$ on both 24-hydroxylase and vitamin D receptor expression (Reinhardt and Horst, 1990). Interestingly, PTH leads to opposite effects on 24-hydroxylase and vitamin D receptor expression in proximal and distal tubules. Thus, PTH augments $1,25(OH)_2D_3$-dependent induction of 24-hydroxylase in DCT cells, possibly by increasing expression of the vitamin D receptor (Yang *et al.,* 1999), whereas it inhibits expression of both the 24-hydroxylase and the receptor in proximal tubules, as described earlier.

Other Renal Effects of PTH

A variety of other effects of PTH on renal metabolism, secretion, and membrane function have been described, the physiologic roles of which currently are less clear than those described elsewhere in this chapter. Examples include rapid microvillar shortening in cultured proximal tubular cells (Goligorsky *et al.,* 1986b); increased renin release from perfused rat kidneys (Saussine *et al.,* 1993); increased proximal tubular gluconeogenesis, ammoniagenesis, and phosphoenolpyruvate carboxykinase (PEPCK) mRNA expression (Chobanian and Hammerman, 1988; Wang and Kurokawa, 1984; Watford and Mapes, 1990); activation of an apical Cl^- channel in rabbit proximal tubular cells (Suzuki *et al.,* 1991); and stimulation of ecto-5′-nucleotidase activity in apical membranes of OK cells, an effect that is mimicked by PTH(3-34) but not by forskolin and is blocked by PKC inhibitors (Siegfried *et al.,* 1995).

Renal Expression and Actions of PTHrP

PTHrP is expressed in the glomeruli, distal tubules, and collecting ducts of fetal kidneys and in PCT, DCT, and glomeruli of the adult kidney (Aya *et al.,* 1999; Philbrick *et al.,* 1996). In one study in rats, PTHrP mRNA was found in glomeruli, PCT, and macula densa but not in cTAL, mTAL, DCT, or CD (Yang *et al.,* 1997). It is unlikely that PTHrP is critical for normal renal development, as the kidneys of mice missing functional PTHrP genes appear histologically normal.

When tested, active amino-terminal fragments of PTHrP generally exhibit renal actions identical to those of PTH, including stimulation of cAMP production and regulation of P_i transport, Ca^{2+} excretion, and $1,25(OH)_2D_3$ synthesis (Everhart-Caye *et al.,* 1996; Pizurki *et al.,* 1988; Yates *et al.,* 1988). However, longer PTHrP fragments may possess unique properties. For example, in an assay of HCO_3^- excretion by the perfused rat kidney, hPTHrP(1-34) was equipotent with hPTH(1-34), whereas hPTHrP(1-84), hPTHrP(1-108), and hPTHrP(1-141) were each less active than hPTH(1-34) (Ellis *et al.,* 1990). As discussed in Chapter 25, the PTHrP gene can generate multiple transcripts and protein products, some of which may undergo unique nuclear localization. It is quite possible, therefore, that locally expressed PTHrP may exert actions in the kidney that are not shared with PTH, although this has not yet been addressed adequately.

A possible role for locally produced PTHrP in the renal response to ischemia has been suggested by findings that PTHrP expression is induced by ischemia or following recovery from ATP depletion (Garcia-Ocana *et al.,* 1999; Largo *et al.,* 1999; Soifer *et al.,* 1993). PTHrP is expressed in the intimal and medial layers of human renal microvessels and in the macula densa (Massfelder *et al.,* 1996). PTHrP (like PTH) increases renin release from the juxtaglomerular apparatus and also stimulates cAMP in renal afferent and efferent arterioles, leading to vasodilation and enhanced renal blood flow (Endlich *et al.,* 1995; Helwig *et al.,* 1991; Musso *et al.,* 1989; Nickols *et al.,* 1986; Saussine *et al.,* 1993; Schor *et al.,* 1981; Simeoni *et al.,* 1994). Evidence for involvement of both cAMP and nitric oxide in PTHrP-induced vasorelaxation *in vitro* has been derived from use of specific inhibitors (Massfelder *et al.,* 1996). Thus, enhanced local PTHrP production induced by inadequate renal perfusion or ischemia may be involved in both local and systemic autoregulatory mechanisms, whereby direct local vasodilatory actions are supplemented by the systemic activation of angiotensinogen that increase-arterial pressure and further sustain renal blood flow.

PTHrP and Receptors for PTH and PTHrP in Bone

Expression of PTHrP in Bone

PTHrP is expressed and secreted by osteoblast-like osteosarcoma cells (Rodan *et al.,* 1989; Suda *et al.,* 1996a) and is secreted by rat long bone explants *in vitro* (Bergmann *et al.,* 1990). Messenger RNA for PTHrP is detected in periosteal cells of fetal rat bones (Karmali *et al.,* 1992). *In situ* hybridization and immunohistochemistry have localized PTHrP mRNA and protein to mature osteoblasts on the bone surface of fetal and adult bones from mice and rats (Amizuka *et al.,* 1996; Lee *et al.,* 1995) and to flattened bone-lining cells and some superficial osteocytes (Amizuka *et al.,* 1996) in postnatal mice. In addition, the PTHrP gene is expressed in preosteoblast cells in culture, and in some studies its expression is reduced as preosteoblasts undergo differentiation (Kartsogiannis *et al.,* 1997; Oyajobi *et al.,* 1999; Suda *et al.,* 1996a). PTHrP is also expressed in tissues adjacent to bone, including growth plate cartilage (Amizuka *et al.,* 1996; Lee *et al.,* 1995) and synovium (Funk *et al.,* 1998), sites where the peptide could affect bone during endochondral bone formation or destructive rheumatoid arthritis, respectively.

Receptors and Second Messenger Systems for PTH and PTHrP in Bone

As discussed in detail in Chapter 24, a shared receptor for PTH and PTHrP (PTH/PTHrP receptor or PTH1R) is present on bone cells; this is a G protein-coupled receptor that recognizes PTH and PTHrP equally well. The receptor couples its ligands to two cellular effector systems: the adenylyl cyclase/cAMP/protein kinase A pathway and the phospholipase C/protein kinase C pathway (Chapters 24 and 26). As will become clear as the individual effects of PTH on bone are laid out, PTH and PTHrP utilize cAMP for virtually every action in bone for which a second messenger has been identified, although in some cases the protein kinase C pathway is also used.

The PTH/PTHrP receptor is expressed widely in the osteoblast lineage. In addition to mature osteoblasts on the trabecular, endosteal, and periosteal surfaces (Amizuka *et al.,* 1996; Fermor and Skerry, 1995; Lee *et al.,* 1993, 1995) and osteocytes (Fermor and Skerry, 1995; van der Plas *et al.,* 1994), the receptor mRNA and protein are expressed in marrow stromal cells near the bone surface (Amizuka *et al.,* 1996), a putatively preosteoblast cell population that had been shown previously to bind radiolabeled PTH (Rouleau *et al.,* 1988, 1990). Considering the anabolic effect of PTH on bone formation, it will be important to understand at what point in the osteoblast lineage receptors for PTH are first expressed. Transcripts for the PTH/PTHrP receptor are absent or nonabundant in STRO-1+ positive, alkaline phosphatase negative marrow stromal cells (Gronthos *et al.,* 1999; Stewart *et al.,* 1999), which are thought to represent relatively early osteoblast precursors, and PTH/PTHrP receptor expression can be induced by the differentiation of stromal cells, MC3T3 cells, or C3H10T1/2 cells with dexamethasone or bone morphogenetic proteins (Feuerbach *et al.,* 1997; Hicok *et al.,* 1998; Liang *et al.,* 1999; Stewart *et al.,* 1999; Wang *et al.,* 1999; Yamaguchi *et al.,* 1996). Other data suggest that PTH receptors are limited to a relatively mature population of osteoprogenitor cells that express the osteocalcin gene (Bos *et al.,* 1996). It thus appears that the PTH/PTHrP receptor appears at a point in osteoblast differentiation when the cells are acquiring other markers of the mature osteoblast phenotype.

Whether receptors for PTH or PTHrP are expressed on the osteoclast is controversial. Initial studies using receptor radioautography failed to demonstrate them (Rouleau *et al.,* 1990; Silve *et al.,* 1982), and recent studies have not identified PTH/PTHrP receptor mRNA or protein on mature osteoclasts (Amizuka *et al.,* 1996; Lee *et al.,* 1993, 1995), although PTH/PTHrP receptors are reportedly present on osteoclasts from patients with renal failure (Langub *et al.,* 2001). However, relatively low-affinity binding of radiolabeled PTH peptides to osteoclasts or preosteoclasts has been reported (Teti *et al.,* 1991). The functional importance of such putative receptors is unclear. As discussed in detail later ("Effects of PTH and PTHrP on Osteoclasts"), the presence of osteoblasts or stromal cells seems to be required to elicit effects of PTH on osteoclasts *in vitro* (McSheehy and Chambers, 1986). The effects of PTH treatment are mimicked by expression of a constitutively active PTH/PTHrP receptor on osteoblasts (Calvi *et al.,* 2001), indicating that osteoclast receptors are dispensible. Studies have found a requirement for the RANKL/RANK system of cytokines and receptors for bone resorption by PTH or PTHrP (Fuller *et al.,* 1998; Lee and Lorenzo, 1999; Morony *et al.,* 1999; Yamamoto *et al.,* 1998), consistent with the interpretation that stromal or osteoblastic cells expressing the cytokine RANKL are required for the induction of bone resorption by PTH.

Both PTH and PTHrP have additional receptors besides the PTH/PTHrP receptor. The PTH2R is a G protein-coupled receptor closely related to the PTH1R (Mannstadt *et al.,* 1999; Usdin *et al.,* 1995), which recognizes the amino-terminal domain of PTH but not of PTHrP (Chapter 24). This receptor is expressed predominantly in brain and has yet to be demonstrated in bone. Evidence for actions of carboxyl-terminal PTH peptides on bone has been presented (Murray *et al.,* 1991; Nakamoto *et al.,* 1993; Nguyen-Yamamoto *et al.,* 2001; Sutherland *et al.,* 1994), as discussed elsewhere in this chapter and in Chapter 24, and evidence for a specific receptor for carboxyl-terminal PTH peptides on osteoblasts (Inomata *et al.,* 1995; Nguyen-Yamamoto *et al.,* 2001) and osteocytes (Divieti *et al.,* 2001) has been presented.

As discussed in Chapter 25, the polyhormone PTHrP is cleaved to produce a set of peptides: those that contain the amino terminus activate the shared PTH/PTHrP receptors, and additional peptides representing the midregion and carboxyl terminus of PTHrP appear to have distinct biological actions mediated by their own receptors (Philbrick *et al.,* 1996; Wysolmerski and Stewart, 1998). Receptors that are specific for amino-terminal PTHrP and do not recognize PTH have been identified in brain (Yamamoto *et al.,* 1997) and other tissues (Gaich *et al.,* 1993; Orloff *et al.,* 1992), and midregion peptides of PTHrP have actions on placental calcium transport that imply a distinct receptor (Care *et al.,* 1990; Kovacs *et al.,* 1996), but there is presently no evidence for either receptor in bone. Carboxyl-terminal PTHrP fragments [e.g., PTHrP(107-139)] are reported to inhibit bone resorption (Cornish *et al.,* 1997; Fenton *et al.,* 1991b) and stimulate (Goltzman and Mitchell, 1985) or inhibit (Martinez *et al.,* 1997) the growth of osteoblasts and their function (Esbrit *et al.,* 2000; Gray *et al.,* 1982), and it is thus likely that a specific receptor for this peptide is present on osteoblasts, and conceivably also on osteoclasts.

Effects of PTH and PTHrP on Bone Cells

Effects on Osteoblast Precursor Cells

In view of the anabolic effects of PTH and PTHrP, evidence for a proliferative effect on osteoblast precursors has been sought. Administration of PTH *in vivo* does not increase mRNA for the proliferation marker histone H4 (Onyia *et al.,* 1995). Immediate early gene expression is increased after *in*

vivo administration of PTH in osteoblasts and osteocytes (Lee *et al.,* 1994; Liang *et al.,* 1999), but the immediate early gene response is delayed in stromal cells, suggesting that they may respond secondarily to factors elaborated by osteoblasts (Lee *et al.,* 1994).

Effects on Osteoblasts

TRANSCRIPTION FACTORS

PTH induces the expression of the immediate early gene families c-fos (c-fos, fra-1, fra-2) and c-jun (c-jun, junD) in osteoblastic cell lines and in osteoblasts *in vivo* (Clohisy *et al.,* 1992; Lee *et al.,* 1994; McCauley *et al.,* 1997; McCauley *et al.,* 2001; Stanislaus *et al.,* 2000a). The effect on c-fos is the largest and best studied. PTH induces c-fos mRNA in a fashion that does not require protein synthesis and is mediated by phosphorylation of the transcription factor CREB by protein kinase A (Evans *et al.,* 1996; Pearman *et al.,* 1996; Tyson *et al.,* 1999) to induce binding to a CRE in the c-fos promoter (Evans *et al.,* 1996; Pearman *et al.,* 1996). The protein kinase C signaling pathway does not appear to be involved in this response (Evans *et al.,* 1996; McCauley *et al.,* 1997).

Because many bone cell genes are regulated by PTH, as discussed later, interactions of PTH with osteoblast-specific transcriptional regulation are likely. A splice variant of the runt-domain transcription factor cbfa1 called OSF2 is required for determination of the osteoblast phenotype and confers osteoblast-specific expression on the osteocalcin gene (Ducy *et al.,* 1997; Ducy and Karsenty, 1998). Although it is not known how PTH interacts with cbfa1 at the osteocalcin promoter, a cbfa1 site in the collagenase-3 (MMP-13) promoter is required along with an AP-1 site for the stimulation of collagenase-3 gene transcription by PTH (Porte *et al.,* 1999; Selvamurugan *et al.,* 1998). There were no acute changes in cbfa1 levels in these studies, and it appears that cbfa1 is activated transcriptionally by phosphorylation in its AD3 domain by protein kinase A (Selvamurugan *et al.,* 2000; Winchester *et al.,* 2000). It has also been reported that PTH and other agents that raise cAMP levels in MC3T3 cells reduce the level of cbfa1 and the activity of cbfa1-dependent genes by activating the destruction of the transcription factor by the ubiquitin-proteosome pathway (Tintut *et al.,* 1999).

CYTOKINES

Insulin-like Growth Factors Bone is a rich source of insulin-like growth factors (IGF) secreted by osteoblasts (see Chapter 45), with IGF-I predominating in rodent bone and IGF-II in human bone (Conover, 1996). The secretion of IGF-I by rat (McCarthy *et al.,* 1989) and IGF-I and IGF-II by mouse (Linkhart and Mohan, 1989) osteoblasts *in vitro* and *in vivo* (Watson *et al.,* 1995) is stimulated by PTH. PTH appears to utilize cAMP as the predominant intracellular second messenger to stimulate IGF gene expression because its effects are mimicked by cAMP analogs or agents that increase cAMP, but not by calcium ionophores or phorbol esters (McCarthy *et al.,* 1990).

Two sets of results raise the possibility that effects of PTH on IGF-I secretion may be essential for its overall anabolic effect on bone. Continuous exposure to PTH, which has catabolic effects on bone *in vivo,* inhibited collagen synthesis by isolated rat calvariae, but exposure to PTH for the first 24 hr of a 72-hr experiment increased collagen synthesis markedly (Canalis *et al.,* 1990). The stimulation of collagen synthesis by PTH is blocked by antibodies to IGF-I, but the stimulation of [^{3}H]thymidine incorporation is not (Canalis *et al.,* 1989). Moreover, treatment of intact rats with PTH under conditions where it has an anabolic effect on bone leads to an increase in mRNA for IGF-I (Watson *et al.,* 1995) and the bone matrix content of both IGF-I and TGF-β. Finally, skeletal unloading leads to resistance to the anabolic effect of PTH, and also resistance *in vitro* to IGF-I, a result that was interpreted as suggesting that resistance to IGF-I may account for the resistance of the unloaded skeleton to PTH (Kostenuik *et al.,* 1999).

PTH and PTHrP also affect the secretion of binding proteins for IGF's (Chapter 45). There are six IGF-binding proteins (IGFBP) and all are present in bone (Conover, 1996). IGFBP-4 inhibits IGF action, but IGFBP-5 seems to function predominately to anchor IGFs to the extracellular matrix and may, in some circumstances, have stimulatory effects on IGF action. Exposure of bone cells to PTH or PTHrP increases the secretion of IGFBP-4 (Latour *et al.,* 1990) and IGFBP-5 (Conover *et al.,* 1993) by cAMP-dependent mechanisms and also increases the level of a related protein, IGFBP-RP-1 (Pereira and Canalis, 1999). Both IGFBPs are subject to proteolysis, and there is limited evidence to suggest that IGFBP protease activity may be regulated by PTH (Hakeda *et al.,* 1996; Kudo *et al.,* 1996). It is not clear whether the effects of PTH on IGFBP levels are biologically significant.

Transforming Growth Factor-β PTH and PTHrP increase both the secretion of TGF-β by osteoblast-like bone cells and the release of TGF-β from calvarial explants (see Chapter 49); the latter may represent in part the release of preformed TGF-β during bone resorption (Finkelman *et al.,* 1992; Merry and Gowen, 1992; Oursler *et al.,* 1991; Pfeilschifter and Mundy, 1987). Intermittent PTH treatment of rats increases the bone matrix content of TGF-β1 as well as IGF-I (Watson *et al.,* 1995), raising the possibility that the anabolic effects of PTH observed with intermittent administration could be mediated, at least in part, by increased secretion of this potent osteoblast growth and differentiation factor. The effect of PTH on TGF-β1 may be protein kinase C mediated, whereas its effect on TGF-β2 is protein kinase A mediated (Wu and Kumar, 2000).

Interleukin-6 Family Cytokines The cytokines IL-6, IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M(OSM), and cardiotropin 1 (CT-1) bind to related receptors and share a signal transduction pathway (Horowitz and Lorenzo, 1996; Manolagas *et al.,* 1996) (see Chapter 53). The pathway involves the common receptor subunit gp 130, binding of JAK family protein

kinases, and phosphorylation and nuclear translocation of the STAT family of transcription factors. Of this cytokine family, three members are prominently stimulated by PTH and PTHrP in bone cells: IL-6 (Feyen *et al.,* 1989; Greenfield *et al.,* 1993; Li *et al.,* 1991; Lowik *et al.,* 1989), IL-11 (Ducy *et al.,* 1997; Elias *et al.,* 1995), and LIF (Greenfield *et al.,* 1993). Both PTHrP(1-34) and PTHrP(107-139) are reported to induce the expression of IL-6 (de Miguel *et al.,* 1999). The production of IL-6 is also increased by PTH in mouse calvaria (Huang *et al.,* 1998) and *in vivo* (Onyia *et al.,* 1995; Pollock *et al.,* 1996). PTH activates transcription of the IL-6 gene (Huang *et al.,* 1998; Onyia *et al.,* 1997a) using cAMP as its principal signaling pathway (Greenfield *et al.,* 1995; Huang *et al.,* 1998; Onyia *et al.,* 1997a), although protein kinase C may also play a role (Sanders and Stern, 2000).

It has been suggested from neutralization experiments that the induction by PTH of osteoblast secretion of IL-6 (Greenfield *et al.,* 1995; Grey *et al.,* 1999) or IL-11 (Girasole *et al.,* 1994), both of which are bone-resorbing cytokines, may be one mechanism by which the osteoblast transmits the bone-resorbing signal of PTH to the osteoclast. However, studies have shown that blockade of the intracellular signaling pathway, using dominant-negative STAT factors (O'Brien *et al.,* 1999) or an IL-6 receptor antagonist, (Devlin *et al.,* 1998), fails to inhibit bone resorption by PTH, even though bone resorption by IL-6 is blocked. Compelling evidence is now available to indicate that the principal mediators of the bone-resorbing effect of PTH are another set of cytokines, RANKL and osteoprotegerin, or OPG. This issue is discussed further later, under "RANK Ligand and Osteoprotegerin."

Other Cytokines and Prostaglandins PTH induces osteoblasts to secrete granulocyte–macrophage colony-stimulating factor (GM-CSF) (Horowitz *et al.,* 1989; Weir *et al.,* 1989) (see Chapter 52). As discussed in Chapter 54, PTH also stimulates production of the prostaglandin PGE_2 by mouse calvarial osteoblasts (Klein-Nulend *et al.,* 1990, 1991; Pilbeam *et al.,* 1989). The direct target of PTH is the enzyme prostaglandin G/H synthase (PGHS-2) (Kawaguchi *et al.,* 1994) whose protein levels are increased by PTH. Another isoform, PGHS-1, is expressed constitutively but is not affected by PTH. The effect of PTH is mediated by cAMP as the dominant second messenger (Klein-Nulend *et al.,* 1990). PGE_2, in turn, has diverse effects on bone, which have been summarized (Pilbeam *et al.,* 2000). Knockout of the prostaglandin G/H synthase or the EP2 receptor for PGE_2 markedly inhibits the bone resorption response to PTH *in vitro* (Li *et al.,* 2000; Okada *et al.,* 2000) and the hypercalcemic response to PTH in mice is abrogated in the absence of prostaglandin G/H synthase (Okada *et al.,* 2000).

RANK Ligand and Osteoprotegerin One of the most important new insights into the regulation of bone metabolism in recent years has been the delineation of a new system for osteoblast–osteoclast cross-talk (Chapter 53). It has three major elements. The first is a new member of the TNF family of cytokines that is expressed on the osteoblast and stromal cell surface; this cytokine is variously known as RANK ligand (RANKL), osteoprotegerin ligand (OPGL), osteoclast differentiation factor (ODF), and TNF-related activation-induced cytokine (TRANCE) (Hofbauer *et al.,* 2000). By binding to a receptor on osteoclast precursors, RANKL provides an essential feeder function for osteoclastogenesis, accounting for earlier observations that coculture of bone marrow cells and stromal cells is required for osteoclastogenesis (Suda *et al.,* 1996b); RANKL also activates bone resorption by mature osteoclasts and inhibits osteoclast apoptosis (Burgess *et al.,* 1999; Fuller *et al.,* 1998; Hofbauer, 1999; Lacey *et al.,* 1998; Yasuda *et al.,* 1998b). RANKL is both necessary and, with M-CSF, sufficient for osteoclastogenesis; disruption of the RANKL gene leads to severe osteopetrosis (Kong *et al.,* 1999b).

The second element of this system is the receptor for RANKL on the surface of osteoclast precursors and mature osteoclasts. This receptor is called RANK (receptor activator of NF-κB) or ODAR (osteoclast differentiation and activation receptor). Disruption of the receptor gene also produces severe osteopetrosis (Hsu *et al.,* 1999). The third element is a decoy receptor, osteoprotegerin (OPG) or osteoclastogenesis inhibitory factor (OCIF) (Simonet *et al.,* 1997; Yasuda *et al.,* 1998a,b). Targeted deletion of OPG produces severe osteoporosis (Mizuno *et al.,* 1998; Simonet *et al.,* 1997), whereas overexpression leads to osteopetrosis (Simonet *et al.,* 1997).

Both genetic and cell biological approaches to this system have yielded decisive results: following an early commitment step under the control of M-CSF, binding of RANKL to RANK is both necessary and sufficient for osteoclastogenesis. The system has a second function to regulate the activity of the mature osteoclast, and exposure of osteoclasts to RANKL inhibits their apoptosis. The decoy receptor OPG must also be important to modulate the tone of the system, as elimination of OPG produces a severe form of osteoporosis. It is conceivable that a parallel system exists, as M-CSF-dependent osteoclast formation from cultured mouse bone marrow cells is induced by TNF-α and is blocked by antibodies to its receptor, but not by OPG or antibodies to RANK (Kobayashi *et al.,* 2000).

The bone-resorbing effects of PTH, long known to require the intermediation of osteoblasts (McSheehy and Chambers, 1986), appear to occur principally through activation of the RANKL/RANK system. Exposure to PTH increases the expression of RANKL in murine bone marrow cultures, cultured osteoblasts, and mouse calvariae (Hofbauer *et al.,* 1999; Lee and Lorenzo, 1999; Yasuda *et al.,* 1998b), and simultaneously decreases the expression of OPG (Lee and Lorenzo, 1999), probably via protein kinase A, with the kinetics *in vivo* of an immediate early gene (Onyia *et al.,* 2000). Stimulation of osteoclastogenesis by PTH is blocked by antibodies to RANKL (Tsukii *et al.,* 1998) or by OPG (Lacey *et al.,* 1998; Yasuda *et al.,* 1998b). Infusion of OPG into animals blocks the hypercalcemic response to PTH or PTHrP (Morony *et al.,* 1999; Oyajobi *et al.,* 2001; Yamamoto *et al.,* 1998). Bone resorption by mature osteoclasts in response to PTH has

long been recognized as requiring coculture with osteoblasts or marrow stromal cells (McSheehy and Chambers, 1986), but when purified cultures of isolated osteoclasts that were unresponsive to PTH were exposed to RANKL, the cytokine was sufficient to induce bone resorption (Fuller *et al.,* 1998). It thus appears that both the stimulation of new osteoclast formation and the activation of the mature osteoclast by PTH and PTHrP take place by binding of the ligand to receptors on osteoblasts, followed by simultaneous induction of the presentation of RANKL on the osteoblast surface and inhibition of secretion of OPG. It is conceivable that the effect of PTH on RANKL and OPG is indirect, involving other cytokines as intermediate steps. It is also possible that a parallel pathway exists in which other cytokines such as IL-6 or IL-11 could mediate part of the effect of PTH on bone resorption, but if so it is likely to be of secondary importance. In the resorption of alveolar bone during eruption of teeth, PTHrP is secreted by stellate reticulum cells and appears to act by binding to receptors on dental follicle cells, which express RANKL and in turn secrete an osteoclast-activating factor likely to be RANKL, as its effects can be neutralized by OPG (Nakchbandi *et al.,* 2000).

CELL PROLIFERATION AND APOPTOSIS

Continuous exposure to PTH(1-34) or PTHrP(1-34) exerts an antiproliferative action on osteoblast-like UMR-106 osteosarcoma cells (Civitelli *et al.,* 1990; Kano *et al.,* 1991; Onishi and Hruska, 1997). This effect is cAMP mediated and results, at least in part, from increased levels of p27Kip1, a regulator of G1 phase cyclin-dependent kinases (Onishi and Hruska, 1997). However, in some cell lines (Finkelman *et al.,* 1992; Onishi *et al.,* 1997; Somjen *et al.,* 1990) and primary cultures (van der Plas *et al.,* 1985), PTH appears to increase osteoblast or preosteoblast proliferation. In the preosteoblast cell line TE-85, the mitogenic response to PTH requires an increase in levels of the the cyclin-dependent kinase cdc2, probably brought about by increased levels of E2F (Onishi *et al.,* 1997).

Treatment of rats with intermittent injections of PTH is reported in some studies to increase the number of osteoprogenitor cells (Kostenuik *et al.,* 1999; Nishida *et al.,* 1994), but not the proliferation of osteoprogenitors (Onyia *et al.,* 1995, 1997b). These data are compatible with the conclusion that PTH increases entry of cells into an osteoprogenitor compartment, e.g., commitment, without an effect on their proliferation. In an important study, continuous labeling of bone with [^{3}H]thymidine during a period of intermittent treatment with PTH(1-34) resulted in no increase in labeled osteoblasts, despite a marked increase in osteoblast number (Dobnig and Turner, 1995). This indicates that the anabolic effect of PTH does not require the proliferation of osteoblast precursors or of mature osteoblasts on the bone surface. The large increase in osteoblast number produced in this study by intermittent treatment with PTH was attributed to the activation of preexisting bone-lining cells to osteoblasts (Dobnig and Turner, 1995), but is also possible that PTH induces the commitment of late osteoprogenitors to the osteoblast lineage without a requirement for mitosis. Another alternative explanation for the increase in osteoblast number with intermittent PTH treatment is provided by recent work that indicates that treatment of mice with intermittent PTH inhibits osteoblast apoptosis (Jilka *et al.,* 1999) (see Chapter 10). Prolongation of the osteoblast life span by PTH could account for the observed increase in osteoblast number, although it is not clear how large a quantitative effect on osteoblast survival would result from the observed inhibition of apoptosis. The integrated effects of PTH on bone formation are discussed further later and in Chapter 75.

EFFECTS ON ION CHANNELS

In several bone cell types, PTH induces multiphasic changes in membrane potential, most often depolarization followed by sustained hyperpolarization (Edelman *et al.,* 1986; Ferrier *et al.,* 1987, 1988; Fritsch *et al.,* 1988). Depolarization has been attributed to cAMP-dependent inactivation of quinine-sensitive K channels (Ferrier *et al.,* 1988). Depolarization of bone cells induces calcium entry through L-type voltage-sensitive Ca channels (Barry *et al.,* 1995; Ferrier *et al.,* 1987; Fritsch and Chesnoy-Marchais, 1994; Yamaguchi *et al.,* 1987). Sustained hyperpolarization may result in return from opening of Ca-sensitive K channels (Moreau *et al.,* 1996), which may be identical to mechanosensitive cation-selective channels also activated by PTH (Duncan *et al.,* 1992). PTH enhances the $[Ca^{2+}]_i$ response of osteoblast-like MC3T3-E1 cells to mechanical stimulation, as well as the COX-2 response to stimulation (Ryder and Duncan, 2000, 2001).

EFFECTS ON CELL SHAPE

PTH treatment of cultured osteoblasts induces a marked retraction of the cell (Miller *et al.,* 2000), and similar changes have been observed with treatment *in vivo* (Matthews and Talmage, 1981). The change in cell shape is cAMP mediated (Babich *et al.,* 1997) and is associated with the disassembly of actin stress fibers (Egan *et al.,* 1991). It can be blocked by inhibitors of the protease calpain (Murray *et al.,* 1995). The significance of changes in cell shape is unknown, but it has been suggested that osteoblast retraction could have a role in bone remodeling by baring portions of the bone surface in response to PTH.

EFFECTS ON GAP JUNCTIONS

PTH increases intercellular communication of bone cells by increasing connexin-43 gene expression (Schiller *et al.,* 1997) and opening gap junctions (Donahue *et al.,* 1995; Schiller *et al.,* 1992). The significance of intercellular communication to the overall effects of PTH on osteoblasts is not clear, although it is reported that the reduction of connexin-43 levels by transfection of antisense cDNA markedly inhibited the cAMP response to PTH (Vander Molen *et al.,* 1996) and blocked the effect of PTH on mineralization by osteoblast-like cells (Schiller *et al.,* 2001).

Effects on Bone Matrix Proteins and Alkaline Phosphatase

The most abundant protein of bone matrix is type I collagen. Given acutely, PTH consistently inhibits collagen synthesis in cultured rat calvaria and in cultured bone cells (Kream *et al.,* 1986; Partridge *et al.,* 1989) by decreasing transcription of the pro-α1(I) gene (Kream *et al.,* 1980) (see Chapter 12). PTH treatment of calvaria inhibits transcription of a 2.3-kb fragment of the pro-α1(I) promoter, indicating that at least one major *cis*-acting element required for the inhibition of gene transcription resides in this portion of the promoter (Kream *et al.,* 1993). PTHrP and agents that increase cAMP have effects similar to PTH (Kano *et al.,* 1992; Pines *et al.,* 1990). Acute infusion of PTH into humans also inhibits collagen synthesis (Simon *et al.,* 1988). In contrast, treatment of calvaria with PTH intermittently can stimulate collagen gene expression (Canalis *et al.,* 1990). The stimulatory effect of PTH on collagen synthesis in calvaria is attributed to the stimulation of IGF-1 production because it is blocked by IGF-1 antibodies (Canalis *et al.,* 1989). Moreover, when given intermittently in an anabolic regimen, treatment with PTH *in vivo* increases bone collagen gene expression (Opas *et al.,* 2000). The reversal of direction of the PTH effect *in vivo* can probably be attributed, at least in part, to increased bone remodeling and increases in osteoblast number induced by the chronic regimen.

Treatment of osteosarcoma cells with PTH has a stimulatory effect on several other bone matrix proteins, including osteocalcin (BGP) (Noda *et al.,* 1988; Theofan and Price, 1989; Towler and Rodan, 1995; Yu and Chandrasekhar, 1997); administration of PTH or PTHrP acutely inhibits osteocalcin release from isolated rat hindlimb, but chronic administration of PTH is stimulatory (Gundberg *et al.,* 1995). The primary signaling pathway is protein kinase A, but protein kinase C may mediate part of the effect of PTH on osteocalcin gene transcription (Boguslawski *et al.,* 2000). Exposure to PTH stimulates bone sialoprotein gene expression in embryonic chick bone cells (Yang and Gerstenfeld, 1997). In ROS 17/2.8 cells, PTH appears to stimulate BSP gene transcription by blocking an inhibitory pit-1 site in the promoter through the agency of protein kinase A (Ogata *et al.,* 2000). However, PTH inhibits deposition of bone sialoprotein in mineralizing osteoblast cultures (Wang *et al.,* 2000). PTH treatment inhibits expression of the osteopontin gene in rat osteosarcoma cells (Noda and Rodan, 1989).

Amino-terminal peptides derived from PTH can either stimulate or inhibit secretion of alkaline phosphatase from bone cells, depending on the cell line (Jongen *et al.,* 1993; Kano *et al.,* 1994; Majeska and Rodan, 1982; McPartlin *et al.,* 1978; Thomas and Ramp, 1978; Yee, 1985). It is reported that carboxyl-terminal PTH fragments can stimulate alkaline phosphatase (Murray *et al.,* 1991; Sutherland *et al.,* 1994), and PTHrP(107-139) is reported to inhibit alkaline phosphatase (Valin *et al.,* 1999). Treatment of women with anabolic regimens of intermittent PTH(1-34) injections increases alkaline phosphatase (Finkelstein *et al.,* 1998), presumably at least in part due to an increase in osteoblast number.

Effects on Proteases of Bone

PTH stimulates the secretion of a number of proteases from osteoblasts (Partridge *et al.,* 1996; Partridge and Winchester, 1996) (see Chapter 16). These include stromelysin (Meikle *et al.,* 1992), gelatinase B (Meikle *et al.,* 1992), the disintegrin and metalloprotease ADAMTS-1 (Miles *et al.,* 2000), and collagenase-3 (MMP-13) (Partridge *et al.,* 1987; Quinn *et al.,* 1990; Scott *et al.,* 1992; Walker *et al.,* 1964; Winchester *et al.,* 1999). Stimulation of the collagenase-3 promoter by PTH requires interactions of an AP-1 site and a binding site for runt-domain transcription factors such as Osf-2; PTH phosphorylates CREB through protein kinase A to activate AP-1 (Porte *et al.,* 1999; Selvamurugan *et al.,* 1998) and phosphorylates cbfa1/osf2, also through protein kinase A (Selvamurugan *et al.,* 2000); cooperation of these transcription factors is required for expression of bone sialoprotein (Winchester *et al.,* 2000). Bone resorption by PTH is markedly abrogated in mice with a mutation in the Collal gene that renders the helical domain of type I collagen resistant to cleavage by collagenase (Zhao *et al.,* 1999b). It has been suggested that collagenase action on a hypomineralized layer of collagen on bone surfaces may be necessary for osteoblast attachment, although multiple other explanations for the observation are also possible. PTH treatment also increases secretion of the inhibitor TIMP (Meikle *et al.,* 1992). Finally, activity of the serine protease plasminogen activator is increased by PTH in bone cell cultures (Hamilton *et al.,* 1985; Leloup *et al.,* 1991). Whether the plasminogen activator is urokinase or tissue-type plasminogen activator and whether the effect of PTH is to increase the level of the protease or decrease the level of its inhibitor PAI-1 are controversial (Catherwood *et al.,* 1994; Fukumoto *et al.,* 1992; Partridge and Winchester, 1996).

Effects on Osteocytes

As noted earlier, PTHrP and the PTH/PTHrP receptor appear to be expressed on osteocytes (Amizuka *et al.,* 1996; Fermor and Skerry, 1995; van der Plas *et al.,* 1994). A receptor specific for the carboxyl-terminal domain of PTH is also expressed on osteocytic cells (Divieti *et al.,* 2001). Exposure to PTH induces ultrastructural changes in osteocytes (Krempien *et al.,* 1978). Although it was long thought that osteocytes, together with bone-lining cells, participate in the acute release of calcium from bone in response to PTH (Talmage *et al.,* 1976), this remains conjectural and seems unlikely in view of evidence that the RANK/RANKL system in osteoclasts is involved (Morony *et al.,* 1999). The current view of osteocytes has for them a predominant role in mechanotransduction. Mechanical loading of rat caudal vertebrae induces an increase in c-fos expression in osteocytes that requires the presence of PTH (Chow *et al.,* 1998). In rat osteocytes, PTH enhances calcium influx through mechanosensitive calcium channels in response to stretch (Miyauchi *et al.,* 2000).

However, it is not known in detail how PTH interacts with the mechanotransduction system (Burger and Klein-Nulend, 1999) (see also Chapter 6).

Integrated Effects of PTH and PTHrP on Bone

PTH, PTHrP, and Bone Resorption

CELLULAR BASIS OF PTH ACTION

PTH and PTHrP increase bone resorption by stimulating both the appearance of new osteoclasts and the activity of existing osteoclasts. The mechanistic details of osteoclastogenesis (Suda *et al.,* 1996b) and osteoclast activation (Duong and Rodan, 1999) are beyond the scope of this chapter, but have been summarized elsewhere; in neither case does PTH have a distinctive effect, rather the distal cellular responses of osteoclast precursors and mature cells to all bone resorbing agents seem to represent a final common pathway.

Both the stimulation of osteoclastogenesis and the activation of the mature osteoclast appear to require the participation of stromal cells or osteoblasts (Akatsu *et al.,* 1989; McSheehy and Chambers, 1986; Suda *et al.,* 1996b). To recapitulate what has been summarized in previous sections of this chapter, osteoclasts have not been shown to possess high-affinity PTH/PTHrP receptors (Amizuka *et al.,* 1996; Lee *et al.,* 1993, 1995; Rouleau *et al.,* 1990; Silve *et al.,* 1982), although several groups have identified low-affinity receptors (Teti *et al.,* 1991). It appears that the effects of PTH are mediated predominately by increased expression of the cytokine RANKL (OPGL, ODF, TRANCE) on the cell surface of stromal cells (Hofbauer *et al.,* 1999; Lacey *et al.,* 1998; Lee and Lorenzo, 1999; Tsukii *et al.,* 1998; Yasuda *et al.,* 1998b), perhaps together with a decrease in expression of the decoy receptor OPG (Yasuda *et al.,* 1998b). The precise target cell in the osteoclast lineage responsible for mediating the bone-resorbing effects of PTH and PTHrP has not been identified, but various marrow stromal cell lines will suffice *in vitro* (Suda *et al.,* 1996b) and bone resorption is still active when mature osteoblasts have been ablated (Corral *et al.,* 1998).

By binding to its cognate receptor (RANK) on osteoclast precursors and mature osteoclasts, RANKL stimulates both osteoclastogenesis and the activity of mature osteoclasts. Osteoclast activation by RANKL is apparently responsible for both bone resorption at the cellular level and for hypercalcemia, as both are blocked by the decoy receptor OPG (Morony *et al.,* 1999; Yamamoto *et al.,* 1998). Although it was suggested previously that the early phase of the increase in the plasma concentration of ionized calcium, e.g., within 1-2 hr, might have an osteoclast-independent mechanism, involving release of calcium by bone-lining cells (Talmage *et al.,* 1976), even early responses to PTH in animal models are blocked by inhibiting the RANK/RANKL system (Morony *et al.,* 1999).

COMPARATIVE EFFECTS OF PTH AND PTHRP

The bone-resorbing effects of amino-terminal PTH and PTHrP are essentially indistinguishable when studied using isolated osteoclasts (Evely *et al.,* 1991; Murrills *et al.,* 1990), bone explant systems (Raisz *et al.,* 1990; Yates *et al.,* 1988), or infusion into the intact animal (Kitazawa *et al.,* 1991; Thompson *et al.,* 1988). PTHrP may be somewhat less potent than equimolar infusions of PTH to induce hypercalcemia in humans, probably due to differences in plasma half-life (Fraher *et al.,* 1992).

As discussed in Chapter 3, PTHrP is a polyhormone, the precursor of multiple biologically active peptides. Carboxyl-terminal peptides that are predicted to arise from cleavage of PTHrP in the polybasic region PTHrP(102-106) have been synthesized and shown to inhibit bone resorption in several explant systems (Fenton *et al.,* 1991a,b, 1993), although not all (Sone *et al.,* 1992), and also *in vivo* (Cornish *et al.,* 1997). On this basis, the minimal peptide that inhibits bone resorption, PTHrP(107-111), has been called osteostatin.

Effects of PTH and PTHrP on Bone Formation

The anabolic effects of PTH and PTHrP are discussed in Chapter 75, and their involvement in the pathogenesis of bone changes in primary hyperparathyroidism is presented.

This section synthesizes a view of the effects of PTH and PTHrP on bone formation from the perspective of the individual cellular actions of the hormones that have been summarized in the preceding sections of this chapter.

Continuous exposure to PTH leads to a coupled increase in bone formation and bone resorption, with a net loss of bone mass in most circumstances, whereas intermittent treatment with injections of PTH once daily, or less frequently, produces a net anabolic effect (Tam *et al.,* 1982) (see Chapter 75 for a review). In contrast, the initial interpretation of bone histomorphometry in malignancy-associated hypercalcemia was that, unlike primary hyperparathyroidism, bone resorption was uncoupled from bone formation (Stewart *et al.,* 1982), raising the possibility that the effects of PTHrP on bone formation differed radically from the effects of PTH. However, in animal models of humoral hypercalcemia, increases in bone resorption were appropriately coupled to increases in bone formation (Strewler *et al.,* 1986). It has been shown that intermittent administration of PTHrP(1-36) into humans for 2 weeks leads to increases in biochemical markers of bone formation and a decrease in markers of bone resorption (Plotkin *et al.,* 1998). PTHrP(1-36) is somewhat less effective than PTH(1-34) as an anabolic agent in the ovariectomized rat (Stewart *et al.,* 2000). Moreover, a carboxyl-substituted analog of PTHrP(1-34) also mimicks the anabolic action of PTH in the rat (Frolik *et al.,* 1999; Vickery *et al.,* 1996). Thus, the anabolic effects of PTH and PTHrP, administered intermittently, appear similar.

Any attempt to understand the cellular basis for the anabolic actions of PTH and PTHrP must take into account

their histomorphological effects. The increase in bone formation is best correlated with marked increases in bone formation surfaces and activation frequency (Boyce *et al.,* 1996; Dempster *et al.,* 1999; Lane *et al.,* 1996; Manolagas, 2000; Shen *et al.,* 1993). Thus, a major effect of PTH is to increase the number of active, bone-forming osteoblasts. Increases in the mineral apposition rate are also seen but tend to be smaller (Boyce *et al.,* 1996; Dempster *et al.,* 1999; Lane *et al.,* 1996; Shen *et al.,* 1993). Duration of the active bone formation phase is not prolonged in dogs treated with PTH (Boyce *et al.,* 1996) but is increased in primary hyperparathyroidism (Dempster *et al.,* 1999).

An increase in the number of active osteoblasts could occur in several ways, and PTH may not have the same effect in all circumstances—its predominant effect on growing bone in a young rodent may differ from its predominant effect in aged bone. First, PTH could increase the birth rate or proliferation of osteoblast precursors in bone marrow. In the rat, an anabolic regimen of PTH does not increase the proliferation of osteoblast precursors based on the absence of an increase in labeled nuclei on the bone surface after continuous labeling with [^{3}H]thymidine (Dobnig and Turner, 1995). This is compelling evidence against the view that a proproliferative effect of PTH is decisive in increasing osteoblast number. However, intermittent exposure to PTH could increase homing to the bone surface of late, postmitotic osteoblast precursors in the bone marrow, which are recognized as having PTH receptors (Amizuka *et al.,* 1996; Rouleau *et al.,* 1990).

Second, PTH treatment could activate bone-lining cells to again become active osteoblasts. There is no direct evidence for or against this hypothesis. However, bone-lining cells cover a relatively large bone surface per cell because of their flattened, spread shape, and it is not clear that the numbers of bone-lining cells are adequate to account for the increase in osteoblast number that is observed with PTH treatment.

Third, an anabolic PTH regimen could increase the life span of the active osteoblast. In the mouse, intermittent treatment with a high dose of PTH reduces the rate of osteoblast apoptosis (Jilka *et al.,* 1999), although PTH treatment actually increased the number of apoptotic osteoblasts in metaphyseal bone of young rats (Stanislaus *et al.,* 2000b). However, it is not clear whether the reduction in cell death is quantitatively sufficient to account for the anabolic activity of PTH. If the life span of an active osteoblast is several months, as surmised (Manolagas, 2000), then the turnover rate of osteoblasts would be considerably too slow for a reduction in the rate of apoptosis to account for a rapid expansion of the osteoblast pool—one that occurs within a week of the onset of PTH administration in rodent models—e.g., if the osteoblast life span is 100 days, their turnover rate is 1% per day, and the maximal increase in osteoblast pool size to be expected from complete abolition of apoptosis would also be 1% per day. If a reduction in apoptosis rate were the primary effect of PTH, increases not only in mean wall thickness but also in the duration of the active formation period would be expected. It is reasonably clear that mean wall thickness is increased by anabolic PTH regimens or in primary hyperparathyroidism, but whether the duration of the active formation period is also increased has not been fully resolved (Boyce *et al.,* 1996; Dempster *et al.,* 1999). In order to determine the mechanism by which PTH or PTHrP increases osteoblast number, and thereby has its anabolic effect, it will ultimately be necessary to learn the origin and fate of osteoblasts that participate in the anabolic effects by determining their precise cellular kinetics.

Perspectives on PTH and PTHrP in Bone

As evident from the previous section on anabolic effects of PTH and PTHrP, there is much to be learned about how the individual effects of the hormones on bone cells are integrated to produce the final effects of the hormones on the physiology of the skeleton. Moreover, there is large lacune in our understanding of the skeletal role of PTHrP. Although bone cells both secrete and respond to PTHrP, PTHrP is a major regulator of cartilage (the precursor of endochondral bones), and it is tantalizing to speculate that PTH evolved as a systemic hormone to overdrive the local regulation of bone metabolism by its sister peptide, the physiology of PTHrP in the skeleton has been refractory to study. Genetic models, so powerful in unraveling the role of PTHrP in the cartilaginous phase of endochondral bone formation (Chapter XX), have yielded little information about bone per se because any changes observed in bone when the PTHrP/receptor system are perturbed are potentially explained by perturbations in endochondral bone formation. To apply genetic methods to the study of PTHrP in bone, what is now necessary is tissue-specific targeting of PTHrP and its receptor in bone, and such studies are underway. By ablating PTHrP or the PTH/PTHrP receptor in bone only and ultimately restoring sequence-specific portions of the polyhormone PTHrP to such animals, it will eventually be possible to determine what is the local role of PTHrP in bone and how PTH and PTHrP interact as regulators of skeletal physiology.

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CHAPTER 29

Physiological Actions of Parathyroid Hormone (PTH) and PTH-Related Protein

Epidermal, Mammary, Reproductive, and Pancreatic Tissues

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Introduction

Documentation of the skeletal abnormalities in mice that either overexpressed parathyroid-related protein (PTHrP) in their skeletons or had the genes for PTHrP and the PTH receptor ablated by the techniques of homologous recombination provided an exciting impetus for the rapid accumulation of knowledge regarding the mechanisms by which PTHrP regulates bone and cartlilage development and physiology. These findings are reviewed in Chapters 13 and 15. Since the mid-1990s, increasing evidence has accumulated that PTHrP and the PTH/PTHrP receptor family also contribute to the development and functioning of several nonskeletal organs. Data regarding the action of PTHrP in the vascular system and the central nervous system are reviewed in Chapter 16. In this, the last of the series of chapters on the physiologic actions of PTHrP, we review data regarding the function(s) of PTHrP in several other nonskeletal sites. We first consider the functions of PTHrP in skin. Next, we review its functions in the mammary gland, placenta, and other reproductive tissues. Finally, we examine its role in the endocrine pancreas.

Skin

PTHrP and PTHrP Receptor Expression

Normal human keratinocytes were the first nonmalignant cells shown to produce PTHrP (Merendino *et al.,* 1986), and multiple studies have confirmed that rodent and human keratinocytes in tissue culture express the PTHrP

gene and secrete bioactive PTHrP (reviewed in Philbrick *et al.,* 1996). PTHrP expression has also been examined in skin *in vivo* using both immunohistochemistry and *in situ* hybridization. During fetal development in rats and mice, PTHrP is expressed principally within the epithelial cells of developing hair follicles (Karmali *et al.,* 1992; Lee *et al.,* 1995). In mature skin, PTHrP has been found at low levels throughout the epidermis from the basal layer to the granular layer. Some studies have suggested that PTHrP is more highly expressed in the superbasal keratinocytes (Danks *et al.,* 1989; Hayman *et al.,* 1989), although not all studies have reported this pattern (Atillasoy *et al.,* 1991; Grone *et al.,* 1994). A variety of factors have been reported to regulate PTHrP production by cultured keratinocytes (see Philbrick *et al.,* 1996, for review). For example, glucocorticoids and 1,25 $(OH)_2D$ have been shown to downregulate PTHrP production, whereas fetal bovine serum, matrigel, and an as yet unidentified factor(s) secreted from cultured fibroblasts have been shown to upregulate PTHrP production. The upregulation of PTHrP production by fibroblast-conditioned media is particularly interesting, as PTHrP, in turn, acts back on dermal fibroblasts, suggesting that it may function in a short regulatory loop between keratinocytes and dermal fibroblasts (Shin *et al.,* 1997; Blomme *et al.,* 1999a). Finally, *in vivo,* PTHrP expression has been shown to be upregulated at the margins of healing wounds in guinea pigs (Blomme *et al.,* 1999b). Interestingly, in this study, PTHrP was also detected in myofibroblasts and macrophages, suggesting that keratinocytes may not be the only source of PTHrP in skin.

It is now clear that keratinocytes do not express the type I PTH/PTHrP receptor (PTHR1), but dermal fibroblasts do (Hanafin *et al.,* 1995; Orloff *et al.,* 1995). PTHrP has been shown to bind to skin fibroblasts and to elicit biochemical and biological responses in these cells (Shin *et al.,* 1997; Blomme *et al.,* 1999a; Wu *et al.,* 1987). In addition, studies utilizing *in situ* hybridization have demonstrated that PTHR1 mRNA in fetal skin, is absent from the epidermis, yet abundant in the dermis, especially in those cells adjacent to the keratinocytes (Karmali *et al.,* 1992; Lee *et al.,* 1995; Dunbar *et al.,* 1999a). There are fewer data concerning the expression patterns of the PTHR1 in more mature skin, but, in mice, it appears that the relative amount of PTHR1 mRNA in dermal fibroblasts is reduced in adult as compared to fetal skin (J. P. Zhang and J. J. Wysolmerski, unpublished data). Although keratinocytes do not express the classical PTH/PTHrP receptor, studies have shown that these cells bind and respond to PTHrP by inducing calcium transients, suggesting that there may be other receptors for PTHrP expressed on these cells (Orloff *et al.,* 1992, 1995). However, to date, no such receptors have been isolated, so their existence remains uncertain.

Biochemistry of PTHrP

As described in Chapters 3 and 4, during transcription, the PTHrP gene undergoes alternative splicing to generate multiple mRNAs, which in human cells give rise to three main protein isoforms. In addition, each of these isoforms is subject to posttranslational processing to generate a variety of peptides of varying length. Human keratinocytes have been shown to contain mRNA encoding for each of the three main isoforms, although, as in other systems, no clearly defined or unique role has yet emerged for any of the three individual isoforms (Philbrick *et al.,* 1996). Keratinocytes have also been shown to process full-length PTHrP into a variety of smaller peptides, including PTHrP(1-36) and a midregion fragment beginning at amino acid 38 (Soifer *et al.,* 1992). These cells have also been shown to secrete a large (~10 kDa) amino-terminal form that is glycosylated (Wu *et al.,* 1991). There is currently no specific information regarding the secretion of COOH-terminal peptides of PTHrP in skin, but keratinocytes are also likely to produce these peptides.

Function of PTHrP

Several studies suggest that PTHrP is involved in the regulation of hair growth. As noted earlier, the PTHrP gene in embryonic skin is expressed most prominently in developing hair follicles, and overexpression of PTHrP in the basal keratinocytes of skin in transgenic mice leads to a severe inhibition of hair follicle morphogenesis during fetal development (Wysolmerski *et al.,* 1994). The effects of PTHrP overexpression appear to act early during hair follicle induction, implicating PTHrP in the regulation of epidermal patterning during embryogenesis. However, any such function of PTHrP during hair follicle morphogenesis is not critical because disruption of the PTHrP or PTHR1 genes does not seem to affect hair follicle formation or patterning in mice (Karaplis *et al.,* 1994; Lansk *et al.,* 1996; Foley *et al.,* 1998).

In addition to effects on hair follicle morphogenesis, it has also been suggested that PTHrP may participate in regulation of the hair cycle. It has been reported that the systemic administration of PTHR1 antagonists to young mice perturbs the hair cycle by prematurely terminating telogen, prolonging anagen growth, and inhibiting catagen (Schilli *et al.,* 1997). These findings imply that PTHrP acts to inhibit hair follicle growth by pushing growing hair follicles into the growth-arrested or catagen/telogen phase of the hair cycle. If this hypothesis were correct, one would expect PTHrP knockout mice to exhibit findings similar to PTHR1 antagonist-treated mice. However, this does not appear to be the case. In mice that lack PTHrP in their skin, the hair cycle appears to be normal (Foley *et al.,* 1998). In fact, rather than a promotion of hair growth, these mice demonstrate a thinning of their coat over time. These conflicting results are difficult to rationalize at this point, but they raise the intriguing possibility that the PTHR1 antagonist might be inhibiting the function of another member of the PTH receptor family and that there may be ligands for such a receptor in skin other than PTHrP.

PTHrP has also been implicated in the regulation of keratinocyte proliferation and/or differentiation. Although

studies in cultured cells have alternately suggested that PTHrP either enhances or inhibits keratinocyte proliferation, data from studies *in vitro* have consistently suggested that PTHrP promotes the differentiation of keratinocytes (reviewed in Philbrick *et al.,* 1996). In contrast, studies *in vivo* have suggested that PTHrP inhibits keratinocyte differentiation (Foley *et al.,* 1998). A careful comparison of the histology of PTHrP-null and PTHrP-overexpressing skin demonstrated reciprocal changes. In the absence of PTHrP, it appeared that keratinocyte differentiation was accelerated, whereas in skin exposed to PTHrP overexpression, keratinocyte differentiation appeared to be retarded (Foley *et al.,* 1998). Therefore, in a physiologic context, PTHrP appears to slow the rate of keratinocyte differentiation and to preserve the proliferative, basal compartment. Remarkably, these changes in the rate of keratinocyte differentiation are exactly analogous to those noted for chondrocyte differentiation in the growth plates of mice overexpressing PTHrP as compared to PTHrP- and PTHR1–null mice (Philbrick *et al.,* 1996) (see Chapter 15). Again, at present, it is difficult to rationalize conflicting data regarding the effects of PTHrP on keratinocyte differentiation, but studies in genetically altered mice clearly indicate that PTHrP participates in the complex regulation of these processes *in vivo*. Further research will be needed to understand its exact role.

An important but still unresolved question is whether the effects of PTHrP on keratinocyte proliferation, differentiation, and hair follicle growth are the result of its effects on keratinocytes directly or via its effects on dermal fibroblasts. At present there are more data to support the paracrine possibility. PTHR1 is expressed on dermal fibroblasts *in vivo* and in culture (Lee *et al.,* 1995; Hanafin *et al.,* 1995). Dermal fibroblasts have been demonstrated to show biochemical and biological responses to PTHrP (Shin *et al.,* 1997; Blomme *et al.,* 1999a; Wu *et al.,* 1987). Furthermore, PTHrP has been shown to induce changes in growth factor and extracellular matrix production that could, in turn, lead to changes in keratinocyte proliferation and/or differentiation and hair follicle growth (Shin *et al.,* 1997; Blomme *et al.,* 1999a; Insogna *et al.,* 1989). Of course, the autocrine and paracrine signaling pathways are not mutually exclusive, but any direct autocrine effects of PTHrP on keratinocytes, as discussed earlier, would require the presence of PTHrP receptors other than the PTHR1 on these cells. Although preliminary biochemical evidence has suggested that this possibility exists, no such receptors have been identified on keratinocytes (Orloff *et al.,* 1992, 1995). An alternative possibility by which PTHrP might have cell autonomous effects on keratinocytes is via an intracrine pathway involving its translocation to the nucleus (Philbrick *et al.,* 1996). Clearly, much research is needed to define the receptors and signaling pathways by which PTHrP acts in skin. Only when this information is available will we be able to understand the mechanisms leading to the skin phenotypes that have been observed in the various transgenic models discussed earlier.

Pathophysiology of PTHrP

To date, PTHrP has not been implicated in any diseases of the skin. It has been suggested that skin and skin appendage findings in the rescued PTHrP-null mice are reminiscent of a series of disorders collectively known as the ectodermal dysplasias (Foley *et al.,* 1998), but PTHrP has not been formally linked to any of these diseases. The most common tumors causing humoral hypercalcemia of malignancy (HHM) are those of squamous histology, but these tumors rarely arise from skin keratinocytes. In fact, the most common skin tumors, basal cell carcinomas, do not overexpress PTHrP and are not associated with hypercalcemia (Philbrick *et al.,* 1996). Although PTHrP appears to participate in the normal physiology of the skin, it is not clear at this juncture if it will be involved in skin pathophysiology.

Mammary Gland

PTHrP was reported to be expressed in mammary tissue and to be secreted into milk very soon after its discovery (Thiede and Rodan, 1988; Budayr *et al.,* 1989). It is now known that PTHrP is critically important for the proper development and functioning of the mammary gland throughout life. In addition, it has been implicated as an important modulator of the biological behavior of breast cancer. The mammary gland develops in several discrete stages and only reaches its fully differentiated state during pregnancy and lactation. PTHrP appears to serve different functions during these different stages of mammary development; therefore, we will organize our discussion around three principal stages of mammary development: embryonic development, adolescent growth, and pregnancy and lactation. For each stage, we will first outline the pertinent developmental events in rodents, as data regarding the function(s) of PTHrP largely come from studies in mice and rats. Next, we will discuss the localization of PTHrP and PTHrP receptors and the regulation of the expression of PTHrP and its receptors. Finally we will address the function of PTHrP.

Embryonic Mammary Development

In mice, there are two phases of embryonic mammary development. The first involves the formation of five pairs of mammary buds, each of which consists of a light bulb-shaped collection of epithelial cells surrounded by several layers of fibroblasts known as the mammary mesenchyme (Sakakura, 1987). After the formation of these buds, mouse mammary development displays a characteristic pattern of sexual dimorphism. In male embryos, in response to androgens, the mammary mesenchyme destroys the epithelial bud and male mice are left without mammary glands or nipples (Sakakura, 1987). In female embryos, however, the mammary buds remain quiescent until embryonic day 16 (E16) when they undergo a transition into the second step

of embryonic development, formation of the rudimentary ductal tree. This process involves the elongation of the mammary bud, its penetration out of the dermis and into a specialized stromal compartment known as the mammary fat pad, and the initiation of ductal branching morphogenesis. At the time of birth, the gland consists of a simple epithelial ductal tree consisting of 15–20 branched tubes within a fatty stroma (Sakakura, 1987). This initial pattern persists until puberty at which time the mature virgin gland is formed through a second round of branching morphogenesis, regulated by circulating hormones (discussed later).

The PTHrP gene is expressed exclusively within epithelial cells of the mammary bud, soon after it begins to form. PTHrP mRNA continues to be localized to mammary epithelial cells during the initial round of branching morphogenesis, as the bud grows out into the presumptive mammary fat pad and begins to branch (Dunbar *et al.,* 1998, 1999a; Wysolmerski *et al.,* 1998). At some point after birth, PTHrP gene expression is downregulated, and in the adult virgin gland PTHrP mRNA is found only within specific portions of the duct system (discussed later) (Dunbar *et al.,* 1998). In contrast to the PTHrP gene, the PTHR1 gene appears to be expressed within the mesenchyme, but its expression is widespread and is not limited to the developing mammary structures. Transcripts for the PTHR1 gene are found within the mammary mesenchyme but also throughout the developing dermis (Dunbar *et al.,* 1999a; Wysolmerski *et al.,* 1998). It is not clear when the receptor gene is first expressed within the subepidermal mesenchyme. However, it appears already to be present when the mammary bud begins to form and it continues to be expressed within fibroblasts surrounding the mammary ducts as they begin to grow out and branch (Wysolmerski *et al.,* 1998; Dunbar *et al.,* 1998).

Epithelial expression of PTHrP and mesenchymal expression of the PTHR1 are not unique to the developing mammary gland, and this pattern has long led to speculation that PTHrP and its receptor might contribute to the regulation of epithelial–mesenchymal interactions during organogenesis. There is now solid evidence that this is the case during embryonic mammary development, where PTHrP appears to serve as an epithelial signal that influences cell fate decisions within the developing mammary mesenchyme. Data supporting this notion come from studies in several genetically altered mouse models. First, in PTHrP or PTHR1 knockout mice, there is a failure of the normal androgen-mediated destruction of the mammary bud due to the failure of the mammary mesenchyme to differentiate properly and to express androgen receptors (Dunbar *et al.,* 1999a) (see Fig. 1). Second, in PTHrP or PTHR1 knockout mice, the mammary buds fail to grow out into the fat pad and initiate branching morphogenesis, again due to defects in the mammary mesenchyme (Wysolmerski *et al.,* 1998; Dunbar *et al.,* 1998). Finally, in keratin 14 (K14) PTHrP transgenic mice that ectopically overexpress PTHrP within all the basal keratinocytes of the developing embryo, subepidermal mesenchymal cells, which should acquire a dermal fate, instead become mammary mesenchyme (Dunbar *et al.,* 1999a).

As demonstrated by these studies, PTHrP signaling is essential for mammary gland formation in rodents. When the mammary gland begins to form, the PTHR1 is expressed in all the mesenchymal cells underneath the epidermis, but PTHrP is expressed only within the mammary epithelial buds and not within the epidermis itself (Karmali *et al.,* 1992; Thiede and Rodan, 1988; Wysolmerski *et al.,* 1998). As the mammary bud grows down into the mesenchyme, PTHrP produced by mammary epithelial cells interacts over short distances with the PTHR1 on the immature mesenchymal cells closest to the epithelial bud and triggers these cells to differentiate into mammary mesenchyme. In this way, PTHrP acts as a patterning molecule contributing to the formation of small patches of mammary-specific stroma around the mammary buds and within the surrounding sea of presumptive dermis. The process of differentiation set in motion by PTHrP signaling is critical to the ability of the mammary-specific stroma to direct further morphogenesis of the epithelium. In the absence of this signaling, the mesenchyme can neither destroy the epithelial bud in response to androgens nor trigger the outgrowth of the bud and the initiation of branching morphogenesis (Dunbar *et al.,* 1998, 1999a; Wysolmerski *et al.,* 1998).

Although the above model just described was developed from studies in mice, it appears that PTHrP is also critical to the formation of breast tissues in human fetuses. It has been demonstrated that a fatal form of dwarfism known as Blomstrand's chondrodyplasia is a result of null mutations of the PTHR1 gene (Jobert *et al.,* 1998) (see Chapter 44). Affected fetuses have skeletal abnormalities similar to those caused by deletion of the PTHrP and PTHR1 genes in mice (see Chapter 15) and, in addition, lack breast tissue or nipples (Wysolmerski *et al.,* 1999). In normal human fetuses, the PTHrP gene is expressed within the mammary epithelial bud, and the PTHR1 gene is expressed in surrounding mesenchyme (Wysolmerski *et al.,* 1999). Therefore, in humans, as in mice, epithelial-to-mesenchymal PTHrP–PTHR1 signaling is essential to the formation of the embryonic mammary gland.

Adolescent Mammary Development

Following birth, the murine mammary gland undergoes little development until the onset of puberty. At that point, in response to hormonal changes, the distal ends of the mammary ducts form specialized structures called terminal end buds. These structures serve as the sites of cellular proliferation and differentiation for a period of active growth that gives rise to the typical branched duct system of the mature virgin gland (Daniel and Silberstein, 1987). Once formed, the ductal tree remains relatively unchanged until another round of hormonal stimulation during pregnancy induces the formation of the lobuloalveolar units that produce milk.

Similar to findings in the embryonic mammary gland during puberty, PTHrP appears to be a product of mammary

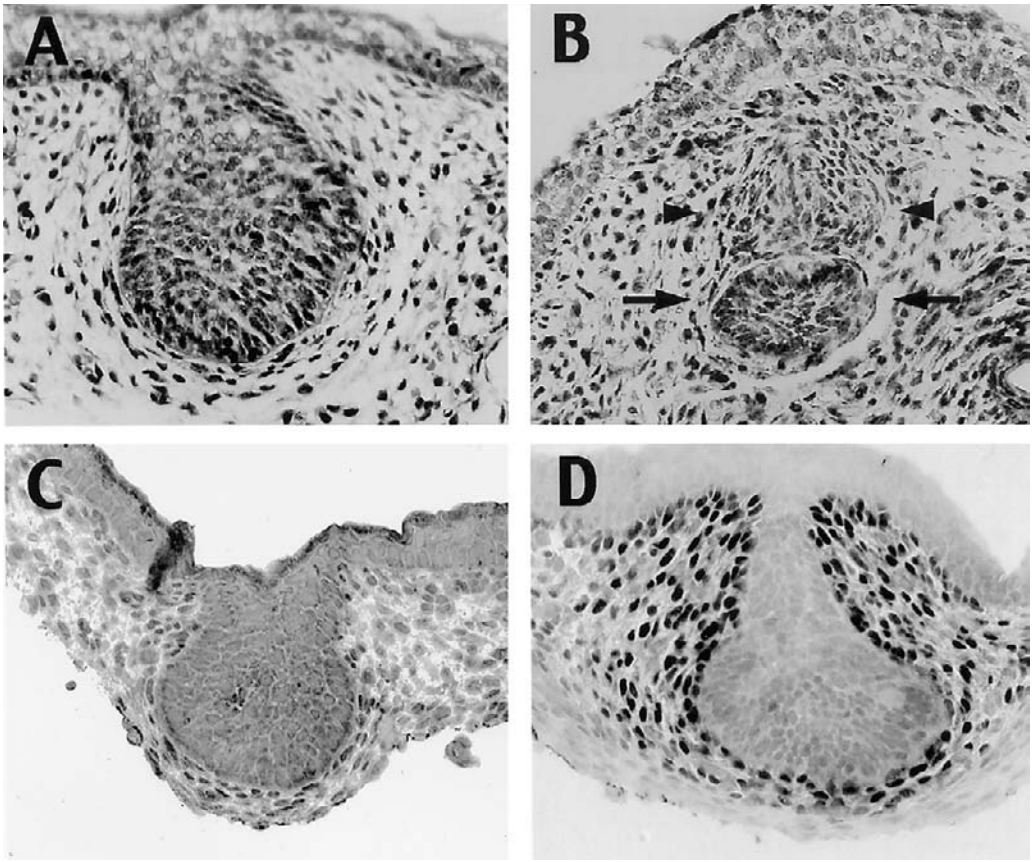

Figure 1 Failure of sexual dimorphism during embryonic mammary development. Mammary buds from PTHrP knockout (A and B) and wild-type (B and D) embryos at E15. Note the destruction of the mammary bud in normal male embryos (B) as evidenced by the mesenchymal condensation that has obliterated the bud stalk (arrowheads) and the degenerating epithelial remnant (arrows). In PTHrP knockout males (A), this process is completely absent. The destruction of the mammary bud is an androgen-dependent phenomenon, and mesenchymal cells are the targets for androgen action, as evidenced by positive staining for androgen receptors seen in the mammary mesenchyme surrounding a normal female mammary bud in D. In PTHrP knockout mammary buds, this process fails due to the failure of androgen receptor expression in the mammary mesenchyme as shown in C. Modified from Dunbar *et al.* (1999a), with permission.

epithelial cells and the PTHR1 appears to be expressed in stromal cells (Dunbar *et al.,* 1998). However, the structure of the pubertal gland is more complex than that of the embryonic gland and, here, there are conflicting data regarding the localization of PTHrP and the PTH receptor. Although there is general agreement that PTHrP is expressed in epithelial cells in the postnatal gland, there is some disagreement regarding the specific epithelial compartments in which PTHrP is found. Studies employing *in situ* hybridization in mice have suggested that, after birth, the overall levels of PTHrP gene expression in mammary ducts are reduced except for in the terminal end buds during puberty (Dunbar *et al.,* 1998). In these structures, appreciable amounts of PTHrP mRNA were detected in the peripherally located cap cells. In other parts of the gland there was little, if any, specific hybridization for PTHrP. In contrast, studies looking at mature human and canine mammary glands using immunohistochemical techniques have suggested that PTHrP can be found in both luminal epithelial and myoepithelial cells throughout the ducts (Grone *et al.,* 1994; Liapis *et al.,* 1993). Furthermore, studies using cultured cells have suggested that PTHrP is produced by luminal and myoepithelial cells isolated from normal glands (Ferrari *et al.,* 1992; Seitz *et al.,* 1993; Wojcik *et al.,* 1999). There have been fewer reports looking at the localization of PTHR1 expression in the postnatal mammary gland, but as in embryological development, it is expressed in the mammary stroma (Dunbar *et al.,* 1998). *In situ* hybridization studies have found the highest concentration of PTHR1 mRNA in the stroma immediately surrounding terminal end buds during puberty (Dunbar *et al.,* 1998). This same study found lower levels of PTHR1 mRNA distributed generally within the fat pad stroma, but very little expression in the dense stroma surrounding the more mature ducts. In addition, these investigators found no evidence of PTHR1 mRNA in freshly isolated epithelial cells (Dunbar *et al.,* 1998). However, in contrast to these findings, other studies have suggested that PTHR1 is expressed in cultured luminal epithelial and myoepithelial cells (Seitz *et al.,* 1993; Wojcik *et al.,* 1999), as well as in cultured breast cancer cell lines (Birch *et al.,* 1995). In summary, during puberty,

PTHrP and its receptor are found predominantly within the terminal end buds, with PTHrP localized to the epithelium and PTHR1 localized in the stroma. It remains an open and interesting question whether, at some time during mammary ductal development, epithelial cells express low levels of PTHR1.

Studies in transgenic mice have suggested that PTHrP is an important regulator of mammary morphogenesis during puberty. Overexpression of PTHrP in mammary epithelial cells using the K14 promoter results in an impairment of ductal branching morphogenesis (Wysolmerski *et al.,* 1995). There are two aspects to the defect. First, the terminal end buds advance through the mammary fat pad at a significantly slower rate than normal. Second, there is a severe reduction in the branching complexity of the ductal tree. As seen in Fig. 2, this results in a spare and stunted epithelial duct system. Experiments altering the timing and duration of PTHrP overexpression in the mammary gland using a tetracycline-regulated K14-PTHrP transgene have demonstrated that the two aspects of this pubertal phenotype appear to represent separate functions of PTHrP. The branching (or patterning) defect results from embryonic overexpression of PTHrP, whereas the ductal elongation defect is a function of overexpression of PTHrP during puberty (Dunbar *et al.,* 1999b). These effects on ductal patterning provide further evidence of the importance of PTHrP as a regulator of embryonic mammary development. In addition, the localization patterns for PTHrP and PTHR1 during puberty, combined with the effects of pubertal overexpression of PTHrP on ductal growth, suggest that PTHrP also functions later in mammary development. During puberty it appears to modulate epithelial–mesenchymal interactions that govern ductal elongation.

Pregnancy and Lactation

Mammary epithelial cells only reach their fully differentiated state during lactation. Under hormonal stimulation during pregnancy, there is a massive wave of epithelial proliferation and morphogenesis that gives rise to terminal ductules and lobuloalveolar units. During the later stages of pregnancy the epithelial cells fully differentiate and then begin to secrete milk during lactation. By the time lactation commences, the fatty stroma of the mammary gland is completely replaced by actively secreting lobuloalveoli. Upon the completion of lactation, there is widespread

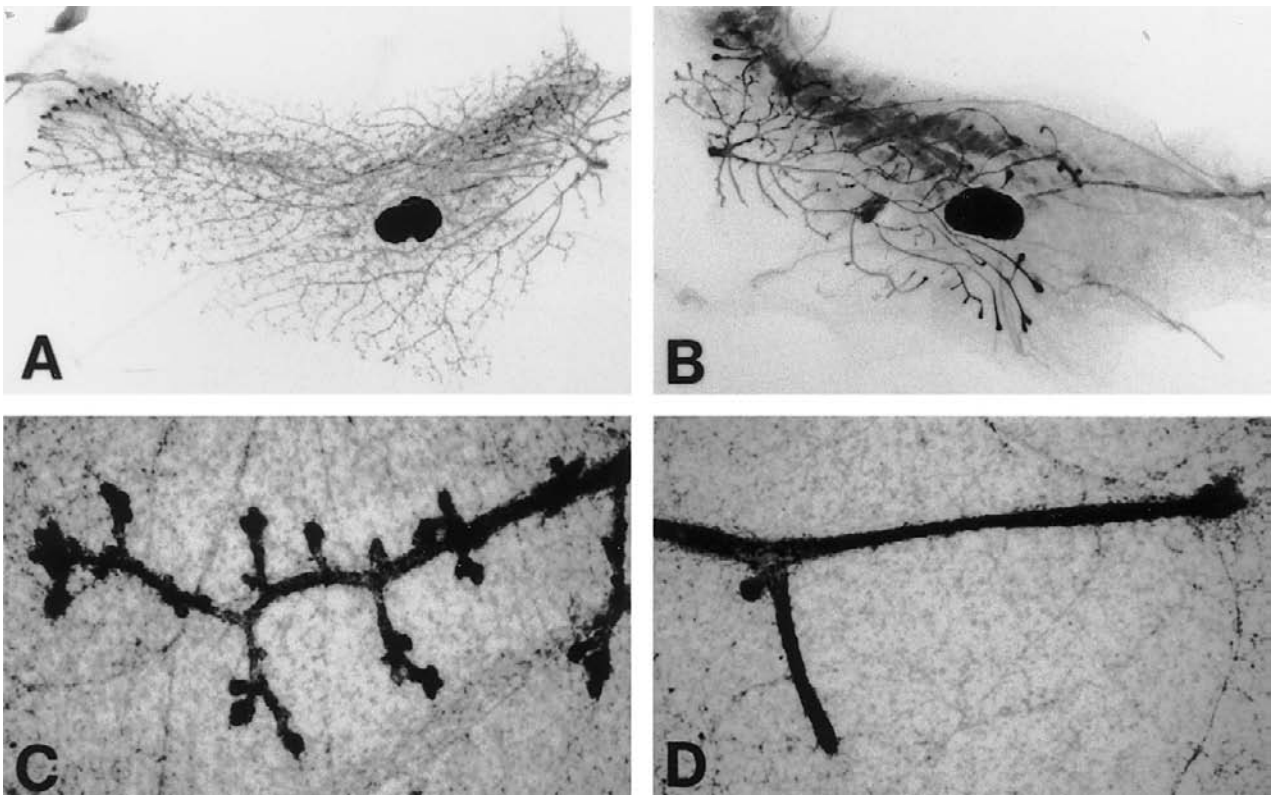

Figure 2 Overexpression of PTHrP in the mammary gland of K14–PTHrP transgenic mice antagonizes ductal elongation and branching morphogenesis during puberty. Typical whole mount analyses of fourth inguinal mammary glands from wild-type (A) and K14–PTHrP transgenic mice (B) at 6 weeks of age. The dark oval in the center of each gland is a lymph node. Growth of the ducts during puberty is directional and each gland is arranged so that the primary duct (the origin of the duct system) is toward the center of the figure. Note that overexpression of PTHrP results in an impairment of the elongation of the ducts through the fat pad, as well as dramatic reduction of the branching complexity of the ductal tree. (C and D) Higher magnifications of a portion of the ducts from wild-type (C) and transgenic (D) glands demonstrating the reduction in side branching caused by the overexpression of PTHrP. Modified from Wysolmerski *et al.* (1995), with permission.

apoptosis of the differentiated epithelial cells and the gland remodels itself into a duct system similar to that of the virgin animal (Daniel and Silberstein, 1987).

Localization studies in humans, rodents, and cows have all noted epithelial cells to be the source of PTHrP in the mammary gland during pregnancy and lactation (Liapis *et al.,* 1993; Wojcik *et al.,* 1998, 1999; Rakopoulos *et al.,* 1990). Based on the assessment of whole gland RNA, PTHrP expression appears to be upregulated at the start of lactation under the control of both local and systemic factors (Philbrick *et al.,* 1996; Thiede and Rodan, 1988; Thiede, 1989; Thompson *et al.,* 1994; Buch *et al.,* 1992). Thiede and Rodan (1988; Thiede, 1989) originally reported that PTHrP expression in rats is dependent on suckling and on serum prolactin concentrations. However, prolactin must serve only as a permissive factor, for additional studies have shown that the suckling response is a local one and that PTHrP only rises in the milked gland (Thompson *et al.,* 1994). Furthermore, PTHrP expression increases gradually over the course of lactation, and in later stages, its expression becomes independent of serum prolactin levels (Bucht *et al.,* 1992). It is clear that much of the PTHrP made during lactation ends up in milk, in which levels of PTHrP are up to 10,000-fold higher than in the circulation of normal individuals and 1000-fold higher than in patients suffering from humoral hypercalcemia of malignancy (Philbrick *et al.,* 1996). PTHrP concentrations in milk have generally been found to mirror RNA levels in the gland, increasing over the duration of lactation and rising acutely with suckling (Thompson *et al.,* 1994; Yamamoto *et al.,* 1992a; Law *et al.,* 1991; Goff *et al.,* 1991). In addition, evidence shows that PTHrP levels correlate with the calcium content of milk in humans and cows, but not in rodents (Yamamoto *et al.,* 1992a; Law *et al.,* 1991; Goff *et al.,* 1991; Vemura *et al.,* 1997; Kovacs and Kronenberg 1997). Finally, in mice, PTHrP mRNA levels are promptly downregulated during the early stages of involution and then increase to prelactation levels about a week into the remodeling process (M. Dunbar and J. J. Wysolmerski, unpublished data).

In contrast to PTHrP, there has been little study of the expression or regulation of PTHrP receptors during pregnancy and lactation. In early pregnancy, the PTH/PTHrP receptor is expressed at low levels in the stroma surrounding the developing lobuloalveloar units (Dunbar *et al.,* 1998). Studies using whole gland RNA demonstrate a reciprocal relationship between PTHR1 and PTHrP mRNA levels. That is, as PTHrP expression rises during lactation, PTHR1 mRNA levels decrease, and as PTHrP mRNA levels fall during early involution, PTHR1 expression increases to its former level (M. Dunbar and J. J. Wysolmerski, unpublished data). This may represent active downregulation of the receptor by PTHrP or may simply reflect the changing amount of stroma within the gland at these different stages. However, in a study of cells isolated from lactating rats, it was suggested that epithelial cells, as well as stromal cells, express this receptor (Wojcik *et al.,* 1999) so the regulation of receptor expression during pregnancy and lactation may be complex.

Initial reports of the presence of PTHrP in the mammary gland and in milk prompted a great deal of speculation regarding its functions in breast tissue during lactation. These proposals have revolved around four general hypotheses: (1) PTHrP may be involved in maternal calcium homeostasis and the mobilization of calcium from the maternal skeleton; (2) PTHrP may be involved in regulating vascular and/or myoepithelial tone in the lactating mammary gland; (3) PTHrP may be involved in transepithelial calcium transport into milk; and/or (4) PTHrP may be involved in neonatal calcium homeostasis or neonatal gut physiology. Although the true function(s) of PTHrP during lactation remains obscure, some experimental evidence addresses the first two of these possibilities. These data are discussed in the following paragraphs. However, at this point, the latter two ideas remain simple speculation and are not discussed further.

The control of maternal mineral metabolism and the mobilization of skeletal calcium for milk production remain enigmatic. Although a significant proportion of the calcium transported into milk is derived from the maternal skeleton, neither of the established calcium-regulating hormones, PTH nor $1,25(OH)_2D$, seems to be necessary or sufficient to account for this phenomenon (Kovacs and Kronenberg, 1997). Therefore, the finding that PTHrP was produced in the lactating breast aroused interest in this protein as the missing factor acting to mobilize calcium during lactation. Although not every study has concurred in support of such a role, the weight of evidence across species now suggests that PTHrP levels in the systemic circulation are elevated during lactation (Kovacs and Kronenberg, 1997). In addition, circulating PTHrP levels have been shown to correlate with bone density changes in lactating humans (Sowers *et al.,* 1996), and it appears that suckling leads to transient increases in circulating PTHrP levels (Dobnig *et al.,* 1995). Suckling has also been shown to lead to increases in urinary phosphate and cAMP excretion in rodents and in cows (Yamamoto *et al.,* 1991; Barlet *et al.,* 1993), changes that might be expected if PTHrP released from the mammary gland was acting in a systemic fashion. Of course, none of these data actually prove that the source of PTHrP in the circulation of lactating humans or animals is the mammary gland itself. More significantly, passive immunization of lactating mice with anti-PTHrP antibodies has not been found to influence maternal calcium homeostasis or the calcium content of milk (Melton *et al.,* 1960). Therefore, although PTHrP remains an appealing candidate regulator of maternal calcium homeostasis during lactation, such an action remains unproven.

The second potential function of PTHrP during lactation concerns the regulation of vascular and/or myoepithelial cell tone. As discussed in Chapter 16, PTHrP has been shown to modulate smooth muscle cell tone in a variety of organs, including the vascular tree, where it acts as a vasodilator. Consistent with these effects, two studies have shown that PTHrP increases mammary blood flow during lactation (Davicco *et al.,* 1993; Thiede *et al.,* 1992). The

injection of amino-terminal fragments of PTHrP into the mammary artery of dried ewes was shown to increase mammary blood flow and to override the vasoconstrictive effects of endothelin (Davicco *et al.,* 1993). Thiede and colleagues (1992) have demonstrated that the nutrient arteries of the inguinal mammary glands of rats make PTHrP and that its production is responsive to suckling and prolactin. Myoepithelial cells in the breast are similar, in some ways, to vascular smooth muscle cells and are thought to participate in the control of milk ejection by contracting in response to oxytocin (Daniel and Silberstein, 1987). Therefore, it is interesting that myoepithelial cells in culture have been shown to express PTHR1 and to respond to PTHrP by elevating intracellular cAMP (Seitz *et al.,* 1993; Wojcik *et al.,* 1999). Furthermore, mirroring the effects of PTHrP on the endothelin-induced contraction of vascular smooth muscle, PTHrP has been shown to block the rise in intracellular calcium normally induced in response to oxytocin in myoepithelial cells (Seitz *et al.,* 1993). Although much more study is needed, current data support speculation that PTHrP might have effects on mammary blood flow and/or milk ejection.

Pathophysiology of PTHrP in the Mammary Gland

Although PTHrP has not been directly implicated in the pathogenesis of any specific disease of the mammary gland, there are now several instances in which it appears to contribute to pathophysiology in the human breast. First, as noted previously, fetuses afflicted with Blomstrand's chondrodystrophy lack nipples and breast tissue (Wysolmerski *et al.,* 1999). Second, there have been two case reports in which lactational hypercalcemia was noted to be related to elevations in circulating levels of PTHrP (Khosla *et al.,* 1990; Reid *et al.,* 1992). One of these cases was caused by massive breast hyperplasia, and the patient required reduction mammoplasty in order to ameliorate her hypercalcemia (Khosla *et al.,* 1990). Finally, the area with the most potential impact on human health is the relationship of PTHrP production to breast cancer. This is evolving into a complicated topic and will be addressed only briefly here. However, it will be reviewed in more depth in Chapter 43.

It is well documented that PTHrP is produced by a number of primary breast carcinomas and that this sometimes leads to classical humoral hypercalcemia of malignancy (Isales *et al.,* 1987). A potentially more widespread role may be the involvement of PTHrP in the osteotrophism of breast cancer (Guise *et al.,* 1996; Yin *et al.,* 1999). Animal models have suggested that PTHrP production by breast tumor cells is important to their ability to form skeletal metastases (Guise *et al.,* 1996; Yin *et al.,* 1999). However, there is conflicting evidence as to whether PTHrP production by a primary breast tumor is predictive of bone metastases in patients (Bundred *et al.,* 1996; Henderson *et al.,* 2001). The largest and most carefully controlled study to date suggested that PTHrP production by the primary tumor is actually a negative predictor, not a positive predictor, of skeletal metastases (Henderson *et al.,* 2001). It may be that PTHrP production does not enable a tumor cell to get into the skeleton, but once there, the ability of tumor cells to upregulate PTHrP production within the bone microenvironment becomes important to their ability to grow in the skeleton (Yin *et al.,* 1999). These are important issues, and ongoing studies should provide us with more information in the near future.

Reproductive Tissues

PTHrP and Placental Calcium Transport

Nearly all of the calcium, and a large proportion of the inorganic phosphate (85%) and magnesium (70%), transferred from the mother to the fetus is associated with development and mineralization of the fetal skeleton (Grace *et al.,* 1986). The concentrations of both total and ionized Ca in all mammalian fetuses studied during late gestation have been observed to be higher than maternal levels. As a result of studies in which the sheep was used extensively for the study of fetal calcium control, one of the first suggested physiological roles of PTHrP was that of regulating the transport of calcium from mother to fetus in the mammal, thereby making calcium available to the growing fetal skeleton (Rodda *et al.,* 1988).

Immunoreactive PTH levels were found to be low in fetal lambs, whereas PTH-like biological activity in serum was high (Care *et al.,* 1985), suggesting the presence of another PTH-like substance. Parathyroidectomy in the fetal lamb resulted in loss of the calcium gradient that exists between mother and fetus, as well as impairment of bone mineralization, implicating parathyroids as the source of the regulatory agent. Crude, partially purified or recombinant PTHrP, but neither PTH nor maternal parathyroid extract that contained no immunoreactive PTHrP, restored the gradient (Rodda *et al.,* 1988). Thus, PTHrP appeared to be the active component of the fetal parathyroid glands responsible for maintaining fetal calcium levels and suppressing fetal PTH levels. In support of this hypothesis, immunoreactive PTHrP was found to be readily detectable in sheep fetal parathyroids from the time they form (MacIsaac *et al.,* 1991) and was also found in early placenta, suggesting that the latter tissue may be a source of PTHrP for calcium transport early in gestation.

The portion of PTHrP that appears to be responsible for regulating placental calcium transport lies between residues 67 and 86 (Care *et al.,* 1990), but the responsible receptor has not yet been identified. While syncytiotrophoblasts are believed to be central in the transport of calcium to the fetus, cytotrophoblasts (which differentiate to form the syncytium) are believed to be the calcium-sensing cells, and raising the extracellular calcium concentration has been shown to inhibit PTHrP release from these cells (Hellman *et al.,* 1992). The calcium-sensing receptor (CaR) has been localized to cytotrophoblasts of human placenta (Bradbury

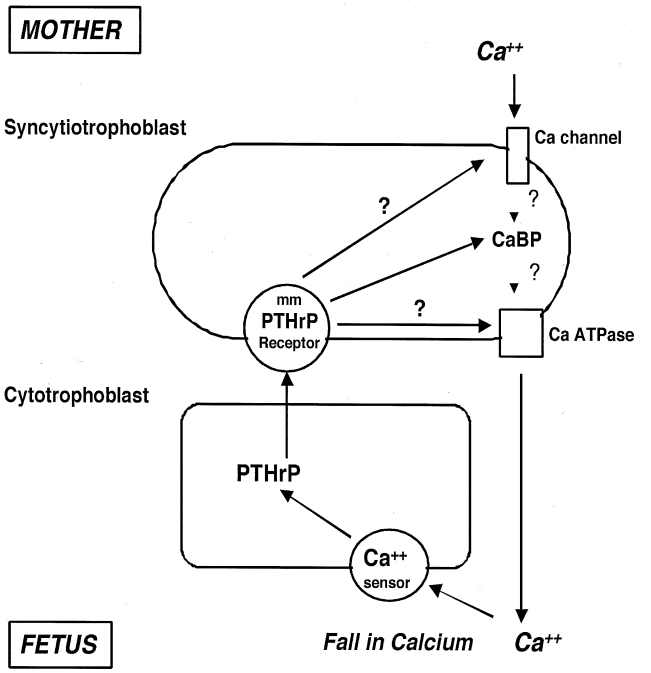

Figure 3 A potential model for the interactions of PTHrP and the CaR in regulating placental calcium transport. A fall in circulating calcium in the fetus activates the calcium-sensing receptor (CaR) on the cytotrophoblast. This leads to PTHrP secretion by the cytotrophoblast, which may then act via the putative midmolecule PTHrP receptor to promote calcium transport from maternal to fetal circulation. The nature and cellular location of the midmolecule PTHrP receptor are unknown, but are likely to be on the syncytiotrophoblast implicated in calcium transport across the placenta. Midmolecule PTHrP signaling could involve several mechanisms as indicated and may include participation of a calcium-binding protein (CaBP). (Modified from Bradbury (1999), with permission).

et al., 1997), and the work of Kovacs *et al.* (1998) has implicated it in placental calcium transport. Furthermore, a calreticulin-like, calcium-binding protein has been isolated from trophoblast cells and its expression is increased by treatment with PTHrP(67-84) but not with N-terminal PTHrP (Hershberger and Tuan, 1998). A working hypothesis for how the CaR, midregion PTHrP, and a midregion PTHrP receptor might interact to regulate transplacental calcium transport is presented in Fig. 3.

Although these observations are strongly suggestive of involvement of PTHrP and the CaR, the mechanisms of placental calcium transport are still not fully understood. Support for the role of PTHrP also comes from the PTHrP gene knockout mouse in which placental calcium transport is severely impaired (Kovacs *et al.,* 1996). In mice homozygous for deletion of the PTHrP gene, fetal plasma calcium and maternal–fetal calcium gradient were significantly reduced. When fetuses were injected *in utero* with fragments of PTHrP or PTH, calcium transport was significantly restored only by treatment with a midmolecular region of PTHrP that does not act via the PTHR1. Furthermore, in mice rendered null for the PTHR1 gene, placental calcium transport was increased, and PTHR1 null fetuses had plasma PTHrP levels more than 10 times higher than controls (Kovacs *et al.,* 2001). The circulating PTHrP in the fetal mice was found to be derived from several tissues, including liver and placenta, but the parathyroids were excluded as a source of PTHrP in this setting (Kovacs *et al.,* 2001).

Thus, conclusions from the murine studies are similar to those in the sheep, namely that PTHrP contributes to fetal skeleton calcium supply by controlling maternal–fetal calcium transport through actions mediated by a midmolecule portion of the PTHrP molecule.

Uterus and Extraembryonic Tissues

The uterus, both pregnant and nonpregnant, is another of the many sites of production and action of PTHrP. The relaxing effect of PTH on uterine smooth muscle had been long recognized (Shew *et al.,* 1984), and it was not surprising that PTHrP had the same effect (Shew *et al.,* 1991). The finding that expression of mRNA for PTHrP in the myometrium during late gestation in the rat was controlled by intrauterine occupancy by the fetoplacental unit raised the possibility of a role for PTHrP in regulating uterine muscle tone (Thiede *et al.,* 1990).

In studies in rats with or without estrogen treatment, protein and mRNA for PTHrP were localized not only in the myometrium, as had been shown in pregnancy (Thiede *et al.,* 1990), but also in the epithelial cells lining the endometrium and endometrial glands. Indeed, the strongest PTHrP production appeared to be in these sites (Paspaliaris *et al.,* 1992), suggesting that the endometrium and endometrial glands might be the major uterine site of PTHrP production and that PTHrP might be a local regulator of endometrial function and myometrial contractility. Estrogen treatment enhanced uterine production of PTHrP, but most significantly, the relaxing effect of PTHrP on uterine contractility *in vitro* was enhanced greatly by the pretreatment of noncycling rats with estrogen. In keeping with this observation, uterine horns from cycling rats in proestrous and estrous phases of the cycle showed a greater responsiveness to PTHrP than those from noncycling rats. These findings are consistent with a role for PTHrP as an autocrine and/or paracrine regulator of uterine motility and function. Furthermore they suggest that PTHrP belongs to a class of other locally acting peptides such as oxytocin, vasoactive intestinal peptide, and relaxin, for which pretreatment of animals with estrogen increases the response of the uterus (Ottesen *et al.,* 1985; Mercado-Simmen *et al.,* 1982; Fuchs *et al.,* 1982).

Further evidence for a specific and regulated role of PTHrP in the uterus during gestation comes from the observation of a temporal pattern in the relaxation response to PTHrP by longitudinal uterine muscle during pregnancy in the rat, with maximal responses at times when estrogen levels would be high. In contrast, the circular muscle did not respond at any stage during gestation (Williams *et al.,* 1994). The inability of PTHrP to relax uterine muscle in the last stages of gestation does not support a direct role in the

onset of parturition. It has been hypothesized that PTHrP may be involved in keeping the uterine muscle relaxed to accommodate the fetus during pregnancy, with the demonstration (Thiede *et al.,* 1990) that expression of mRNA was dependent on the presence of the fetus and that levels increased throughout pregnancy and decreased sharply after delivery. It seems likely, therefore, that the observed fall in PTHrP reflects the recontracted state of the uterine muscle, consistent with the observation in the bladder (Yamamoto *et al.,* 1992b), and that the level of expression is functionally related to contractility. The temporal expression of PTHrP in endometrial glands and blood vessels (Williams *et al.,* 1994) also supports roles in other regulated functions that might include uterine growth during pregnancy and the regulation of uterine and placental blood flow (Mandsager *et al.,* 1994).

Placenta and Fetal Membranes

PTHrP mRNA and protein have been detected in rat and human placenta in various cell types (Hellman *et al.,* 1992; Germain *et al.,* 1992; Bowden *et al.,* 1994). In addition, neoplastic cells of placental origin secrete PTHrP, including hydatidiform moles and choriocarcinomas *in vitro* (Deftos *et al.,* 1994). The presence of PTH/PTHrP receptor mRNA has been demonstrated in rat (Urena *et al.,* 1993) and human (Curtis *et al.,* 1998) placenta and infusion of PTHrP(1-34) into isolated human placental lobules stimulates cyclic AMP production (Williams *et al.,* 1991). Three further sets of observations lend support to the hypothesis that PTHrP is involved in placental/uterine interactions and that its most likely role in the placenta and placental membranes is related to the growth and maintenance of the placenta itself during pregnancy. First, PTHrP production by cultured amniotic cells has been shown to be regulated by prolactin, human placental lactogen, transforming growth factor-β (TGFβ), insulin, insulin-like growth factor, and epidermal growth factor (Dvir *et al.,* 1995). Second, PTHrP has been shown to regulate epidermal growth factor receptor expression in cytotrophoblast cultures (Alsat *et al.,* 1993), an event associated with placental development. Third, studies of vascular reactivity in isolated human placental cotyledons preconstricted with a thromboxane A_2 mimetic showed PTHrP to be a very effective vasodilator (Macgill *et al.,* 1997). The narrow concentration range to which the tissue responded, together with the desensitization in response to repeated PTHrP infusions, was consistent with a paracrine and/or autocrine action of PTHrP in human gestational tissues. Adequacy of the fetoplacental circulation is essential for the nutritional demands of the growing fetus, and both humoral and local factors are likely to be important in its control. It is possible that alterations of the expression, localization, and/or action of PTHrP might contribute to the genesis of conditions such as preeclampsia and intrauterine growth retardation in which placental vascular resistance is increased (Gude *et al.,* 1996). Another related and potentially interacting influence is angiotensin II, known to be a powerful enhancer of PTHrP production in the vasculature and in human placental explants (Li *et al.,* 1998). The ability of angiotensin II to stimulate estradiol production in human placental explants through actions upon its AT_1 receptor (Kalenga *et al.,* 1995) provides a further link with PTHrP control.

The most likely source of increased amniotic fluid PTHrP concentrations during pregnancy is the amnion itself, as PTHrP mRNA expression is also highest at term and greater in the amnion than in choriodecidua or placenta (Bowden *et al.,* 1994; Ferguson *et al.,* 1992; Wlodek *et al.,* 1996). In tissue from women with full-term pregnancies and not in labor, the concentration of N-terminal PTHrP has been found to be higher in amnion covering the placenta than in the reflected amnion covering the decidua parietalis (Bowden *et al.,* 1994). Nevertheless, the concentration of N-terminal PTHrP in reflected amnion (the layer apposed to the uterus) was inversely related to the interval between rupture of the membranes and delivery. The observation that PTHrP levels in the amnion decrease after rupture of the fetal membranes has led to the proposal that PTHrP derived from the membranes may inhibit uterine contraction and that labor may occur following loss of this inhibition. Plasma levels of PTHrP increase during pregnancy and at 6 weeks postpartum (Gallacher *et al.,* 1994; Ardawi *et al.,* 1991;) with the likely sources being placenta and breast, respectively. Human fetal membranes have been shown to inhibit contractions of the rat uterus *in vitro* (Collins *et al.,* 1993) so this tissue does appear to produce factors that can modulate uterine activity. Furthermore, primary cultures of human amniotic cells secrete PTHrP into the medium (Germain *et al.,* 1992). Thus, while the physiological function(s) of amnion-derived PTHrP is currently unknown, preliminary evidence suggests that it may play a role in regulation of the onset of labor. It is also possible that it is a source of PTHrP ingested by the fetus, with a growth factor role in lung and/or gut development.

In summary, although many functional studies remain to be completed, potential roles for PTHrP produced by fetal membranes and placenta include transport of calcium across the placenta, accommodation of stretch of membranes, growth and differentiation of fetal and/or maternal tissues, vasoregulation, and the regulation of labor.

Implantation and Early Pregnancy

Some physiological functions other than control of myometrial activity were suggested by findings of Beck *et al.* (1993), who identified PTHrP mRNA as being limited to epithelial cells of implantation sites. This pregnancy-related expression appeared at day 5.5 in the rat fetus in the antimesometrial uterine epithelium of implantation sites, raising the possibility of a further function of PTHrP, playing a part in the localization of implantation or initial decidualization. Decidual cells produced mRNA for PTHrP both in normal gestation and after the induction of deciduomata. Expression of the gene in these cells followed epithelial expression by 48 hr. It was concluded from this work that

the location of PTHrP gene expression in the uterus, together with the time of its expression, suggests that it plays a part in implantation of the blastocyst. Further evidence for a function of PTHrP in the implantation process came from Nowak *et al.* (1999), who showed that PTHrP and TGFβ were potent stimulators of trophoblast outgrowth by mouse blastocysts cultured *in vitro*. The TGFβ effect appeared to be mediated by PTHrP, which itself was acting through a mechanism distinct from the PTHR1.

Thus, both the timing and the localization of PTHrP gene expression suggested that it might play a part in the implantation of the blastocyst (Beck *et al.,* 1993). Upon finding substantial levels of immunoreactive PTHrP in uterine luminal fluid of estrogen-treated immature rats, and because the PTH/PTHrP receptor was known to be expressed in rat uterus (Urena *et al.,* 1993), Williams *et al.* (1998) investigated the role of PTHrP acting through this receptor in influencing early pregnancy in the rat. Infusion of either a PTHrP antagonist peptide or a monoclonal anti-PTHrP antibody into the uterine lumen during pregnancy resulted in excessive decidualization. The latter appeared to be the result of a decrease in the number of apoptotic decidual cells in the antagonist-infused horn. In pseudopregnant rats, infusion of receptor antagonist into the uterine lumen resulted in increases in wet weight of the infused horn compared with the control side, indicating an effect on decidуoma formation.

These observations suggest that activation of the PTH/PTHrP receptor by locally produced PTHrP might be crucial for normal decidualization during pregnancy in rats, probably not by being involved in the initiation of the decidual reaction, but rather in the maintenance of the decidual cell mass.

Summary

The multiple roles of PTHrP in the reproductive tissues and cycle and in the placenta largely reflect its roles as a paracrine/autocrine/intracrine regulator. Of the many functions it exerts in these systems, probably the only endocrine one is that in which PTHrP in the fetal circulation regulates placental calcium transport. There remains much to be learned of the place of PTHrP in reproductive and placental physiology and pathology.

Endocrine Pancreas

PTHrP and Its Receptors

The presence of PTHrP in the pancreatic islet became apparent shortly following the identification of PTHrP in 1987. Asa *et al.* (1990) demonstrated that PTHrP was present in islet cells and demonstrated that it was present in all four cell types of the islet, including the α, β, δ and PP cells. PTHrP mRNA was demonstrated to be present in isolated islet RNA as well (Drucker *et al.,* 1989), demonstrating that the peptide could be produced within the islet. Gaich and collaborators (1993) confirmed these findings, demonstrating that PTHrP was indeed present in islet cells of all four types and that it was also present in pancreatic ductular epithelial cells. The peptide is not present in adult pancreatic exocrine cells. Plawner and colleagues (1995) demonstrated that PTHrP is present in individual β cells in culture and showed that PTHrP colocalized with insulin in the Golgi apparatus, as well as in insulin secretory granules. Interestingly, in a perifusion system employing a β cell line, PTHrP was shown to be cosecreted with insulin from β cells following depolarization of the cell (Plawner *et al.,* 1995). The secreted forms of PTHrP included amino-terminal, midregion, and carboxy-terminal forms of PTHrP (see later).

With regard to receptors for PTHrP on β cells, little direct evidence has been provided for the presence of the PTHR1, although its presence has not been sought rigorously. However, there can be no question as to the presence of some type of PTHrP receptor on the pancreatic β cell, as it is clear that PTHrP(1-36) elicits prompt and vigorous responses in intracellular calcium in cultured β cell lines. For example, Gaich *et al.* (1993) have demonstrated that PTHrP(1-36) in doses as low as 10^{-12} *M* stimulates calcium release from intracellular stores. Interestingly, unlike events observed in bone and renal cell types where PTHrP receptor activation is associated with activation of cAMP/PKA, as well as the PKC/intracellular calcium pathways, only the latter is observed in cultured β cells in response to PTH or PTHrP(1-36) (Gaich *et al.,* 1993). Whether this reflects the presence of a different type of receptor on β cells or differential coupling of the PTHR1 to subsets of specific G proteins or catalytic subunits in β cells as compared to bone and renal cells has not been studied.

Regulation of PTHrP and PTHrP Receptors

There is little information describing how or to what degree PTHrP or the PTH receptor family is regulated in the pancreatic islet. As will become clear from the sections that follow, there are physiologic reasons why such regulation might occur under normal circumstances, but this area remains unexplored.

Biochemistry of PTHrP

PTHrP undergoes extensive posttranslational processing as described in Chapters 3 and 4. Most of what is known or inferred regarding PTHrP processing is derived from studies in the rat insulinoma line, RIN-1038 (Soifer *et al.,* 1992; Yang *et al.,* 1994; Wu *et al.,* 1991). These cells have served as a model of PTHrP processing, as they have been shown to serve as a model for authentic processing of other human neuroendocrine peptides, such as insulin, proopiomelanocortin, glucagon, and calcitonin. Using a combination of untransfected RIN-1038 cells, RIN-1038 cells overexpressing hPTHrP(1-139), hPTHrP(1-141), or hPTHrP(1-173), and

a panel of region-specific radioimmunoassays and immunoradiometric assays, RIN cells have been shown to secrete PTHrP(1-36), PTHrP(38-94), PTHrP(38-95), and PTHrP(38-101) (Soifer *et al.,* 1992; Yang *et al.,* 1994; Wu *et al.,* 1991). In addition, RIN 1038 cells have been shown to secrete a form of PTHrP that is recognized by a PTHrP(109-138) radioimmunoassay (Yang *et al.,* 1994), and another form that is recognized by a PTHrP(139-173) radioimmunoassay (Burtis *et al.,* 1992).

As described earlier, PTHrP(1-36) stimulates intracellular calcium increments in cultured β cells (Gaich *et al.,* 1993). PTHrP (38-94) has also been shown to stimulate intracellular calcium release in these cells (Wu *et al.,* 1991). PTHrP (38-94) does not activate adenylyl cyclase in cultured β cells, and other PTHrP species have not been explored in β cells in functional terms.

Function of PTHrP

Pancreas development in rodents begins at approximately day E9-10, and by day E18-19, clusters of β cells have begun to coalesce and form immature islets (Edlund, 1998). These islet cell clusters continue to increase in number, in size, and in density of β cells in the week following delivery and then decline abruptly in number through a wave of β-cell apoptosis (Finegood *et al.,* 1995).

The role of PTHrP in β-cell development and function is poorly understood at present. The pancreas of PTHrP-null mice (Karaplis *et al.,* 1994) develops normally in anatomic terms (R. C. Vasavada and A. F. Stewart, unpublished observations), but nothing is known about the function of these islets. PTHrP-null mice die immediately after delivery, so nothing is known of islet function or development following birth in the absence of PTHrP. "Rescued" PTHrP mice do exist (Wysolmerski *et al.,* 1998) and they survive to adulthood. These mice have normal appearing pancreata and islets (R. C. Vasavada and A. F. Stewart, unpublished observations), but they have dental abnormalities, are undernourished, and grow poorly. Therefore, it is difficult to characterize their islets in functional terms, as islet mass, proliferation, and function are heavily dependent on fuel availability. Streuker and Drucker (1991) have suggested that PTHrP may play a role in β-cell differentiation, as it is upregulated in β-cell lines in the presence of the islet-differentiating agent, butyrate.

In an effort to understand the role of PTHrP in the pancreatic islet, Vasavada and collaborators have developed transgenic mice that overexpress PTHrP under the control of the rat insulin-II promoter (RIP) (Vasavada *et al.,* 1996; Porter *et al.,* 1998). RIP-PTHrP mice display striking degrees of islet hyperplasia and an increase in islet number, as well as the size of individual islets. This increased islet mass is associated with increased function: RIP-PTHrP mice are hyperinsulinemic and hypoglycemic as compared to their littermates (Vasavada *et al.,* 1996; Porter *et al.,* 1998). They become profoundly and symptomatically hypoglycemic with fasting. Interestingly, RIP-PTHrP mice are also resistant to the diabetogenic effects of β-cell toxin, streptozotocin. Following the administration of streptozotocin, normal mice readily develop diabetes, but RIP-PTHrP mice either fail to become diabetic or develop only mild hyperglycemia (Porter *et al.,* 1998).

The mechanism(s) responsible for the increase in islet mass in the RIP-PTHrP mouse remains undefined. There are two levels at which this question can be addressed: identification of the source of the cells responsible for the increase in islet mass and the signaling mechanisms that are responsible for the increase. With respect to the first, islet mass can, in theory, be increased by three pathways: (a) the recruitment of new islets from the pancreatic duct or its branches distributed throughout the exocrine pancreas, in a process referred to as "islet neogenesis"; (b) induction of proliferation of existing β cells within islets; and/or (c) prolongation of the life span of existing β cells. Of these options, there is clear evidence against the second possibility (Vasavada *et al.,* 1996), suggesting that islet neogenesis [e.g., PTHrP is present in the normal pancreatic duct and is upregulated during ductular differentiation into β cells (Gaich *et al.,* 1993; Mashima *et al.,* 1999)] or inhibition of islet cell death (as occurs in the presence of PTHrP in other cell types) the likely explanation. These processes are under active study.

At the signaling level, little is known regarding the mechanism of action of PTHrP on β cells. While it is known that PTHrP can stimulate intracellular calcium in cultured β-cell lines (Gaich *et al.,* 1993; Wu *et al.,* 1991), it is not known whether this occurs *in vivo* in normal, nontransformed β cells within intact islets. Nor is it known if PTHrP stimulates adenylyl cyclase in normal β cells *in vivo* or if it participates in nuclear or intracrine signaling in β cells as it appears to in chondrocytes, osteoblasts, vascular smooth muscle cells, or other cell types (Aarts *et al.,* 1999; Massfelder *et al.,* 1997; Lam *et al.,* 1999) (see Chapter 6). These processes, too, are under study.

Finally, and importantly, the results of overexpression studies do not demonstrate that PTHrP plays β cell mass-enhancing roles *in vivo* under normal circumstances. In the absence of meaningful data from knockout or rescued knockout mice, it is difficult to be sure if PTHrP is important in normal islet biology. This question, too, will need to await further studies such as the conditional or islet-specific deletion of the PTHrP gene.

Pathophysiology of PTHrP

From the discussion given earlier, it is clear that the normal physiologic role of PTHrP in the pancreatic islet remains undefined. In contrast, PTHrP plays clear pathophysiologic roles in at least some pancreatic islet neoplasms. PTHrP overexpression with resultant development of humoral hypercalcemia of malignancy has been demonstrated on multiple occasions in multiple investigators' hands (Asa *et al.,* 1990; Stewart *et al.,* 1986; Wu *et al.,* 1997; Skrabanek *et al.,* 1980). In the only large series of malignancy-associated

hypercalcemia in which tumors have been fully subdivided based on histology (Skrabanek *et al.,* 1980), islet cell carcinomas, which are not particularly common, produce humoral hypercalcemia of malignancy fully as often as pancreatic adenocarcinomas, a very common neoplasm. Historically, islet tumors were among the first in which PTHrP bioactivity was identified (Stewart *et al.,* 1986; Wu *et al.,* 1997). Furthermore, patients with islet carcinomas regularly demonstrate increases in circulating PTHrP as determined by radioimmunoassay or immunoradiometric assays (Lansk *et al.,* 1996). When assessed by immunohistochemistry, these tumors also demonstrate increased staining for PTHrP (Asa *et al.,* 1990; Drucker *et al.,* 1989).

The significance of these findings for islet tumor oncogenesis is not known. Is this simply a random derepression of the PTHrP gene or is it a specific upregulation of the PTHrP gene? Is there a pathologic role for PTHrP in the development of pancreatic islet tumors, corresponding to the mass enhancing effects of PTHrP in the islets of the RIP-PTHrP mouse? These questions remain interesting but unanswered at present.

Conclusion

Advances in mouse genetics and in transgenic technology have been a boon to the study of physiology. This has certainly been the case for the PTHrP field, where studies in genetically altered mice have provided a starting place for the study of the physiology of a protein that was discovered out of its natural context. This chapter outlined the current state of knowledge regarding the physiological roles of PTHrP in skin, the mammary gland, placenta, uterus, and pancreas. Much of this information (although not all) has come from studies performed in a variety of transgenic mice. These studies have shown that PTHrP is important to both the development and the physiologic functioning of these organs. However, at this point, we have only the rudiments of an understanding of the functions of PTHrP at these sites. Thus, we continue to have more questions than answers. There will be many challenges to be overcome before we truly comprehend all the nuances of the functions of PTHrP at these sites. There will also be new tools with which to investigate these questions (as this chapter is being written, several eagerly anticipated experiments ablating the PTHrP and PTHR1 genes in organ-specific fashion are in the pipeline). The next several years promise to be an exciting time for the investigation of the nonskeletal effects of this remarkable molecule.

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CHAPTER 30

Vascular, Cardiovascular, and Neurological Actions of Parathyroid-Related Protein

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Vascular and Cardiovascular Actions

Historical Perspectives

The origins of parathyoid hormone (PTH) as a putative cardiovascular regulatory factor date to the early 1900s when the calcemic properties of the hormone were first identified. In classic studies, Collip and Clark (1925) demonstrated that systemic injection of extracts of parathyroid glands lowered systemic blood pressure into dogs (Fig. 1). The first formal characterization of the cardiovascular activity of PTH was conducted by Charbon (1968a,b, 1969,) in the early 1960s. These investigators quantified the vasodilatory effects of a purified parathyroid extract in the rabbit and cat and also showed that a synthetic N-terminal fragment displayed similar actions in the dog. Relaxant activity was not blocked by pharmacological antagonists of other known vasoactive agents, suggesting a direct action of the hormone. Since then, numerous studies have unequivocally established the hypotensive/vasodilatory and cardiac effects of PTH (Mok *et al.,* 1989) that can be summarized broadly as follows: First, the hypotensive and vasorelaxant actions of PTH occur in the absence of a change in blood calcium and are mediated by PTH activation of the type 1 PTH/PTH-related protein (rP) (PTH1R) receptor expressed in the smooth muscle layer of the vessel wall. Second, although all vascular beds are relaxed by PTH, resistance vessels appear to be more responsive than conduit vessels. Third, PTH can reduce the pressor effects of other vasoactive agents that exert their action through different mechanisms. Finally, PTH exerts both ionotropic and chronotropic effects on the heart (Ogino *et al.,* 1995).

Although the cardiovascular effects of PTH are undisputed, their physiological significance has been frequently debated. This is in part because the concentrations of PTH required to produce vasodilation (10 to 100 n*M*) are substantially above those that normally circulate (low p*M*). Consequently, it has been difficult to conceptualize how physiological levels of this systemic hormone, which is synthesized only in the parathyroid gland, could function in the local control of vascular tone. Also enigmatic is the fact that patients with primary hyperparathyroidism and elevated circulating PTH levels often have high (not low) blood pressure that sometimes returns to normal after parathyroidectomy. A plausible explanation for the seemingly enigmatic regulatory effects of PTH on the cardiovascular system emerged with the discovery of PTHrP in 1987.

As discussed in Chapter 29 of this volume, PTHrP was identified as the factor responsible for the paraneoplastic syndrome termed humoral hypercalcemia of malignancy. Almost immediately after its cloning, expression of PTHrP was detected in many normal fetal and adult tissues but was

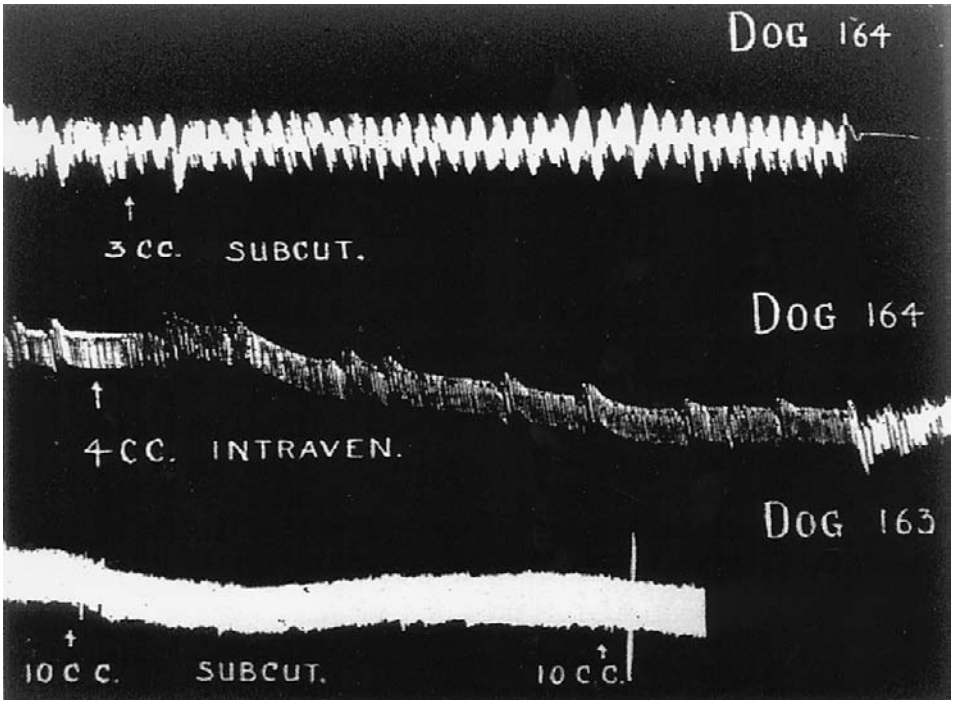

Figure 1 Effects of extracts of parathyroid glands on blood pressure in thyroparathyroidectomized dogs. From Collip and Clark (1925), with permission.

undetectable in the circulation, suggesting that the protein functioned in an autocrine/paracrine mode. Although many functions have been ascribed for PTHrP, three main physiological themes have emerged. Observations in gene knockout mice have demonstrated that PTHrP is required for the development of cartilage, morphogenesis of the mammary gland, and tooth eruption (reviewed in Philbrick *et al.,* 1996, and elsewhere in this volume). PTHrP also appears to participate in maternofetal calcium transfer across the placenta. The third physiological role for PTHrP is in smooth muscle in which the protein functions to regulate contractility and proliferation. Although this chapter focuses on the physiology of PTHrP in *vascular* smooth muscle, it is relevant to begin with a brief review of its physiological effects in other smooth muscle-containing tissues.

In all smooth muscle cell beds studied to date, induction of PTHrP expression occurs in close association with normal physiological stimuli. In the smooth muscle layer of the chicken oviduct, induction of PTHrP expression coincides temporally with egg movement and its arrival in the shell gland (Thiede *et al.,* 1991). In the rat uterus, PTHrP expression is localized to the myometrium and is markedly upregulated by fetal occupancy (Thiede *et al.,* 1990) or by mechanical distention of the uterine horn using a balloon catheter (Daifotis *et al.,* 1992). PTHrP expression is increased in prelabor human amnion and abruptly falls with the onset of labor and rupture of the amniotic sac (Ferguson *et al.,* 1992). In the urinary bladder, induction of PTHrP mRNA occurs during filling in proportion to bladder distension (Yamamoto *et al.,* 1992). Finally, as discussed in detail later, PTHrP is also expressed in vascular smooth muscle, in which it is induced by vasoconstrictor agents and mechanical stimuli. In each of these smooth muscle beds, application of PTHrP to precontracted smooth muscle preparations induces relaxant activity, precisely mimicking the actions described previously for PTH. It would therefore appear that PTHrP rather than PTH represents the physiologically important regulator of smooth muscle tone. Consequently, the remainder of this chapter focuses primarily on the physiology of PTHrP in the cardiovascular system.

PTHrP in the Vasculature

Vascular Anatomy and Contractile Mechanisms

Blood vessels are composed of three principal cell types: the intima, which consists of a single epithelial cell layer; the muscularis layer, made up of vascular smooth muscle cells embedded in a connective tissue matrix; and an outer adventitial layer, which receives input from the cholinergic and adrenergic nervous system. The relative composition and contribution of each of these cell types to vascular growth and tone varies during development and among different vascular beds. For example, during development, blood vessels form initially as simple tubular structures consisting entirely of endothelial cells into which smooth muscle cells migrate to form the vascular wall. In the mature mammal, the large conduit vessels (e.g., aorta) are highly elastic to accommodate high capacity blood flow, whereas resistance vascular beds (e.g., mesentery) typically contain more smooth muscle cells and are densely innervated. Changes in the cellular and connective tissue constituents within the vasculature occur with normal aging

and in particular during pathological conditions such as atherosclerosis. The regulation of vascular growth, remodeling, and smooth muscle cell tone is achieved through a coordinated network of both systemic and local factors, as well as input from adrenergic, cholinergic, peptidegic, and sensory neurons.

Mechanisms regulating vascular smooth muscle cell contractility have been studied in detail (reviewed in Somlyo *et al.,* 1999). The intracellular-free calcium concentration is the major determinant of vascular tone. Depolarization of vascular smooth muscle cells opens L-type voltage-sensitive calcium channels (L-VSCCs), enabling calcium to enter the cell. These events trigger the release of much larger quantities of calcium from the sarcoplasmic reticulum. Alternatively, pharmacologic or ligand activation of G protein receptors (e.g., angiotensin II) activate phospholipase (PLC), which catalyzes phosphoinositol hydrolysis and causes calcium release from intracellular stores. The increases in cytoplasmic calcium achieved by either of these mechanisms activate myosin light chain kinase through the calcium–calmodulin complex and phosphorylation of the 20-kDa regulatory light chain of myosin, with subsequent cross-bridge cycling and force development. The mechanisms of vascular smooth muscle cell relaxation are less well understood. In the most simple scheme, a reduction of cytoplasmic calcium with a fall in myosin light chain kinase activity would suffice to account for dephosphorylation of the regulatory light chain and relaxation. However, other mechanisms have been implicated in cyclic nucleotide-dependent relaxation in vascular and other smooth muscle tissues (McDaniel *et al.,* 1994).

The demonstration of a calcium-sensing receptor in vascular smooth muscle with pharmacological properties similar to those of the parathyroid calcium-sensing receptor (discussed in Chapter 23) has prompted speculation that it might also participate in the regulation of contractile events (Bukoski *et al.,* 1995). Alterations of extracellular calcium over the physiological concentration range depress contractility of precontracted vascular smooth muscle. This effect of extracellular calcium has been shown to be mediated by activation of a calcium-dependent potassium channel and is associated with alterations in myofilament calcium sensitivity. These activities were mimicked by gadolinium, neomycin, and lanthanum, all factors that activate the calcium-sensing receptor. However, the structure of this putative calcium-sensing receptor is unknown and it remains unclear whether it bears homology to the renal or parathyroid or kidney calcium-sensing receptor.

Expression and Regulation of PTHrP

PTHrP is expressed in blood vessels in essentially all vascular beds from a broad range of species, including rodent and human fetal blood vessels (Moniz *et al.,* 1990), adult rat aorta (Burton *et al.,* 1994; Pirola *et al.,* 1994), vena cava (Burton *et al.,* 1994), kidney afferent arterioles, artery and microvasculature (Nickols *et al.,* 1990), the arterial and venous supply of the mammary gland (Thiede, 1994), the serosal arterioles in avian egg shell gland (Thiede *et al.,* 1991), and blood vessels of the rat penis (Lang *et al.,* 1999). The protein appears to be expressed predominantly in the smooth muscle layer of the vessel, although its expression has also been reported in cultured endothelial cells (Rian *et al.,* 1994; Ishikawa *et al.,* 1994a). The regulation of PTHrP mRNA expression has been studied in detail using cultured vascular smooth muscle cells. In primary rat aortic vascular smooth muscle cells, expression of PTHrP is induced rapidly (2–4 hr) but transiently by exposure of quiescent cells to serum (Hongo *et al.,* 1991) (Fig. 2). This mode of tight regulation is reminiscent of the behavior of cytokine mRNAs and would appear to constitute a mechanism that would restrict the activity of PTHrP to a narrow window of time. Among the most potent inducers of PTHrP are vasoconstrictors, including angiotensin II, seritonin, endothelin, norepinephrine, bradykinin, and thrombin, each of which induces PTHrP mRNA and protein levels over the same time course as that observed for serum (Pirola *et al.,* 1993). The induction of PTHrP mRNA by angiotensisn II is dependent on protein kinase C activation and is mediated by both transcriptional and posttranscriptional mechanisms (Pirola *et al.,* 1993). Prior addition of saralaysin and captopril, which inhibit angiotensin II action or generation, respectively, inhibit the serum-induced increase in PTHrP in vascular smooth muscle cells. This finding suggests that the angiotensin II present in serum represents a significant component of the serum induction of PTHrP.

PTHrP is also induced in vascular smooth muscle in response to mechanical stimuli. PTHrP mRNA is increased transiently in rat aorta following distension with a balloon catheter (Pirola *et al.,* 1994). Flow motion-induced

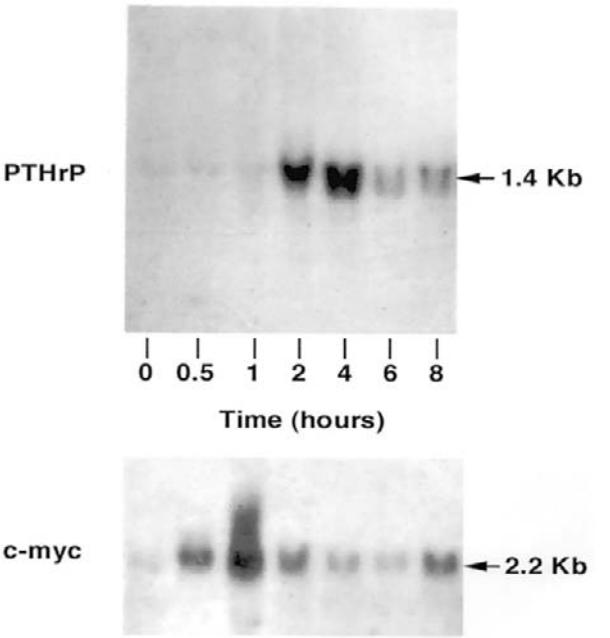

Figure 2 Time course of serum induction of PTHrP mRNA in aortic vascular smooth muscle cells. From Hongo *et al.,* (1991), with permission.

mechanical events induced by rocking or rotation of monolayer cultures of rat aortic vascular smooth muscle cells result in increased PTHrP mRNA expression (Pírola *et al.,* 1994; Noda *et al.,* 1994). The inductive effects of mechanical stretch and angiotensin II on PTHrP mRNA appear to be synergistic, suggesting that they occur through distinct mechanisms (Noda *et al.,* 1994). PTHrP mRNA is also produced in capillaries of slow-twitch soleus and fast-twitch skeletal muscle, and its expression is increased in response to low-frequency stimulation (Schnoider *et al.,* 1999). This maneuver was associated with enhanced capillarization of the muscle, indicating that PTHrP might function to promote to new capillary growth in response to increased contractile activity.

VASCULAR ACTIONS OF PTHRP

Shortly after the identification of PTHrP, a number of studies demonstrated that synthetic N-terminal fragments of the peptide replicated many of the vascular actions of PTH, including its vasrelaxant actions in aorta (Crass and Scarpace, 1993), portal vein (Shan *et al.,* 1994), coronary artery (Nickols *et al.,* 1989), renal artery (Wingulst *et al.,* 1987; Musso *et al.,* 1989), placenta (Macgill *et al.,* 1997; Mandsager *et al.,* 1994), and mammary gland (Prosser *et al.,* 1994). In general, the vasodilatory potency of PTHrP is comparable to that of PTH when examined in organ bath systems. In contrast, in mouse portal vein preparations, PTHrP(1-34) was shown to be a more potent vasorelaxant than PTH(1-34) (Shan *et al.,* 1994). In perfused rabbit kidney (Musso *et al.,* 1989) and in rat aorta (Nickols *et al.,* 1989) the vasorelaxant effects of PTHrP do not appear to require the presence of an intact endothelium. However, in mouse aortic rings, endothelium denudatation attenuates the relaxant activity of PTHrP markedly (Sutliff *et al.,* 1999), possibly reflecting a species difference.

In addition to its effects on vascular tone, PTHrP also modulates vascular smooth muscle cell proliferation. The peptide decreases serum and platelet-derived growth factor (PDGF)-activated DNA synthesis in primary arterial vascular smooth muscle cells (Hongo *et al.,* 1991; Jiang *et al.,* 1995) and in A10 vascular smooth muscle cells stably expressing the PTH1R receptor (Maeda *et al.,* 1996). In both of these cell types, antimitogenic effects require the PTH-like N-terminal portion of the molecule and are mimicked by dibutyryl cAMP or forskolin. The mechanism for the antiproliferative effect of PTHrP involves the induction of the cyclin-dependent kinase inhibitor, $p27^{kip1}$, and impairment of phosphorylation of the retinoblastoma gene product (Rb), which results in cell cycle arrest in mid-G_1 phase (Maeda *et al.,* 1997). However, Massfelder *et al.,* (1997) reported that overexpression of PTHrP in A10 cell vascular smooth muscle cells was associated with an increase in DNA synthesis coincident with an increased nuclear localization of the protein. However, in these studies, exogenous application of PTHrP inhibited A10 cell growth in agreement with the studies cited earlier. A putative nuclear targeting motif was found to be required for the nuclear import of PTHrP in vascular smooth muscle cells in accordance with previous studies in chondrocytes (Henderson *et al.,* 1995). Therefore, the ability of PTHrP to influence the proliferation of vascular smooth muscle cells either positively or negatively appears to depend on where the protein is trafficked in the cell. Cellular levels of PTHrP fluctuate during the cell cycle and reach their highest levels in G_2/M (Okano *et al.,* 1995). It is possible that the protein is directed to the nucleus in the later stages of the cell cycle to participate in mitotic events.

PTHrP also inhibits PDGF-directed migration of vascular smooth muscle cells *in vitro* (Ishikawa *et al.,* 1998). The antimigratory effects of PTHrP are mediated through a cAMP-dependent mechanism that leads to diminished PDGF signaling through the PI3 kinase cascade.

The effects on vascular smooth muscle cell growth and migration *in vitro* are likely to be physiologically relevant to conditions under which VSMC growth and migratory behavior is altered *in vivo*. For example, Ozeki *et al.* (1996) have reported that PTHrP protein and mRNA expression were upregulated markedly in neointimal smooth muscle in rat carotid arteries following experimental balloon injury. Moreover, immunoreactive PTHrP is increased in human arterial tissue removed from patients undergoing angioplasty. In light of the possibility of opposing effects of PTHrP on vascular smooth muscle cell growth cited earlier, these observations can be viewed in one of two ways: either upregulation of PTHrP is a primary stimulus for growth under these conditions or, alternatively, it represents an antiproliferative signal. Consistent with the latter possibility, the local administration of 3′,5′-cyclic AMP or the phosphodiesterase inhibitors aminophylline or amrinone inhibit neointimal formation following experimental balloon injury in rat carotid arteries *in vivo* (Indolfi *et al.,* 1997). Moreover, other studies using a similar model of arterial injury showed high levels of $p27^{kip1}$ expression in media within 2 weeks after angioplasty (Tanner *et al.,* 1998).

The ability of PTHrP to modulate vascular smooth muscle cell growth suggests that the protein might function during the development of the cardiovascular system. Although the cardiovascular system appears to develop normally in the PTHrP knockout mouse, homologous deletion of the PTH1R receptor results in a higher incidence of early fetal death at approximately embryonic day 9–10, coincident with development of the heart and major blood vessels (Lanske *et al.,* 1996). Furthermore, transgenic mice expressing high levels of PTHrP and its receptor in vascular smooth muscle, created by crossing the ligand and receptor overexpressing mice, die at day E9.5 with severe thinning of the ventricle and disruption of ventricular trabeculae (Qian *et al.,* 1999) (Fig. 3, see also color plate). Additional anecdotal evidence for a role of PTHrP in heart and vascular development is evident from the abnormalities seen in patients with the rare fatal condition known as Blomstrand chondrodysplasia caused by an inactivating mutation of PTH1R receptor (Karaplis *et al.,* 1998). These patients die prenataly with coartation of the aorta and hydrops fetalis, the latter condition typically caused by high output heart failure.

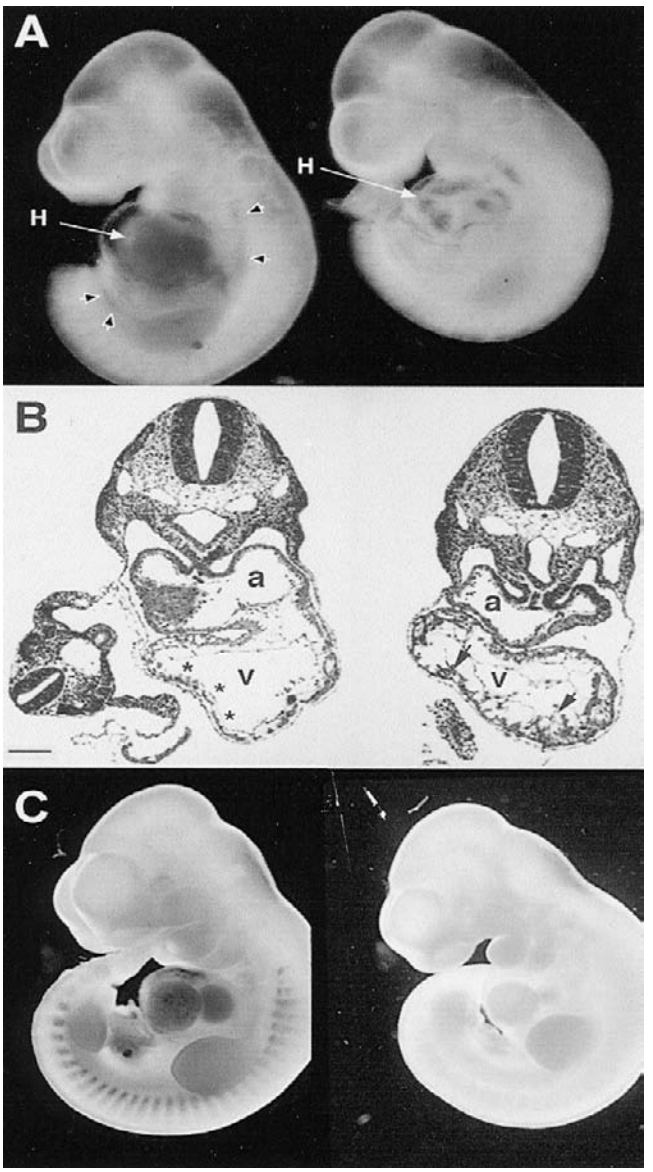

Figure 3 Overexpression of PTHrP and PTH1R disrupts heart development. (A) Whole mounts at E9.5 of double transgenic (left) and wild-type (right) embryos. The double transgenic embryo exhibits a greatly enlarged heart with pericardial effusion and vascular pooling (arrows). (B) Histologic sections of double transgenic (left) and wild-type (right) embryos at E9.5. Trabeculae within the ventricular cavity (v) of the wild-type embryo are prominent (large arrows), whereas in the double transgenic embryo, trabeculae are reduced severely or absent (asterisks). Prominent gaps are also evident between the cardiomyocytes in the double transgenic hearts (small arrowheads). a, atria. Bar: 100 μm, (C, left). Localization of SMP8 lacZ transgene in a 9.5-day-old embryo. Staining is apparent in heart, hind gut, and somites. (Right) An unstained control. From Qian *et al.,* (1999), with permision. (See also color plate).

MODE OF ACTION/RECEPTOR INTERACTIONS

PTHrP exerts its vasodilatory actions by activating the PTH1R. This receptor is expressed in rat vascular smooth muscle beds (Urena *et al.,* 1993), and relaxation of aortic preparations is accompanied by an increased accumulation of cAMP (Ishikawa *et al.,* 1994b). Cultured rat aortic smooth muscle cells also express the PTH1R and respond to N-terminal PTHrP peptide fragments with an increase in cAMP formation (Wu *et al.,* 1993). Moreover, relaxation responses to PTH in aortic strip preparations are potentiated by phosphodiesterase inhibitors and forskolin (Nickols and Cline, 1987). Although the PTH1R receptor appears to be primarily coupled to adenylate cyclase, linkage to calcium–phosphoinositol pathways is suggested by studies by Nyby *et al.* (1995), who demonstrated a transient increase in cytosolic calcium and cAMP in response to PTHrP(1-34) in primary arterial rat vascular smooth muscle cells. However, other studies using similar preparations of primary rat aortic smooth muscle cells showed that PTHrP consistently stimulated cAMP accumulation but had no effect on intracellular calcium (Wu *et al.,* 1993). Furthermore, in A10 embryonic aortic vascular smooth muscle cells stably expressing recombinant PTH1R receptors, PTHrP induced large increases in cAMP accumulation but did not increase cytoplasmic calcium (Maeda *et al.,* 1996) despite the presence of detectable levels of expression of G_q, known to be required for functional coupling of the receptor to the PLC–phosphoinositide calcium pathway. However, when G_q was overexpressed in these cells, PTHrP evoked a calcium transient. It therefore appears that under most conditions the PTH1R receptor couples preferentially to G_s and adenylate cyclase to raise intracellular cAMP, which would be consistent with the established vasodilatory properties of this cyclic nucleotide. This does not, however, preclude the possibility that under certain physiological conditions (or in specific vascular smooth muscle cell beds), PTHrP might also activate PLC, which could mediate other as yet unidentified activities of the protein.

Vasorelaxation induced by cyclic nucleotides in arterial smooth muscle has also been reported to be associated with a reduction in intracellular calcium. In addition, in rat tail artery, PTH relaxes KCl-induced contraction; this effect is inhibited by nifedipine, suggesting an inhibition of the L-VSCC (Wang *et al.,* 1991a). Subsequent patch-clamp experiments (Wang *et al.,* 1991b) confirmed a decrease in L-type voltage-dependent calcium currents in vascular smooth muscle cells in response to PTH. Although not yet formally tested, it is likely that PTHrP also inhibits the L-VSCC activity in vascular smooth muscle, as is the case in cultured neuroblastoma cells.

As discussed in detail elsewhere in this volume (Chapter 29), PTHrP is subject to posttranslational processing to produce both N-terminal peptides, midregion PTHrP fragments, and possibly also C-terminal forms. PTHrP peptides that lack the PTH-like N-terminal region likely activate receptors distinct from the PTH1R and would be expected to exhibit a biological profile different from N-terminal PTHrP peptides. To date, however, there is no evidence that these midregion or C-terminal forms of PTHrP are biologically active either in cultured vascular smooth muscle cells (Wu *et al.,* 1993) or in intact vessel preparations (Sutliff *et al.,* 1999).

Although PTHrP is capable of relaxing vascular preparations devoid of endothelium, studies in mouse aortic

preparations suggest that the endothelial layer may serve to amplify relaxant effects of PTHrP and PTH (Sutliff *et al.,* 1999). The mechanism accounting for the endothelium-dependent relaxant effects of PTH and PTHrP remains unclear, but does not appear to require nitric oxide formation. The recent demonstration of expression of a novel PTH-2 receptor (PTH2R, see later) in endothelial and smooth muscle cells in blood vessels and heart (Usdin *et al.,* 1996) suggests an additional pathway through which PTH-related peptides could alter vascular reactivity.

As with other G-coupled receptors, prolonged exposure of vessel preparations (Nyby *et al.,* 1995) or cultured aortic smooth muscle cells (Okano *et al.,* 1994) to PTHrP is associated with desensitization. Angiotensin II, which induces PTHrP expression in cultured aortic vascular smooth muscle cells, also rapidly desensitizes cells to PTHrP and downregulates the PTH1R receptor mRNA expression (Okano *et al.,* 1994), indicating cross-talk in the signaling circuitry among these vasoactive peptides.

From the studies just summarized, it is possible to construct a simple model for the mode of PTHrP action in vascular smooth muscle (Fig. 4, see also color plate). In response to mitogenic, vasoconstrictor, or mechanical signals, PTHrP is released and acts locally via a short feedback loop to activate the PTH1R receptor and stimulate adenylate cyclase in adjacent cells. Effector pathways downstream of cAMP impact on specific sets of genes, which function to oppose the pressor (contraction coupling) and mitogenic (cell cycle) events. As mentioned earlier, induction of $p27^{kip1}$ with consequent inhibition of Rb phosphorylation would represent one such target for cAMP-induced cell cycle arrest. With regard to relaxant activity, stimulation of cAMP-dependent protein kinase (PKA) is associated with a reduction in cytoplasmic calcium and attenuated myosin light chain kinase activity (McDaniel *et al.,* 1994).

Because PTH and PTHrP activate the same receptor, how does the smooth muscle PTH1R receptor distinguish between these two ligands? A likely possibility is that the sensitivity of a given tissue to PTH or PTHrP is governed by the relative abundance of each ligand and the number of PTH1R receptors. For example, in tissues such as vascular smooth muscle, which express high levels of PTHrP but relatively low numbers of the PTH1R receptor, the fraction of receptor occupancy must be high in order to achieve a response, thus favoring the local (PTHrP) regulator. In contrast, in bone cells, PTHrP expression is low and the receptor expression is high, enabling preferential receptor activation by PTH arriving from the systemic circulation.

PTHrP in the Heart

PTHrP and the PTH1R receptor are expressed in fetal and adult heart from a number of different species (Burton *et al.,* 1994). PTHrP has been immunolocalized to atrial natriuretic peptide-containing granules of rat atria. One interpretation of this finding is that PTHrP, like atrial natriuretic peptide, is released in response to stretch, but this concept has yet to be tested. Both PTH and PTHrP exert pronounced effects on cardiac function (reviewed in Schluter and Piper, 1998).

Infusion of physiological levels of N-terminal fragments of PTH and PTHrP induce hypotension and tachycardia in intact rats (Mok *et al.,* 1989). In isolated perfused hearts, PTHrP induces chronotropic and ionotropic effects that are independent of perfusion pressure (Nickols *et al.,* 1989).

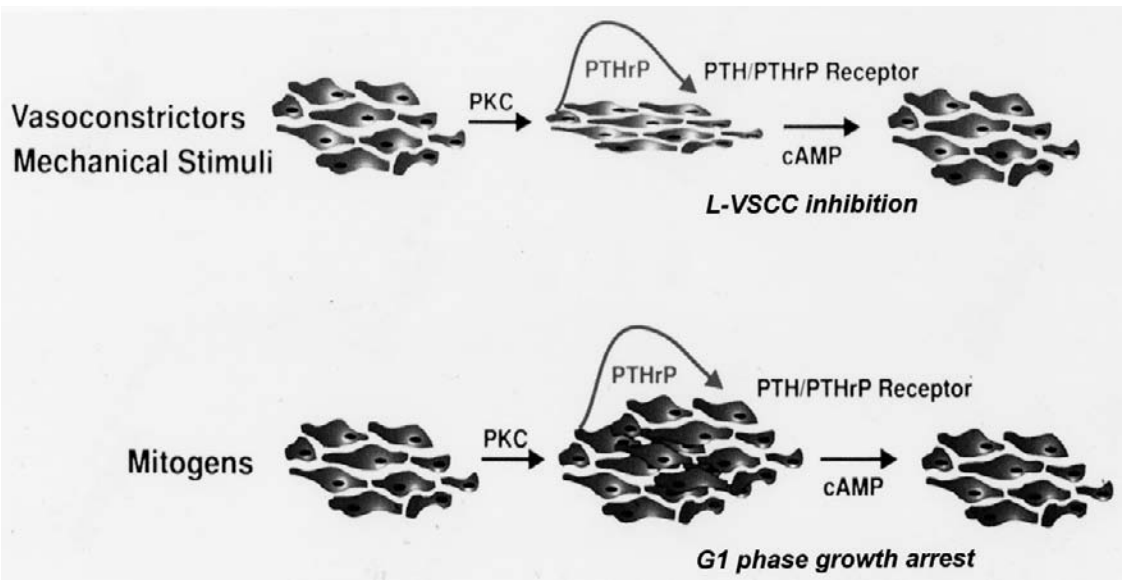

Figure 4 Model for PTHrP production and action in vascular smooth muscle cells (see text for description). (See also color plate.)

More recently it has been established that the inotropic activity of PTHrP occurs indirectly in response to increased coronary blood flow (Ogino *et al.,* 1995). The mechanisms responsible for the chronotropic effects of PTH and PTHrP have been examined in cultured cardiomyocytes (reviewed in Schluter and Piper, 1998). In neonatal cardiomyocytes, PTH increases beating frequency through a cAMP-dependent pathway. These effects are associated with increased L-type calcium currents, precisely the opposite of what is observed in vascular smooth muscle cells. In contrast, in adult rat cardiac myocytes, both PTH(1-34) and PTHrP(1-34) increase the rate of spontaneous contraction, but only PTHrP was found to stimulate cAMP accumulation. The reason for this difference is unclear but may relate to the coupling of the PTH1R receptor to different G proteins. PTH has also been shown to elicit a hypertrophic response in adult rat cardiomyocytes characterized by increased protein synthesis, cell mass, and the reexpression of embryonic cardiac proteins. These effects, together with clinical observations of patients with elevated PTH and increased left ventricular mass, have been interpreted as evidence for a pathogenic role of PTH in ventricular hypertrophy. Finally, as discussed earlier, the timing (E9-10) of embryonic death occurring in PTH/PTHrP receptor-null mice raises the possibility that PTHrP functions during heart development.

Insight into the global actions of PTHrP in the cardiovascular system has come from studies in genetically manipulated mice. Transgenic mice overexpressing either PTHrP or PTH1R in smooth muscle have reduced systemic blood pressure consistent with the prediction that PTHrP acts as a local vasodilator (Qian *et al.,* 1999). In aortic ring preparations from PTHrP-overexpressing mice, the relaxant effects of both PTHrP and acetylcholine seen in nontransgenic mice were attenuated markedly in aortas from PTHrP-overexpressing mice. This finding suggests that local overexpression of PTHrP not only desensitizes the vasculature to PTHrP, but also dampens relaxation to acetylcholine and perhaps other vasorelaxants. Thus it appears that prolonged stimulation of the PTH1R and the consequent increase in cAMP converge on signaling circuitry used by acetylcholine.

PTH-Related Proteins and Hypertensive States

Several lines of circumstantial evidence suggest that PTH and PTHrP alter vascular tone in hypertensive humans and animals. For example, primary hyperparathyroidism is commonly associated with hypertension which may be corrected upon removal of the parathyroid lesion (Young *et al.,* 1988). However, because alterations in circulating PTH also influence other regulators of vascular tone (e.g., ionized calcium), it is probable that the hypertension seen in long-term hyperparathyroidism is a secondary event. Alternatively, prolonged exposure to elevated PTH concentrations in these patients could desensitize vascular tissue to PTH or PTHrP, thereby increasing vascular tone (Nyby *et al.,* 1995). A similar scenario appears to occur in two rat models of hypertension. For example, removal of the parathyroid glands in the spontaneously hypertensive rat (SHR) and the DOCA salt hypertensive rat attenuates the development of hypertension (Schleiffer, 1992). Moreover, the PTH-induced changes in urinary cAMP, magnesium, calcium, and phosphorus responses are blunted in the SH rats, again suggesting a desensitization of the PTH1R receptor. The apparent resistance to PTH and PTHrP in humans and rats with hypertension described earlier prompted Pang and co-workers (1991) to propose the existence of an additional "hypertensive" factor made in the parathyroid gland. However, despite over a decade of work on this putative hypertensive factor, its precise structure is still unknown.

Neurological Actions

Introduction

As noted earlier, interest in potential regulation of excitable cells by PTH/PTHrP began with Collip and Clark's demonstration in 1925 that parathyroid extracts had hypotensive effects in the dog. For the next 60 years, PTH was the focus of work in both vascular and nonvascular smooth muscle and in neurons. In smooth muscle, it now seems quite clear that the physiological regulator is actually PTHrP, acting on the PTH1R. In the central nervous system (CNS), the best functional evidence also involves PTHrP acting on the PTH1R. In addition, evidence shows that PTH may influence pituitary function, and the recently described TIP39 acting on the PTH2R may prove to be an important CNS regulatory system.

There are two aspects of the PTH-smooth/cardiac muscle literature that are relevant to PTHrP function in the CNS. The first is that it is the L-VSCC that seems to be the pivotal target of PTH/PTHrP regulation. The second is that PTH and/or PTHrP appears to be capable of either inhibiting or stimulating L-VSCC-mediated Ca^{2+} influx depending on the cell/tissue in question (Wang *et al.,* 1991a).

PTH/PTHrP Gene Family Expression in the CNS

The CNS was one of the first sites to be examined in detail for PTHrP gene expression, and the gene was found to be widely expressed in neurons of the cerebral cortex, hippocampus, and cerebellum (Weir *et al.,* 1990) (Fig. 5). This work was extended by a second survey, which included the PTH1R as well as PTHrP (Weaver *et al.,* 1995). Both were found to be widely expressed, and they colocalized in a number of sites (Weaver *et al.,* 1995). It was noted at the time that the hot spots for PTHrP gene expression are neuronal populations that have a number of features in common, including high-density L-VSCC expression as well as high-density expression of excitatory amino acid receptors and a known susceptibility to excitotoxicity. The implications of these common features will become clear later in this chapter.

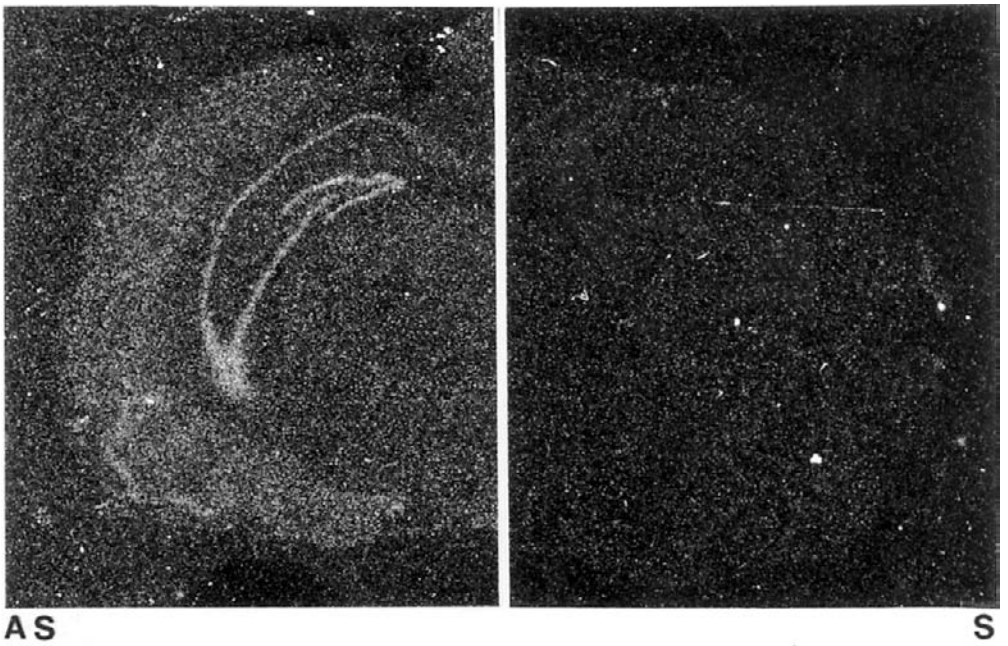

Figure 5 *In situ* hybridization histochemistry of PTHrP mRNA in the CNS of the rat using antisense (AS) and sense (S) oligonucleotides. The dentate gyrus and CA fields of the hippocampus are strongly positive. There are also many PTHrP-positive neurons scattered throughout the cerebral cortex. From Weir *et al.* (1990), with permission.

While early histochemical studies suggested that PTH might be present in a number of neuronal populations, the most careful and reproducible work has localized PTH to nuclei on the hypothalamus with projections into the portal system (Pang *et al.,* 1988; Harvey and Fraser, 1993). The implication is that PTH may regulate pituitary function, specifically including prolactin secretion (Harvey and Fraser, 1993).

Usdin and colleagues identified the PTH2R in a cerebral cortical cDNA library by homology screening in 1995. This receptor is sensitive to PTH(1-34) (EC_{50} about 1 n*M*) but is unresponsive to PTHrP(1-36). The PTH2R is expressed most abundantly in several basal forebrain nuclei and hypothalamic nuclei (Usdin *et al.,* 1996). This group has succeeded in purifying the natural ligand for this receptor using a staggering 50 lbs of bovine hypothalamus as starting material (Usdin *et al.,* 1999). This ligand is a small unmodified peptide of 39 amino acids referred to as tuberoinfundibular peptide 39 (TIP39), and it bears only 9 of 39 amino acids that are identical to those of bovine PTH. Only limited structure–function work has been done, but TIP39 is at least as potent as PTH(1-34) at the PTH2R and may be one or two orders of magnitude more potent than PTH, depending on the species of origin of the PTH2R (Usdin *et al.,* 1999). The sites of PTH2R expression imply potential TIP39 function in regulating the pituitary and in modulating pain sensitivity.

Thus, three ligands and at least two receptors of the PTH/PTHrP gene family are expressed in the CNS. Two of the ligands (PTH and TIP39) are expressed in highly discrete locations, whereas PTHrP is widely expressed in neuronal populations throughout the brain.

Calcium Channels, Neuromodulation, and Signaling Microdomains

CALCIUM CHANNELS

Calcium channels are heteromeric associations of four or five subunits (Walker and De Waard, 1998). The α_1 subunit is the pore-forming structure that is responsible for permeation as well as gating function of the channel. There are a half-dozen classes of calcium channels, each defined by a specific α_1 gene. Given the number of different genes for each subunit and alternate splicing of these gene products, the combinatorial possibilities are enormous (perhaps 1000).

In brief, calcium channels are either L-type or non-L-type (e.g., N, P/Q, T, and R channels) (Walker and De Waard, 1998). L-type channels mediate large and long-lasting Ca^{2+} fluxes (therefore "L") and are composed of three subclasses, defined by their α_1 subunits, as well as by the locations in which they were initially identified. These are S ("skeletal", α_1s), C ("cardiac", α_1c), and D (neuroendocrine, α_1d). L channels are dihydropyridine sensitive, and there are a number of classes of these widely used drugs (e.g., nifedipine, diltiazem).

Virtually every class of calcium channel is expressed in the CNS (Walker and De Waard, 1998). N and P/Q channels are expressed in both pre- and postsynaptic locations and are involved in the regulation of synaptic transmission. L channels are widely expressed in neurons throughout the brain and are found only in postsynaptic locations, specifically on cell bodies and proximal dendrites (Hell *et al.,* 1993). This localization is crucial to L channel function. These channels appear to regulate cytosolic Ca^{2+} levels in the soma and proximal dendrites of neurons as a function of

the integrated excitatory synaptic input into these locations (Hell *et al.,* 1993). Given the location and gating of these channels, it is quite clear that their Ca^{2+} currents are not involved in neurotransmission, but rather with fundamental aspects of neuronal cell biology such as regulation of cellular signaling pathways and regulation of gene expression.

NEUROMODULATION

The clustering of L-VSCCs on neuronal cell bodies is also characteristic of the location of neuropeptide/growth factor receptors. This clustering of receptors is strategically convenient to the nucleus as well as to the regulation of channels of all sorts and the capacity of peptides and growth factors to cross-talk with each other (Hökfelt, 1991). This kind of short-range autocrine/paracrine signaling to the soma and proximal processes of neurons is referred to as "neuromodulation" to emphasize that the regulation and signaling involved are very different from neurotransmission (Hökfelt, 1991).

SIGNALING MICRODOMAINS

Even a generation ago, it was clear that signal transduction corresponded to more than cells simply serving as bags of rising and falling tides of cyclic nucleotides and Ca^{2+}, but the biochemical details that account for the exquisite specificity of signal transduction have become clear only in the past decade. The work of Ghosh and Greenberg (1995) has provided insight into the specificity of neuronal Ca^{2+} signaling. Depending on the specific route of entry into a neuron, Ca^{2+} has highly specific and differential effects on a wide variety of neuronal processes, such as gene expression, learning and memory, modulation of synaptic strength, and Ca^{2+}-mediated cell death (Ghosh and Greenberg, 1995). For example, Ca^{2+} entry via L-VSCCs elicits an entirely different response in terms of gene expression than Ca^{2+} entry mediated via NMDA receptors (Ghosh and Greenberg, 1995). Clearly, every calcium ion entering the cytosol of a neuron is not perceived in the same way. Equally, clearly cAMP generated in a neuron by a voltage-sensitive adenylate cyclase as opposed to a G protein coupled to a hormone receptor is not perceived by the cell in the same way.

A major advance in understanding the specificity of signaling has come from the recognition that microdomains exist at the cell surface that cluster together the receptor/channel in question, the PKA and/or PKC transducers, and the target to be modified. The key recent players that account for this clustering of specific signaling components are the AKAPs (A kinase anchoring proteins) and the RACKs (receptors for activated C kinase) (Mochly-Rosen, 1995). In certain cases, a single AKAP is capable of binding both PKA and PKC, thus serving as a scaffold that brings together all of the early components of a complex regulatory system. The net result of this tethering of signaling receptor, transducer, and target into a microdomain is a tremendous resolution in terms of specificity and speed.

PTHrP Is Neuroprotective

PTHRP GENE EXPRESSION IN NEURONS IS REGULATED BY L-VSCC CA^{2+} INFLUX

It turns out that the regulation of PTHrP gene expression in cerebellar granule cells is a classic example of the kind of specificity of Ca^{2+} signaling described in the previous section. Cerebellar granule cells are a hot spot of PTHrP and PTH1R expression *in vivo* (Weir *et al.,* 1990; Weaver *et al.,* 1995), and cultured cerebellar granule cells are a commonly used neuronal model system *in vitro*. PTHrP gene expression in these cells is a direct function of depolarization, which triggers L-VSCC Ca^{2+} influx that tracks to the PTHrP gene via the calmodulin–CaM kinase cascade (Holt *et al.,* 1996; Ono *et al.,* 1997). Ca^{2+} entry into granule cells by any other granule cells, as in most other cells that express the PTHrP gene, PTHrP is a constitutive secretory product, so

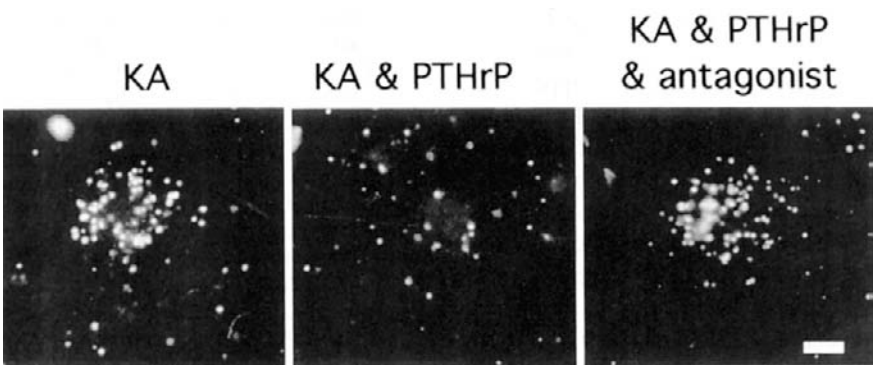

Figure 6 Cell death assessed by propidium iodide staining. Propidium iodide can bind to nuclear DNA only when the cell membrane is not intact; each bright dot therefore represents the nucleus of a dead cell. Kainic acid (KA) alone (left), KA plus PTHrP (center), and KA together with PTHrP and a 10-fold molar excess of a competitive antagonist of PTHrP binding (right). Percentage kill ($\pm$ SEM) under these three conditions was 23 $\pm$ 3% ($n = 10$), 2 $\pm$ 2% ($n = 11$, $P < 0.001$ with respect to KA alone), and 23 $\pm$ 2% ($n = 10$), respectively. Scale bar: 25 μM. From Brines *et al.* (1999), with permission.

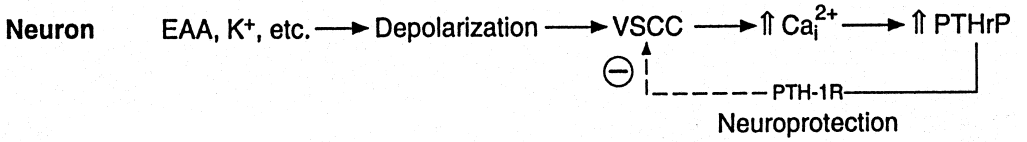

Figure 7 Schema of autofeedback loop in which PTHrP, triggered by L-VCSS Ca^{2+} influx, feeds back via the PTH1R to dampen L-VCSS Ca^{2+} currents.

that the quantity of PTHrP secreted by the cell is a linear function of the level of PTHrP mRNA expression. PTHrP immunolocalizes principally to the granule cell soma (Holt *et al.,* 1996) so that it is presumably secreted by the cell bodies themselves, acting in the autocrine/paracrine fashion typical of a neuromodulatory peptide.

PTHrP Inhibits L-VSCC Ca^{2+} Influx, Defining a Protective Feedback Loop

Overstimulation can lead to neuronal injury or death, a process referred to as excitotoxicity. Excitotoxicity comes in two flavors. High concentrations of the excitory amino acid, glutamate, cause a generalized influx of cations and a collapse in mitochondrial function leading to almost immediate necrosis (Ankarcrona *et al.,* 1995). Lower concentrations of glutamate or exposure to other excitotoxins, such as kainic acid, trigger Ca^{2+} entry via L-VSCCs, which leads to excitotoxicity characterized by a long latency (6–24 hr to cell death) (Ankarcrona *et al.,* 1995; Weiss *et al.,* 1990).

The granule cell system is subject to both immediate and latent forms of excitotoxicity. A low concentration of kainic acid produces about 50% granule cell death at 24 hr, and the calcium channel blocker nitrendipine is capable of fully protecting these cells, thereby defining the central importance of L-VSCC Ca^{2+} influx in long latency excitotoxicity (Brines *et al.,* 1999). It will be recalled that PTH has been shown to inhibit L-VSCCs in smooth muscle and neuroblastoma cells (Wang *et al.,* 1991; Pang *et al.,* 1990). This led to the working hypothesis that PTHrP might be capable of inhibiting L-VSCC Ca^{2+} influx in cerebellar granular cells, which proved to be the case. PTHrP was found to be fully neuroprotective in kainic acid-treated granule cells (Fig. 6) and was as effective as nitrendipine in reducing kainic acid-induced L-VSCC Ca^{2+} influx (Brines *et al.,* 1999). Pang *et al.* (1990) used whole cell patch-clamp techniques to demonstrate that PTH is capable of inhibiting L-VSCC Ca^{2+} influx in mouse neuroblastoma cells and one of us (AEB) has used patch-clamp techniques to demonstrate the same findings with PTHrP in these cells. This effect is mediated by the PTH1R, but nothing is yet known of the mechanism by which the channel is actually regulated.

Taken together, these findings indicate that PTHrP serves as an endogenous L-VSCC regulator that functions in a neuroprotective feedback loop of the sort depicted in Fig. 7. As shown, the L-VSCC itself is the fulcrum of this loop, and the rheostat is L-VSCC Ca^{2+} entry. This loop would provide neuroprotection to individual (autocrine) and neighboring (paracrine) neurons.

As described in Chapter 13, the PTHrP knockout mouse dies at birth as a result of systemic chondrodystrophy. This mouse has been rescued by a genetic strategy, generating a mouse that is PTHrP sufficient in chondrocytes but PTHrP null in all other sites (Wysolmerski *et al.,* 1998). There are no CNS abnormalities per se in the rescued mouse, but it displays a six-fold increase in sensitivity to kainic acid. Thus, as might be predicted from the findings in cultured cerebellar granule cells described above, PTHrP appears to be provided a defense against excitotoxicity that is operative *in vivo* (this work is described briefly because it is as yet unpublished).

Other Potential Calcium Channel Effects

PTHrP increases L-VSCC activity and thereby enhances dopamine secretion in PC-12 cells (Brines and Broadus, 1999). PTH and/or PTHrP has been reported to increase calcium channel-like activity in snail neurons (Kostyuk *et al.,* 1992) and in rat hippocampal neurons (Hirasawa *et al.,* 1998, Fukayama *et al.,* 1995), but these effects are slow and perhaps involve channels other than the classic L-VSCC. UMR-106 osteoblast-like cells contain L-VSCCs that are stimulated by PTH treatment (Barry *et al.,* 1995). L-VSCCs are also widely expressed in a great many other excitable and nonexcitable cells that have thus far not been examined with respect to PTHrP regulation.

Acknowledgments

The authors were asked to write two chapters on PTHrP effects in excitable tissues for separate books texts, the present text and "The Parathyroids" (J. P. Bilezikian, R. Marcus, and M. A. Levine, eds.). Because the chapters were on the same subject and had almost simultaneous due dates, they are virtually identical in context.

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CHAPTER 31

$1\alpha,25(OH)_2$Vitamin D_3

Nuclear Receptor Structure and Ligand Specificities for Genomic and Rapid Biological Responses

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Introduction to Vitamin D and $1\alpha,25(OH)_2D_3$

Structure

The term vitamin D designates a group of closely related seco steroids that possess antirachitic activity (see Fig. 1).[1] The two most prominent members of this group are ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) (Norman, 2001). Ergocalciferol is derived from a common plant steroid, ergosterol, and is the form that was employed for vitamin D fortification of foods from the 1940s to 1960s.

Cholecalciferol is the form of vitamin D obtained when radiant energy from the sun strikes the skin and converts the precursor 7-dehydrocholesterol into vitamin D_3. Because the body is capable of producing cholecalciferol, vitamin D technically does not meet the classical definition of a vitamin, i.e., a substance required by the body, but which cannot be made by the body. A more accurate description of vitamin D is that it is a prohormone. It has been shown that vitamin D is metabolized to a biologically active form, $1\alpha,25(OH)_2$vitamin D_3 [$1\alpha,25(OH)_2D_3$], which functions as a steroid hormone (Norman,1996; Reichel *et al.,* 1989). However, because the parent vitamin D was first recognized as an essential nutrient, it continues to be classified among the fat-soluble vitamins. Indeed, even in the last decade of the 20th century there are classic examples of vitamin D deficiency in many countries, as documented by low serum concentrations of $25(OH)D_3$ [e.g., United States (Dawson-Hughes *et al.,* 1997; Jacques *et al.,* 1997), India (Goswami *et al.,* 2000), and South Africa (Feleke *et al.,* 1999)].

Conformational Flexibility

Vitamin D_3 and all its daughter metabolites, including $1\alpha,25(OH)_2D_3$, are unusually conformationally flexible; (see Fig. 2). Three key aspects of the $1\alpha,25(OH)_2D_3$ molecule confer a unique range of conformational mobility on this

[1] The chemical structures of vitamin D and its daughter metabolites are closely related structurally to their provitamin forms as well as to the four ring nucleus of other classical steroids that are derived from the cyclopentanoperhydrophenanthrene ring system, (see Fig. 1). The official nomenclature proposed for vitamin D by the International Union of Pure and Applied Chemistry (IUPAC) relates it to the steroid nucleus, which is numbered as shown in Fig. 1 for the provitamin. The carbons in vitamin D retain the same number as designated in the provitamin. No vitamin D biological activity becomes apparent until ring B of the provitamin is opened. Thus, vitamin D and its metabolites are simply steroids with a broken B ring as a consequence of rupture of the carbon–carbon bond between C-9 and C-10. The presence of the nonintact B ring in the steroid nucleus is officially designated by the use of the term "seco." The formal chemical name of vitamin D_3 is 9,10-secocholesta-5,7,10(19)-trien-3β-ol and $1\alpha,25(OH)_2D_3$ is 9,10-secocholesta-5,7,10(19)-trien-$1\alpha,3\beta$,25-triol.

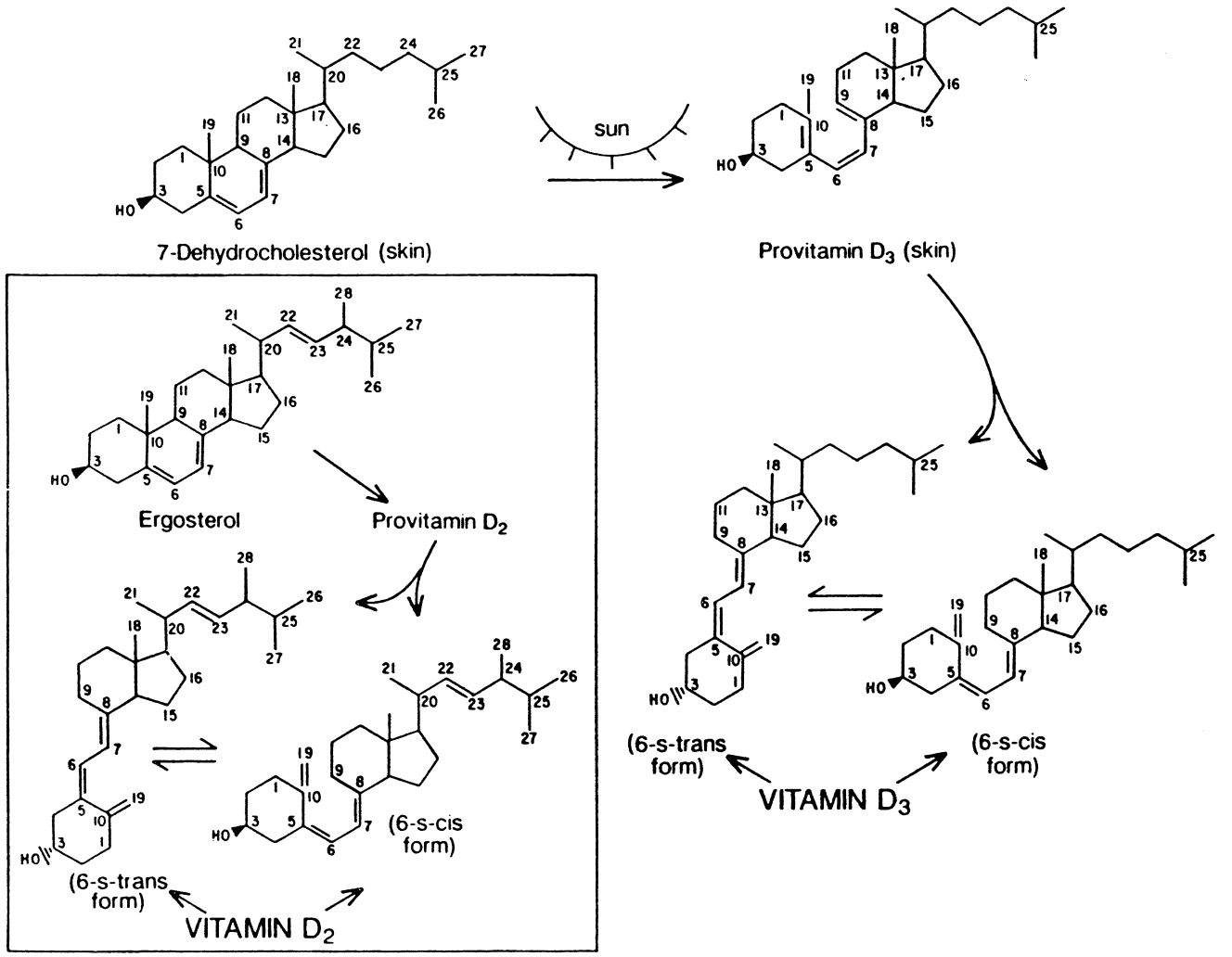
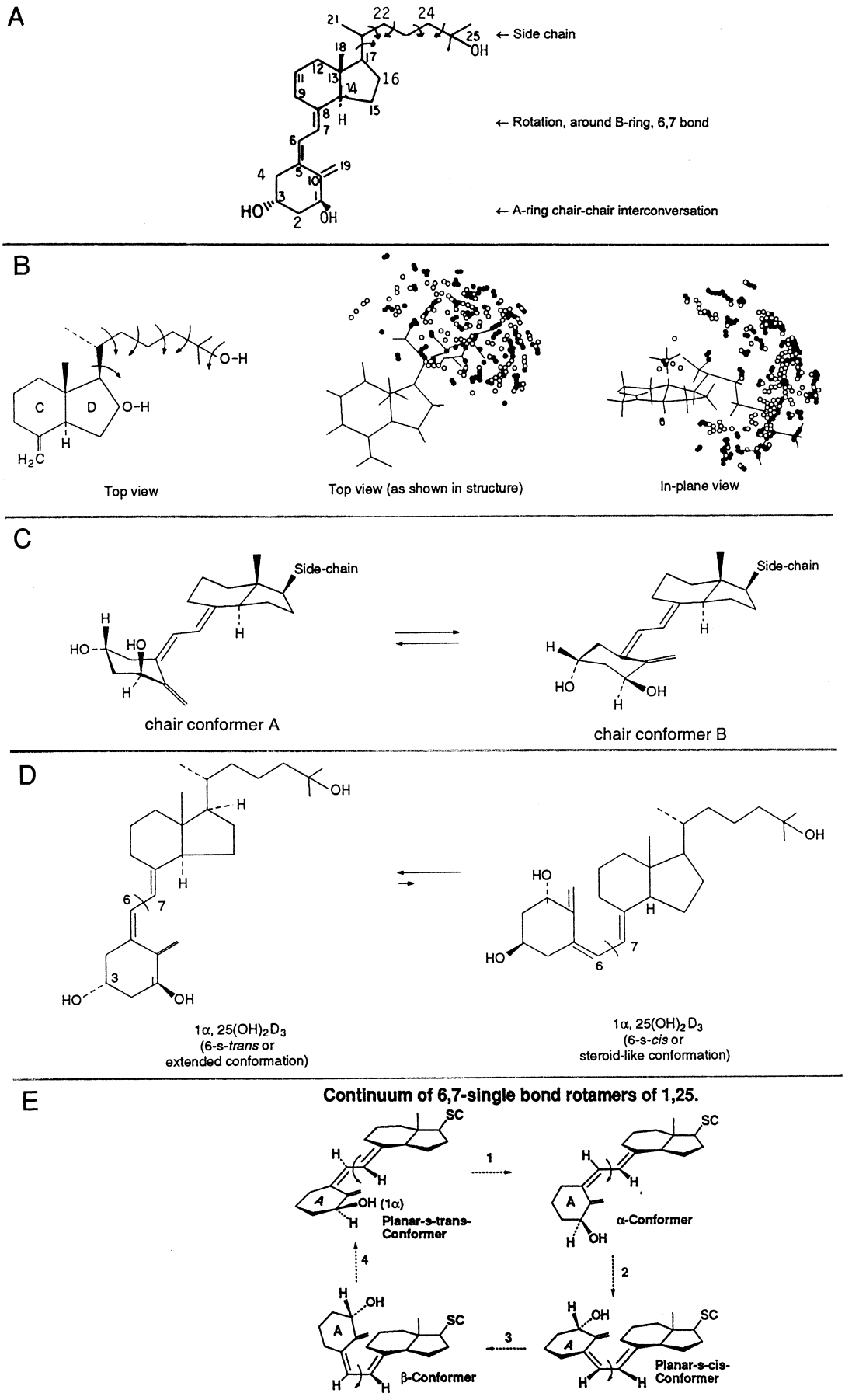


Figure 1 Chemistry and irradiation pathway for production of vitamin D_3 (a natural process) and vitamin D_2 (a commercial process). In each instance the provitamin, which is characterized by the presence of a Δ5, Δ7-conjugated double bond system in the B ring, is converted to the seco B previtamin steroid, where the 9,10 carbon–carbon bond has been broken. Then the previtamin D, in a process independent of ultraviolet light, isomerizes thermally to the vitamin form, which is characterized by a Δ6, Δ8, Δ10, 19-conjugated triple bond system. In solution (and in biological systems), vitamin D is capable of assuming a large number of conformational shapes because of rotation about the 6,7 carbon–carbon single bond of the B ring.

molecule. (a) The intact eight carbon side chain of vitamin D and related seco steroids can easily assume numerous shapes and positions in three-dimensional (3D) space by virtue of rotation about its five carbon–carbon single bonds. A discussion of the consequences of side chain conformational mobility has been presented previously (Midland *et al.,* 1993; Okamura *et al.,* 1992). (b) The cyclohexane-like A ring is free to rapidly interchange (many thousands of times per second) between a pair of chair–chair conformers; this has the consequence of changing the orientation of the key 1α and 3β hydroxyls between either an equatorial or an axial orientation (Wing *et al.,* 1974). (c) Rotational freedom

Figure 2 Structure of 1α,25$(OH)_2D_3$ illustrating the three structural aspects of vitamin D seco steroids that contribute to the conformational flexibility of these molecules. (A) Structure of 1α,25$(OH)_2D_3$ with indication of the three structural features of the molecule, which confer conformational flexibility on this molecule. (B) The dynamic rotation of the cholesterol-like side chain of 1α,25$(OH)_2D_3$ with 360° rotations about the five single carbon–carbon bonds indicated by the curved arrows. Dots indicate the position in three-dimensional space of the 25-hydroxyl group for some 394 readily identifiable side chain conformations. A discussion of the consequences of side chain conformational mobility has been presented (Midland *et al.,* 1993; Okamura *et al.,* 1992, 1994). (C) The rapid (millions of times per second) chair–chair interconversion of the cyclohexane-like A ring of the seco steroid between chair conformer A and chair conformer B; this effectively equilibrates 1α- and 3β-hydroxyls between axial and equatorial orientations. (D) Rapid (millions of times per second) rotational freedom about the 6,7 carbon–carbon bond of the seco B ring generates a population of shapes or conformations ranging from the more steroid-like 6-s-*cis* conformation, to the open and extended 6-s-*trans* form of the hormone (Norman *et al.,* 1993c). (E) Further illustration of the 360° rotation about the 6,7 carbon–carbon bond. Four steps of successive 90° rotation are illustrated. Each intermediate structure has a dramatically *different* shape, particularly with respect to the position of the critical 1α-hydroxyl and the plane of the A ring in relation to the plane of the C/D rings; the preferred shape of the ligand for the VDR_{nuc} is illustrated in Fig. 11.


A
← Side chain
← Rotation, around B-ring, 6,7 bond
← A-ring chair-chair interconversation
B
Top view
Top view (as shown in structure)
In-plane view
C
Side-chain
chair conformer A
chair conformer B
D
1α, 25(OH)2D3
(6-s-trans or
extended conformation)
1α, 25(OH)2D3
(6-s-cis or
steroid-like conformation)
E
Continuum of 6,7-single bond rotamers of 1,25.
Planar-s-trans-
Conformer
α-Conformer
β-Conformer
Planar-s-cis-
Conformer

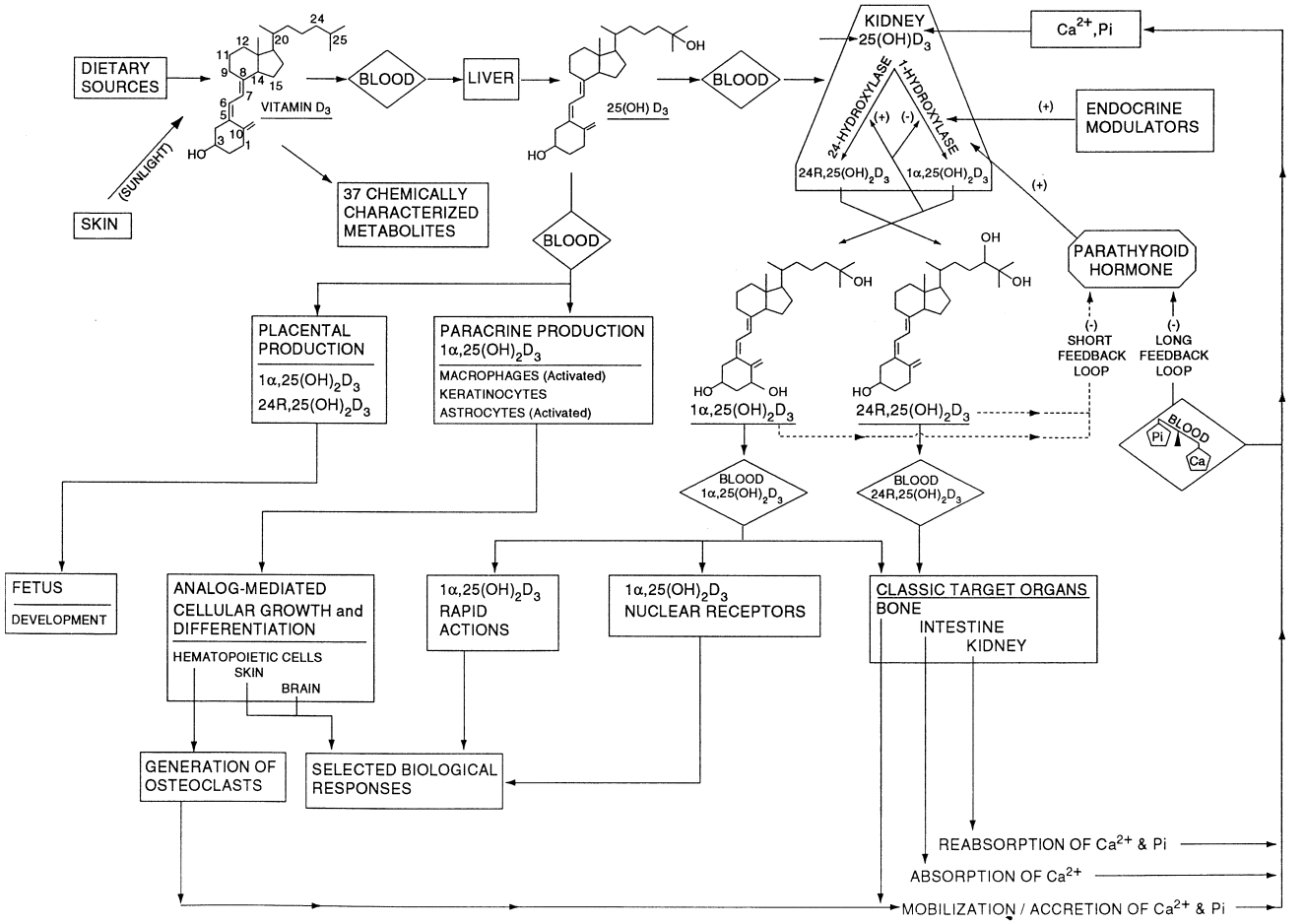


Figure 3 Overview of the vitamin D endocrine system. Target organs and cells for $1\alpha,25(OH)_2D_3$ by definition contain receptors for the VDR_{nuc} that enable them to modulate genomic events. Tissues that possess the VDR_{nuc} are listed in Table I and are discussed in Hannah *et al.,* (1994). In addition, $1\alpha,25(OH)_2D_3$ generates biological effects via rapid response pathways; these sites are listed in Table II and are discussed in Norman (1997). There is emerging evidence that $24R,25(OH)_2D_3$ may also have important biological effects (Seo *et al.,* 1997a).

about the 6–7 carbon–carbon bond of the seco B-ring allows conformations ranging from the more steroid-like 6-s-*cis* conformation to the open and extended 6-s-*trans* form of the hormone (Norman *et al.,* 1993c). It is generally accepted that this conformational mobility of vitamin D seco steroids is displayed by molecules in an organic solvent as well as an aqueous environment similar to that encountered in biological systems. Thus receptors for $1\alpha,25(OH)_2D_3$ have had to accommodate to the reality of binding a highly conformationally flexible ligand.

Vitamin D Endocrine System

The concept of the existence of the vitamin D endocrine system is firmly established (Bouillon *et al.,* 1995; Reichel *et al.,* 1989), (see Fig. 3). The key organ in this endocrine system is the kidney where the renal proximal tubule is responsible for producing the hormonal $1\alpha,25(OH)_2D_3$ in accordance with strict physiological signals (Henry,2000). The parent vitamin D_3 is metabolized to 25(OH)D3 (by the liver) and then to $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ (by the endocrine gland, the kidney), as well as to 34 other metabolites (Bouillon *et al.,* 1995). The seco steroid $1\alpha,25(OH)_2D_3$ has been shown to initiate biological responses via regulation of gene transcription as well as via rapid membrane receptor-initiated pathways. The rapid responses can, for example, involve opening of voltage-gated Ca^{2+} channels (Caffrey *et al.,* 1989) or the rapid stimulation of intestinal Ca^{2+} absorption known as transcaltachia (Nemere *et al.,* 1984) (see Fig. 4).

An additional key participant in the operation of the vitamin D endocrine system is the plasma vitamin D-binding protein (DBP), which carries vitamin D_3 and all its metabolites to their various target organs. The DBP is known to have a specific ligand-binding domain for vitamin D-related ligands, which is different in specificity from the ligand-binding domain of the nuclear vitamin D receptor (Bishop *et al.,* 1994). DBP is similar in function to the corticosteroid-binding globulin (CBG), which carries glucocorticoids, and the steroid hormone-binding globulin (SHBG), which transports estrogens or androgens. DBP is a slightly acidic (p*I* 5.2) monomeric glycoprotein of 53 kDa synthesized and secreted by the liver as a major plasma constituent.

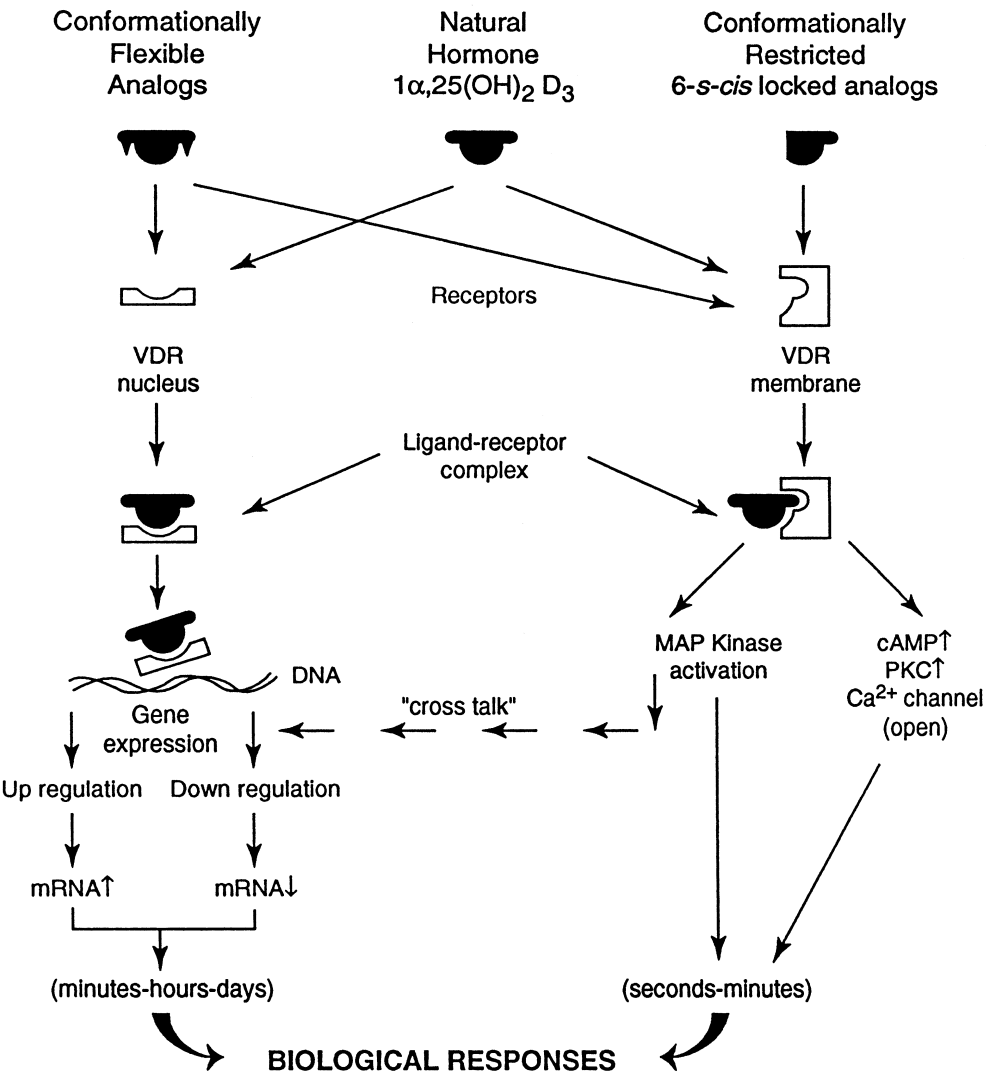


Figure 4 Proposed signal transduction pathways utilized by 1α,25(OH)$_2$D$_3$ ( ) and analogs of 1α,25(OH)$_2$D$_3$ to generate biological responses. Different shapes of 1α,25(OH)$_2$D$_3$ and its conformationally flexible analogs ( ) may interact with either the VDR$_{nuc}$ or the VDR$_{mem}$ to initiate different signal transduction pathways, which result in genomic responses or rapid responses. In contrast, the conformationally restricted 6-s-*cis* analogs ( ) (see Fig.1; JM, JN) can only interact with VDR$_{mem}$. In the genomic pathway (left side), occupancy of the nuclear receptor for 1α,25(OH)$_2$D$_3$ (VDR$_{nuc}$) by a ligand leads to an up- or downregulation of genes subject to hormone regulation. More than 50 proteins are known to be transcriptionally regulated by 1α,25(OH)$_2$D$_3$ [see Table I and Hannah *et al.,* (1994)]. In the membrane-initiated pathway (right side), occupancy of a putative membrane receptor for 1α,25(OH)$_2$D$_3$ by a ligand is believed to lead rapidly to activation of a number of signal transduction pathways, including adenylate cyclase, phospholipase C, protein kinase C (PKC), mitogen-activated protein kinase (MAP kinase), and/or opening of voltage-gated L-type Ca^{2+} channels, which are either individually or collectively coupled to generation of the biological response(s).

From analysis of the cloned cDNA, it has been determined that DBP is structurally homologous to albumin and α-fetoprotein; these three plasma proteins are members of the same multigene family, which likely is derived from the duplication of a common ancestral gene. DBP, originally called group-specific component (Gc), was initially studied electrophoretically as a polymorphic marker in the α-globulin region of human serum (see Cooke *et al.,* 1997; Haddad,1995). Preliminary data describing the X-ray crystal structure of DBP has been presented (Bogaerts *et al.,* 2000).

Signal Transduction Pathways Utilized by 1α,25(OH)$_2$D$_3$ to Generate Biological Responses

Figure 4 presents a schematic model that postulates that the various biologic responses generated by 1α,25(OH)$_2$D$_3$ are dependent on two types of receptors. These are the classic nuclear receptor for 1α,25(OH)$_2$D$_3$ (designated as VDR$_{nuc}$) and a putative membrane receptor (designated as VDR$_{mem}$). It has been postulated that the conformationally flexible steroid hormone 1α,25(OH)$_2$D$_3$ generates biological responses using

Table I Tissue Distribution of Nuclear $1\alpha,25(OH)_2D_3$ Receptor

Adipose	Hair follicle	Parotid
Adrenal	Intestine	Pituitary
Bone	Kidney	Prostate
Bone marrow	Liver (fetal)	Retina
Brain	Lung	Skin
Breast	Muscle, cardiac	Stomach
Cancer cells (many)	Muscle, smooth	Testis
Cartilage	Osteoblast	Thymus
Colon	Ovary, embryonic	Thyroid
Eggshell gland	Pancreas β cell	Uterus
Epididymus	Parathyroid	Yolk sac (bird)

different shapes so as to selectively activate the two general signal transduction pathways (Norman, 1997). (a) One shape of $1\alpha,25(OH)_2D_3$ interacts with the VDR_{nuc} to form a competent receptor–ligand complex that interacts with other nuclear proteins to create a functional gene transcription complex to increase or decrease mRNA coding for selected proteins (Fig. 4, left). (b) A different shape of $1\alpha,25(OH)_2D_3$ interacts with the putative VDR_{mem} that promptly stimulates signal transduction events, which activate the *rapid* appearance of biological responses (Fig. 4, right).

Table I summarizes 33 target organs known to possess the VDR_{nuc} and in which there is an impressive amount of detail concerning the regulation of gene transcription (Hannah *et al.,* 1994).

A wide array of rapid responses stimulated by $1\alpha,25-(OH)_2D_3$ have been reported since the mid-1980s; a summary is given in Table II. Recent additions to the list include demonstration that $1\alpha,25(OH)_2D_3$ can stimulate opening of chloride channels (Zanello *et al.,* 1996) and activation of MAP kinase (Beno *et al.,* 1995; Song *et al.,* 1998). MAP kinase belongs to the family of serine/-threonine protein kinases and can be activated by phosphorylation on a tyrosine residue induced by mitogens or cytodifferentiating agents (Pelech *et al.,* 1992). MAP kinase integrates multiple intracellular signals transmitted by various second messengers and regulates many cellular functions by phosphorylation of a number of cytoplasmic kinases and nuclear transcription factors, including the EGF receptor, c-Myc, and c-Jun (Lange-Carter *et al.,*

Table II Distribution of Rapid Responses to 1α, $25(OH)_2D_3$

Organ/cell/system	Response studied	Reference
Intestine	Rapid transport of intestinal Ca^{2+} (Transcaltachia);	De Boland *et al.* (1990a,b); Nemere *et al.* (1984)
	CaCo-2 cells, PKC, G proteins	Khare *et al.* (1994);
	Activation of PKC	Bissonnette *et al.* (1994); De Boland *et al.* (1990a)
	Activation of MAP kinase	De Boland *et al.* (1998);
	Stimulation of phospholipase C	Khare *et al.* (1997)
Colon	PKC effects	Bissonnette *et al.,* (1995); Simboli-Campbell *et al.* (1992);
	Subcellular distribution	Simboli-Campbell *et al.* (1994)
	Regulation of $25(OH)D_3$-24-hydroxylase	Mandla *et al.* (1990)
Osteoblast	ROS 17/2.8 cells	
	Ca^{2+} channel opening	Caffrey *et al.* (1989)
	Cl^- channel opening	Zanello *et al.* (1996)
Chondrocytes	PKC activation	Schwartz *et al.* (2000); Sylvia *et al.* (1996)
	Phospholipase A_2 activation	Boyan *et al.* (1998)
Liver	Lipid metabolism; activation of PKC and MAP kinase	Baran *et al.* (1989, 1990); Beno *et al.* (1995)
Muscle	PKC and Ca^{2+} effects	De Boland *et al.* (1993); Morelli *et al.* (1993); Selles *et al.* (1991); Vazquez *et al.* (1996)
	Phospholipase D	Fernandez *et al.* (1990)
Promyelocytic	Aspects of cell differentiation	Bhatia *et al.* (1995, 1996); Miura *et al.* (1999a)
Leukemic cells	PKC effects	Biskobing *et al.* (1993)
	Activation of MAP kinase	Berry *et al.* (1996); Song *et al.* (1998)
Keratinocytes	Alter PKC subcellular distribution	Gniadecki *et al.* (1997); Yada *et al.* (1989)
	Sphingomyelin hydrolysis	Gniadecki (1996)
	Activation of Src and Raf	Gniadecki (1996, 1998a)
Pancreas B cells	Intracellular calcium changes	Sergeev *et al.* (1995)
	Insulin secretion	Kajikawa *et al.* (1999)
Parathyroid cells	Phospholipid metabolism	Bourdeau *et al.* (1990)
	Cytosolic Ca^{2+}	Sugimoto *et al.* (1992)
Lipid bilayer	Activation of highly purified PKC	Slater *et al.* (1995)

[a]The reader should compare the information in this table with the concepts illustrated in Figs. 2 and 3, which summarize the vitamin D endocrine system and signal transduction pathways utilized by $1\alpha,25(OH)_2D_3$ for generation of biological responses.

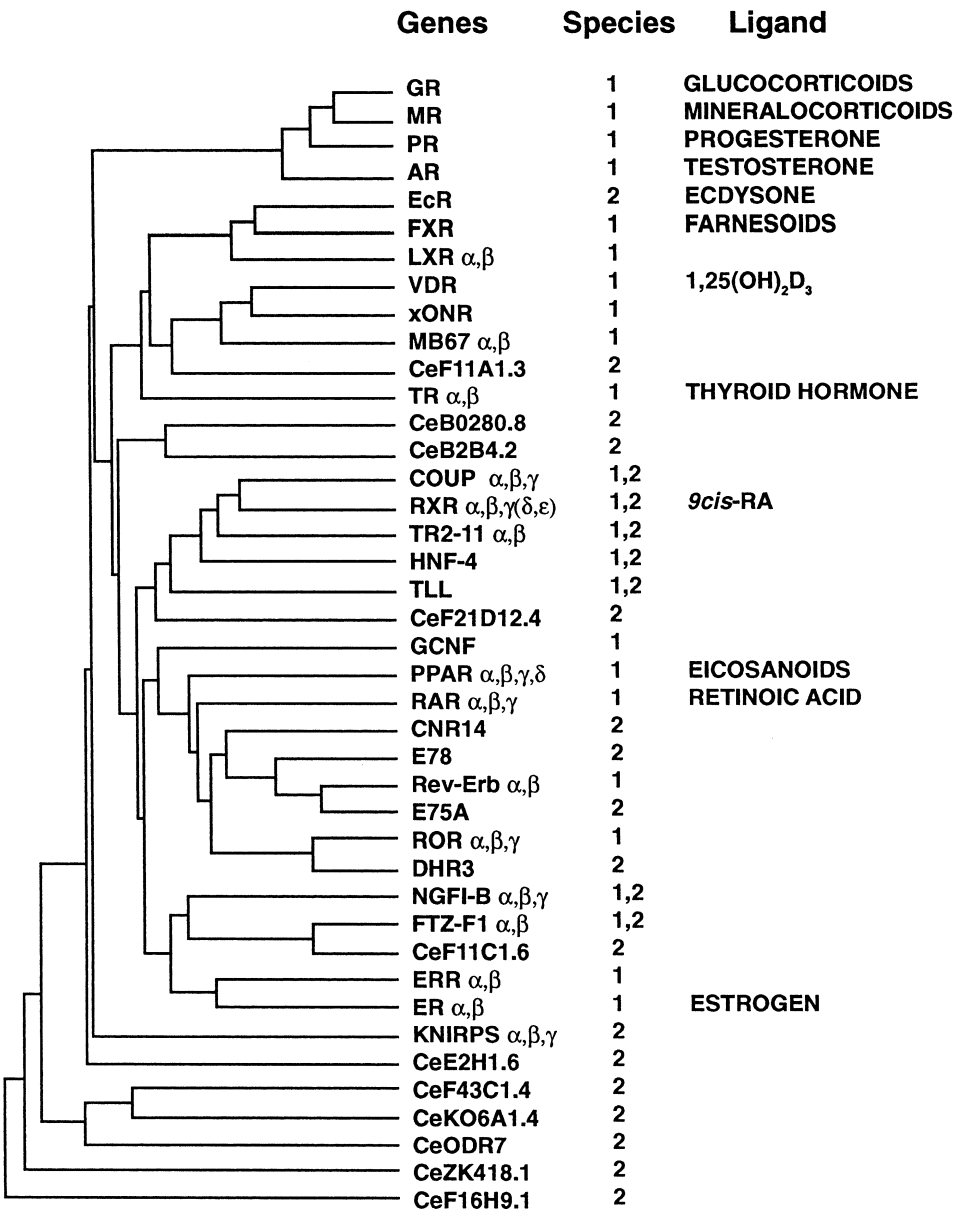


Figure 5 Nuclear receptor superfamily. Hypothesized evolutionary relationships for the extended family of known nuclear receptors and related orphan receptors based on the extent of homology for the nucleotide sequence of the cDNA of the individual protein are summarized. This figure was modified from that presented by Mangelsdorf *et al.* (1995).

1993). These rapid actions of 1α,25(OH)$_2$D$_3$ have been postulated to regulate cell biological function and potentially to interact with other membrane-mediated kinase cascades or to cross-talk with the cell nucleus to control genomic responses associated with cell differentiation and proliferation (Berry *et al.,* 1996).

Nuclear Receptor (VDR$_{nuc}$) for 1α,25(OH)$_2$D$_3$

The VDR$_{nuc}$ belongs to a superfamily of ligand-dependent nuclear receptors, which includes receptors for glucocorticoids (GR), progesterone (PR), estrogen (ER), aldosterone, androgens, thyroid hormone (T$_3$R), hormonal forms of vitamin A (RAR, RXR), vitamin D (VDR), and several orphan receptors (Evans,1988; Lowe *et al.,* 1992; Parker,1991), (see Fig. 5). Figure 5 summarizes the hypothesized evolutionary relationships for the extended family of known nuclear receptors and related orphan receptors in vertebrate and invertebrates (*Caenorhabditis elegans, Drosophila*). For further details, see Carlberg (1996) and Mangelsdorf *et al.* (1995).

Figure 6 illustrates the structural relationship among the gene for the VDR$_{nuc}$, the mRNA, and the protein receptor. At the protein level, comparative studies of the VDR with all the steroid, retinoid, and thyroid receptors reveal that they have a common structural organization consisting of five domains (Krust *et al.,* 1986) with significant amino acid sequence homologies. The different domains act as

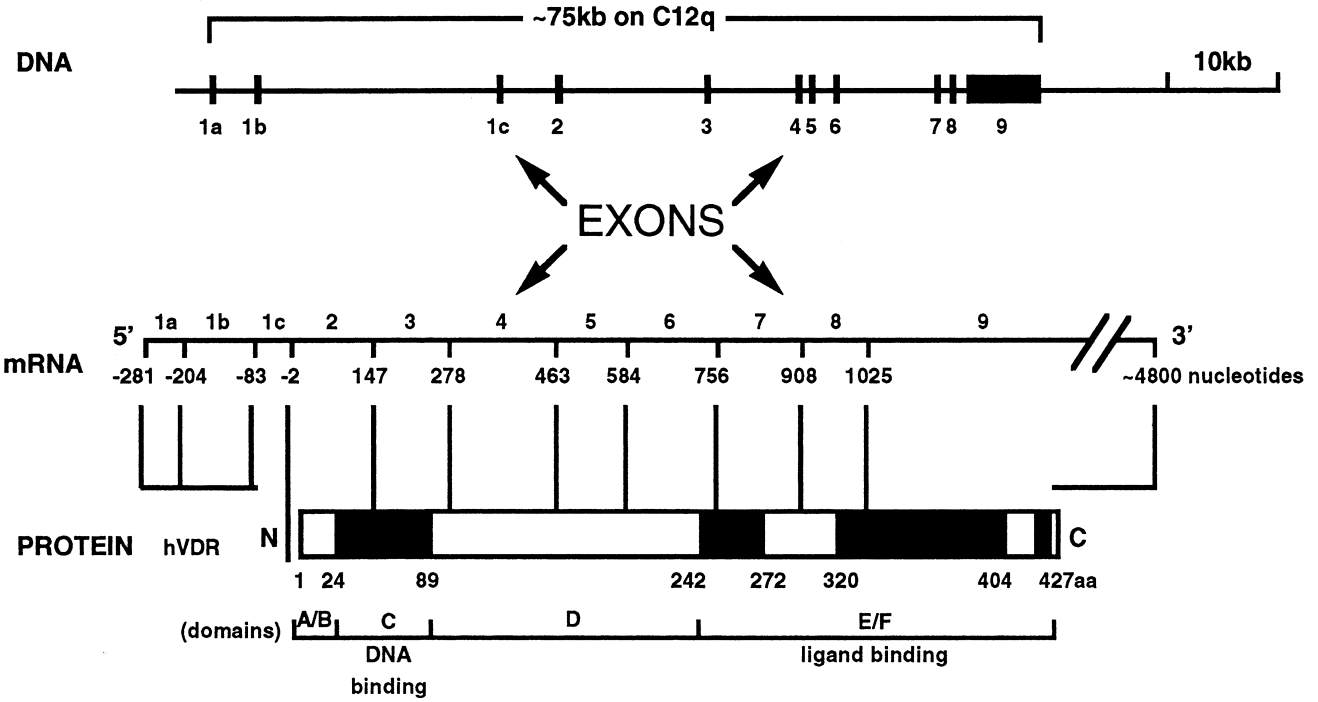


Figure 6 Schematic model of the VDR_{nuc} gene, mRNA, and protein. The gene for the VDR_{nuc} is located on human chromosome 12 and spans approximately 10 kb. The gene has 11 exons, which are processed to yield a full-length mRNA of 4800 nucleotides, the VDR_{nuc} protein is composed of 427 amino acids. Numbers below the hVDR indicate the amino acid residue boundaries for the various domains. Nuclear receptors consist of five domains (A–E) based on regions of conserved amino acid sequence and function. The C domain, the most highly conserved domain, is the DNA-binding domain and defines the superfamily; it contains two zinc finger motifs. The E domain is less conserved and is responsible for ligand binding, dimerization, and transcriptional activation. Subdomains within the domain include ligand 1 (residues 242–272) and ligand 2, or the τ_i or transcriptional inhibition, and dimerization domain (residues 310–404), which contains nine heptad repeats as first described by Forman *et al.* (1990). Domains A/B and D have the least sequence homology. See also information presented in Carlberg *et al.* (1998) and Haussler *et al.* (1998).

distinct modules that can function independently of each other (Beato,1989; Green *et al.,* 1988; Ham *et al.,* 1989). The DNA-binding domain, C, is the most conserved domain throughout the family. About 70 amino acids fold into two zinc finger-like motifs. Conserved cysteines coordinate a zinc ion in a tetrahedral arrangement. The first finger, which contains four cysteines and several hydrophobic amino acids, determines the DNA response element specificity. The second zinc finger, which contains five cysteines and many basic amino acids, is also necessary for DNA binding and is involved in receptor dimerization (Evans, 1988; Forman *et al.,* 1990; Green *et al.,* 1988; Rastinejad *et al.,* 1995).

The next most conserved region is the steroid-binding domain (region E). This region contains a hydrophobic pocket for ligand binding and also contains signals for several other functions, including dimerization (Bourguet *et al.,* 1995; Fawell *et al.,* 1990; Forman *et al.,* 1989; Glass *et al.,* 1989), nuclear translocation, and hormone-dependent transcriptional activation (Beato,1989; Green *et al.,* 1988; Picard *et al.,* 1990a).

The A/B domain is also known as the immuno- or transactivation domain. This region is poorly conserved in amino acids and in size, and its function has not been clearly defined. The VDR has the smallest A/B domain (25 amino acids) of the known receptors, whereas the mineralocorticoid receptor has the largest A/B domain (603 amino acids). An independent transcriptional activation function is located within the A/B region (Evans, 1988; Green *et al.,* 1988; Ham *et al.,* 1989), which is constitutive in receptor constructs lacking the ligand-binding domain (region E). The relative importance of transcriptional activation by this domain depends on the receptor, the context of the target gene promoter, and the target cell type (Tora *et al.,* 1989).

Domain D is the hinge region between the DNA-binding domain and the ligand-binding domain. The hinge domain must be conformationally flexible because it allows the ligand-binding domain and DNA-binding domains some flexibility for their proper interactions. The VDR hinge region contains 65 amino acids and has immunogenic properties (McDonnell *et al.,* 1988).

Receptor Dimerization

The superfamily of nuclear receptors has been classified into subgroups based on their dimerization properties, DNA-binding site preferences, and cellular localization. Group I includes receptors for glucocorticoids, estrogen, mineralocorticoids, progesterone, and androgens. These receptors bind as homodimers to palindromic DNA response elements. Group II includes receptors for VDR_{nuc}, T_3R, RAR, RXR, ecdysone, and several orphan receptors.

These receptors bind as homodimers or heterodimers to direct repeats, palindromic, and inverted palindromic DNA response elements. Group III includes the receptors for reverb A, ROR, SF-1, and NGFI-B. No ligands have yet been identified for these receptors and they bind DNA response elements as monomers or heterodimers.

As a class, group II receptors bind nonsteroid conformationally flexible ligands (where vitamin D is classified as a seco steroid rather than as a steroid). Group II receptors have more flexibility in the types of DNA response elements they can recognize and in the types of dimeric interactions they participate in than group I receptors. All of the group II receptors can form heterodimers with RXR (Kliewer *et al.,* 1992; Yu *et al.,* 1991), and other heterodimeric interactions have also been reported (Carlberg, 1993). The VDR$_{nuc}$ can bind to DNA response elements as homodimers and as heterodimers with RAR, RXR, and T$_3$R (Carlberg,1993; Schräder *et al.,* 1993). The ability to form heterodimers with other receptors allows for enhanced affinity for distinct DNA targets, generating a diverse range of physiological effects, as shown in Fig. 7.

The first zinc finger determines the sequence specificity of the DNA element. The second zinc finger is aligned by the binding of the first finger to the DNA and is involved in the protein–protein contacts responsible for the cooperativity of binding. The spacing of nucleotides between the two half-sites is important for DNA-binding specificity because of the asymmetric dimer interface formed by the DNA-binding domains of a heterodimer pair. Ligand binding may function to modulate receptor dimerization. In fact, VDR$_{nuc}$ has been shown to exist as a monomer in solution either in the presence or in the absence of ligand. When DNA is present, in the absence of ligand, the VDR$_{nuc}$ binds to the DNA both as monomers and homodimers. The addition of ligand stabilizes the bound monomer, which favors the formation of VDR$_{nuc}$-RXR (or other) heterodimers. The presence of the ligand decreases the rate of monomer-to-homodimer conversion and enhances the dissociation of the dimer complex. The presence of the RXR ligand, 9-*cis*-retinoic acid, has the opposite affect on heterodimerization formation; it enhances the binding of RXR homodimers to DR+1 elements (Cheskis *et al.,* 1994). Ligand bound to VDR$_{nuc}$ enhances the binding of RXR-VDR$_{nuc}$ heterodimers to DR+3 elements. Other possible protein–protein interactions can also involve VDR$_{nuc}$, including association with AP-1, EE1A/TFIID; TFIIB. These protein–protein interactions can be determined by the concentration of the protein partner and/or by the concentration of ligand or both, as well as by the nature of the DNA target site itself.

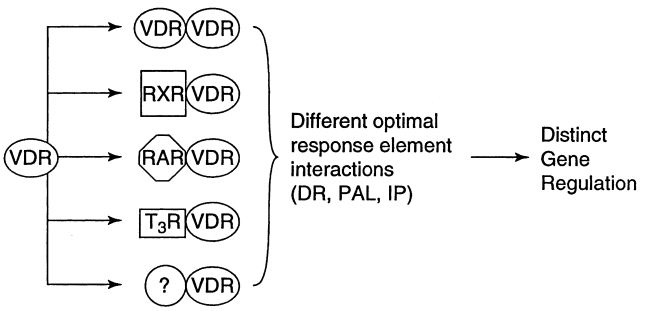


Figure 7 Schematic of possible dimeric interactions of VDR$_{nuc}$ with other receptor members of the superfamily (see Fig. 6). VDR$_{nuc}$ can bind to DNA as a homodimer or as a heterodimer with a variety of other group 2 receptors, i.e., RXR (retinoid X receptor), RAR (retinoid A receptor), T$_3$R (thyroid receptor), and perhaps other receptors or factors not yet identified. Each dimer pair has an enhanced affinity for distinct DNA targets, allowing a small family of receptors to generate a diverse range of physiological effects.

Hormone Response Elements

Each zinc finger appears to be encoded by separate exons as shown by the genomic structure of the ER (Ponglikitmongkol *et al.,* 1988), the PR (Huckaby *et al.,* 1987), and the VDR$_{nuc}$ (Freedman,1992). Most of the knowledge of how zinc fingers interact with DNA response elements has been gained by studies of GR and ER. The palindromic nature of GR and ER response elements suggested that these hormone receptors would bind to DNA as symmetrical dimers. Subsequent studies have confirmed that both GR and ER bind as homodimers to their response elements (Picard *et al.,* 1990b; Schwabe *et al.,* 1990). The principal ER dimerization domain is in its ligand-binding domain (Kumar *et al.,* 1988). Both the ER and the GR contain additional residues in the DNA-binding domain that are also important for dimerization. When the GR and ER DNA-binding domain are translated, they cannot dimerize alone but, in the presence of the correct palindromic response element, they bind to DNA as a dimer in a cooperative manner (Hard *et al.,* 1990). The five amino acid stretch between the first two coordinating cysteines of the second zinc finger is designated the "D" box (Umesono *et al.,* 1988) and mediates spacing requirements critical for cooperative dimer binding to palindromic HREs probably through a dimer interface involving these residues in each monomer (Diamond *et al.,* 1990; Jonat *et al.,* 1990; Schule *et al.,* 1990).

Using the GR and ER as models of receptor–DNA interactions, the binding of VDR$_{nuc}$ to DNA has also been examined. Because VDR$_{nuc}$ can bind to DNA as a heterodimer, often with RXR, VDR$_{nuc}$ and other group II receptors seem to display more variety in how they bind to their response elements (Forman *et al.,* 1989; Freedman,1992; Jones,1990). The primary response element for group II receptors is a direct repeat instead of an inverted palindrome; the protein–protein contacts are nonequivalent. There is an asymmetrical dimerization interface. Amino acid residues, designated the T/A box in the hinge region (domain D) just adjacent to the DNA binding domain, are involved. T/A box residues form an α helix, making backbone and minor groove interactions, which are involved in intramolecular packing against residues in the tip of the first zinc finger and determine the spacing requirements for the heterodimer pair.

Table III summarizes examples of hormone response elements for VDR$_{nuc}$. Natural response elements for group II receptors appear to consist of a direct repeat of the hexamer AGGTCA. The spacing of the direct repeat determines

Table III Hormone Response Elements for the Nuclear Vitamin D Receptor (VDR_{nuc})[a]

Gene	Hormone response element	Reference
hOsteocalcin	GGGTGA acg GGGGCA	Morrison *et al.* (1989)
rOsteocalcin	GGGTGA atg AGGACA	Terpening *et al.* (1991)
mOsteopontin	GGTTCA cga GGGTCA	Noda *et al.* (1990)
rCalbindin D_{9k}	GGGTGA cgg AAGCCC	Darwish *et al.* (1993)
mCalbindin D_{28k}	GGGGGA tgt GAGGAG	Gill *et al.* (1993)
24R-Hydroxylase	AGGTGA gtg AGGGCG	Hahn *et al.* (1994)
DR+3	AGGTCA agg AGGTCA	Umesono *et al.* (1991)
CONSENSUS	GGGTGA nnn *GG*GNCNAA	

[a]A comparison of reported VDREs. The two half-sites are listed as uppercase letters. The sequences are −500 to −486 of human osteocalcin, −456 to −438 of rat osteocalcin, −758 to −740 of mouse osteopontin, −488 to −474 of rat calbindin D_{9k}, and −199 to −184 of mouse calbindin D_{28k}.

the receptor preference: VDR_{nuc} prefers a 3-bp space, T_3R prefers 4 bp, and RAR prefers 5 bp (Umesono *et al.,* 1991). RXR, RAR, T_3R, and VDR_{nuc} spacing optimum on a palindrome is no nucleotides between the half-sites. Spacing on inverted palindromes depends on the overhang of the dimeric partners: 11 for VDR_{nuc}-RAR; VDR_{nuc}-RXR is predicted to be 7 to 8, but actually is 9; RXR appears to use a slightly different contact interface when it heterodimerizes with VDR_{nuc} than with other receptors (Schräder *et al.,* 1994). Free rotation around the hinge (domain D) enables the same interaction of the ligand-binding domains of both receptors on each response element. The steric requirements of T/A boxes give the receptor its asymmetry when binding to direct repeats and inverted palindromes and determine optimal spacing, illustrated in Fig. 8.

Ligand Binding

The ligand-binding domain of group II receptors as exemplified by the VDR has been dissected further (see Fig. 6). Subdomains ligand 1 and ligand 2 are nearly identical among receptors of the same binding specificity, but are different among receptors of different binding specificity (Giguere *et al.,* 1987; Harrison,1991; Thompson *et al.,* 1987). Surprisingly, there is greater homology between the ligand binding subdomains of RAR (α, β, γ) and T_3R (α, β) than between RAR and RXR. The τ_i subdomain is highly conserved among all nuclear hormone receptors and is a putative transcriptional inactivating domain. Inactivation of this domain is relieved by ligand binding.

The dimerization domain consists of eight to nine heptad repeats of hydrophobic amino acids. The heptads contain leucine or other hydrophobic residues such as Ile, Val, Met, or Phe at positions one and eight or charged amino acids with hydrophobic side chains such as Arg or Gln in the fifth position. In an ideal coiled–coil α helix, these amino acids would form a hydrophobic surface along one face of the

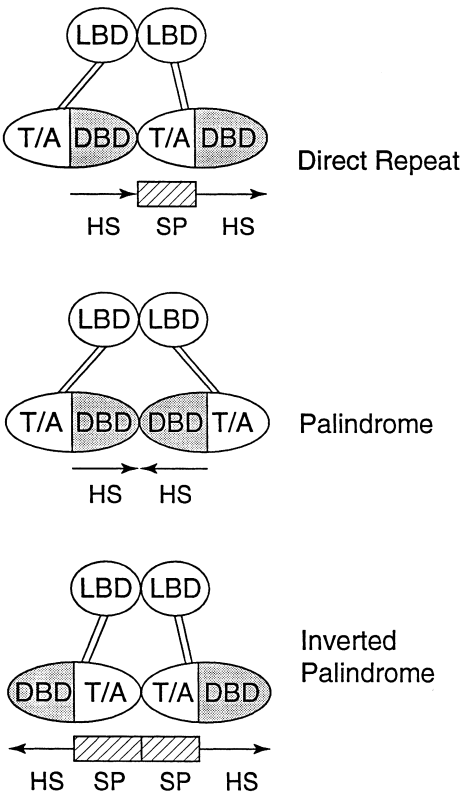


Figure 8 Mechanism of steriod nuclear receptor superfamily dimers binding to DNA response elements. Group II receptors can bind to three types of response elements, which are direct repeats, palindromes, and inverted palindromes. The spacing (SP), number of base pairs between half-sites (HS), is determined by steric constraints of the T/A box. The orintation of the DNA half-sites is shown with arrows. The flexiable hinge domain allows the formation of the same dimerization interface between ligand-binding domains (LBD) regardless of the orientation of the DNA half-sites.

helix that would act as a dimerization interface (Fawell *et al.,* 1990). Deletion/mutation analysis of the VDR$_{nuc}$ ligand-binding domain has shown that Asp-258 and Ile-248 are involved in heterodimerization with RXR. Leu-254 and -262 are critical for heterodimerization. A mutant that is truncated at amino acid 190 becomes constituitively transcriptionally active. Other amino acids identified as being important for heterodimerization are 325–332, 383–390, and 244–263. Residues 403–427 are particularly important for ligand side chain binding [1α,25(OH)$_2$D$_3$] (Nakajima *et al.,* 1994).

VDR Receptor Structure

A dramatic advance in understanding of the 3D structure of the LBD of steroid receptors occurred in the late 1990s with the X-ray crystallographic structure determination of LBD of five hormone receptors. These include LBDs of the thyroid hormone (TR), retinoic acid (RAR), estrogen (ER), progesterone (PR), and PPARγ (see the review by Weatherman *et al.,* 1999). Also, an X-ray structure is available for the LBD of the unoccupied 9-*cis* retinoic acid receptor RXR (Bourguet *et al.,* 1995). Further, ER LBD X-ray structures are known for a ligand (raloxifene), which can act as an *antagonist* of the transcriptional activation function (Brzozowski *et al.,* 1997; Shiau *et al.,* 1998)

The crystal structure of an engineered version of the ligand-binding domain of the nuclear receptor for vitamin D, bound to its natural ligand, has been determined at a 1.8-A resolution (Rochel *et al.,* 2000). The structure of the LBD of the human VDR$_{nuc}$ spans amino acid residues 143–427 (COOH terminus) and is very similar to that of a proposed model of the VDR LBD (Norman *et al.,* 1999), as well as to the LBD of the other five receptor structures (Weatherman *et al.,* 1999). The VDR$_{nuc}$ LBD structure, as does the other five nuclear receptors, consists of 12 α helices that are arranged to create a three-layer sandwich that completely encompasses the ligand 1α,25(OH)$_2$D$_3$ in a hydrophobic core. Impressively, all six X-ray structures are so similar that their ribbon diagrams are virtually superimposable, indicating a remarkable spatial conservation of the secondary and tertiary structures (Weatherman *et al.,* 1999) (see Fig. 9 (see also color plate). In addition, the AF-2 domain of the C-terminal helix 12 contributes to the hormone-binding pocket, suggesting that the ligand could play a role in receptor activation.

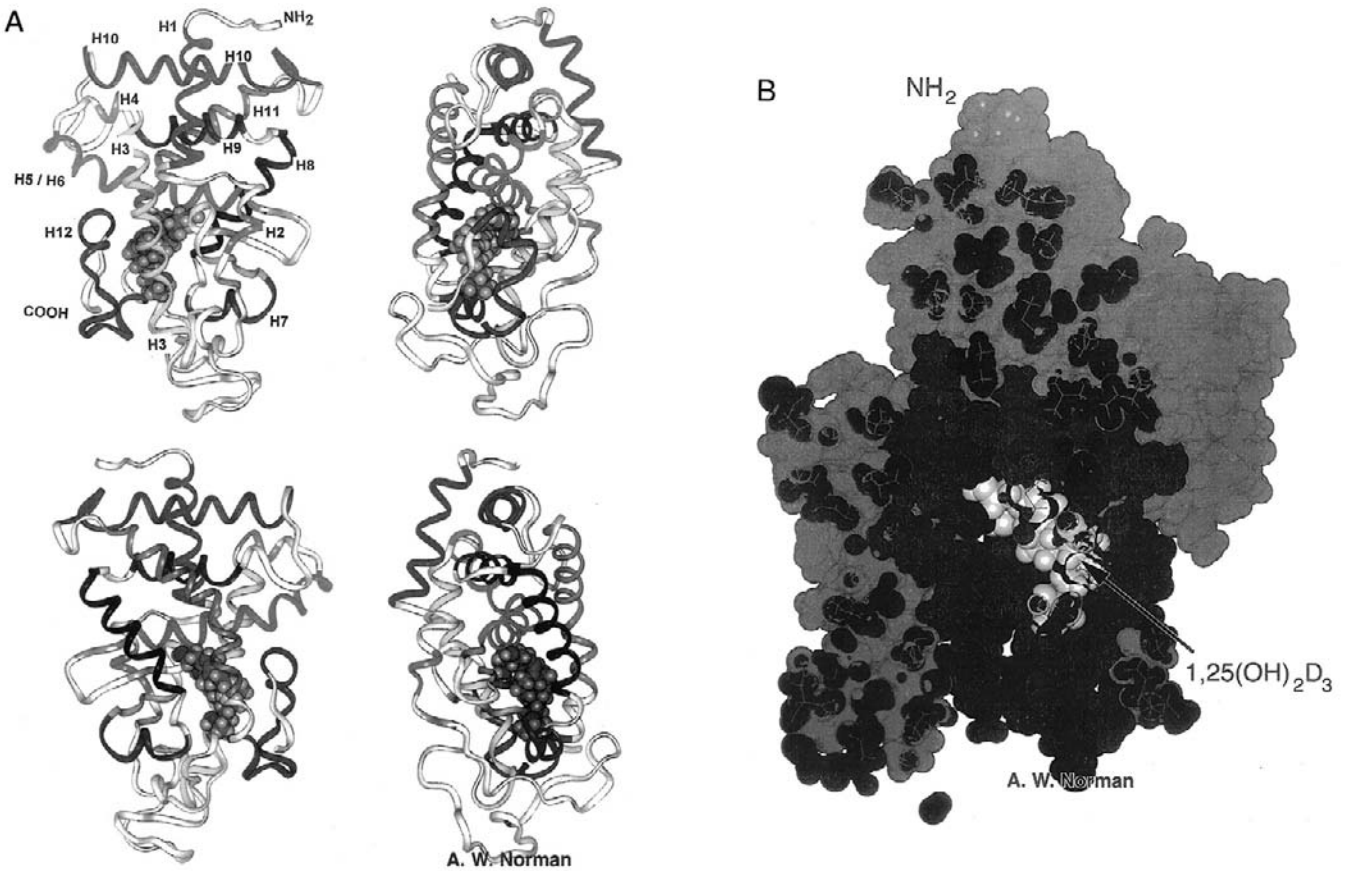


Figure 9 Structural representations of the ligand-binding domain of the nuclear receptor for 1α,25(OH)$_2$D$_3$ derived from X-ray crystallographic analysis (Rochel *et al.,* 2000). The model of the LBD of the human VDR$_{nuc}$ spans amino acid residues 143–427 (COOH terminus) (Baker *et al.,* 1988) and is very similar to that of the LBD of the other five nuclear (TR, ER, P, RaR, and PPAR) receptor structures (Weatherman *et al.,* 1999). Different representations of the same 3D model of hVDR$_{nuc}$ are illustrated. The ligand in each panel is 6-s-*trans* 1α,25(OH)$_2$D$_3$. (A) Ribbon views illustrate four successive 90° rotations of the 12 α helices and β strands that collectively define the VDR$_{nuc}$ model. Helices are numbered in the same order as TR, RAR, and ER structures. (B) An in-plane "slice" exposing the interior so that 1α,25(OH)$_2$D$_3$ is visible. (See also color plate.)

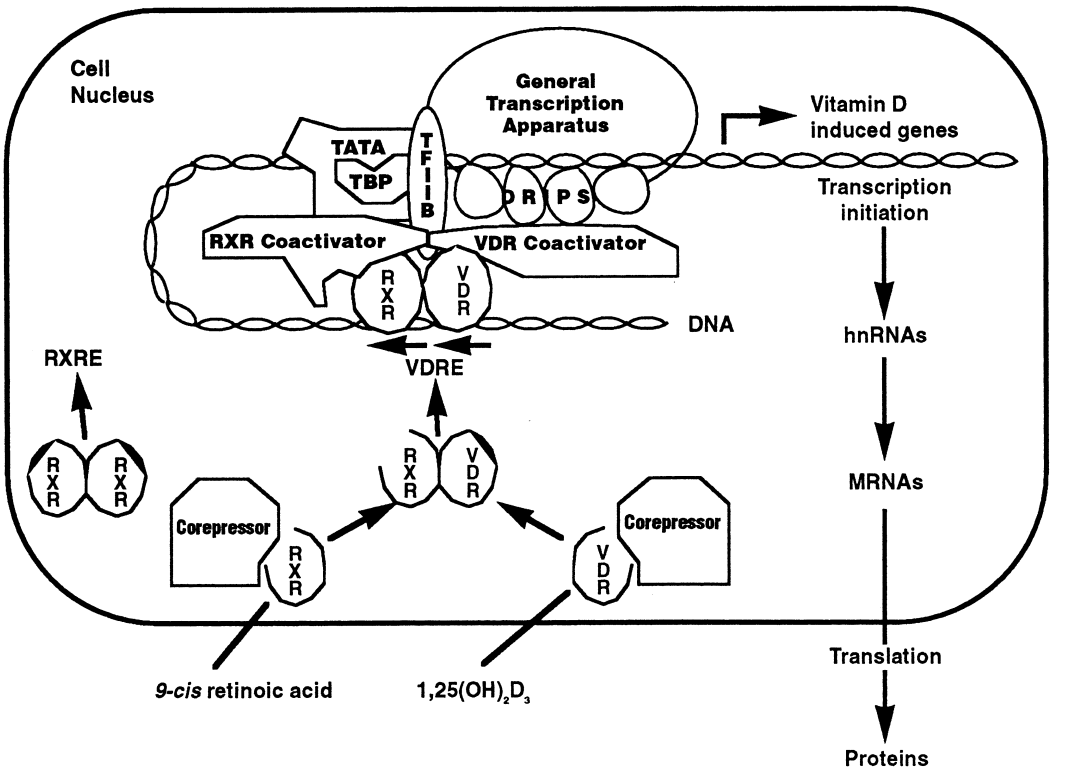


Figure 10 Model of 1α,25(OH) and VDR_{nuc} activation of transcription. The VDR after binding its cognate ligand $1\alpha,25(OH)_2D_3$ forms a heterodimer with RXR. This heterodimer complex then interacts with the appropriate VDRE on the promoter of genes (in specific target cells), which are destined to be up- or downregulated. The heterodimer–DNA complex then recruits necessary coactivator proteins, TATA, TBP, TFIIB, and other proteins to generate a competent transcriptional complex capable of modulating mRNA production.

Figure 10 presents a schematic model of the VDR_{nuc} interaction with its heterodimer partner and their subsequent interaction with the promoter of genes selected for modulation, as well as with other proteins (coactivators, TATA binding protein, etc.) so as to generate a competent transcriptional complex. Over the past decade there has been a continuing evolution of understanding and complexity concerning the details of what constitutes a "competent transcriptional complex." Additional viewpoints and information can be found elsewhere (Barrett *et al.,* 1999; Glass *et al.,* 2000; Leo *et al.,* 2000; Weatherman *et al.,* 1999).

Genetics and the Vitamin D Endocrine System

MUTATIONS IN VDR_{NUC}

Table IV summarizes the 6 natural and 26 experimental mutations in the LBD of the VDR_{nuc} that have identified amino acids critical for normal LBD function. There are also at least 14 other natural mutations in the zinc finger DNA-binding domain C of the VDR_{nuc} [data not presented but reviewed in Haussler *et al.,* (1997b)].

Hereditary vitamin D-resistant rickets (HVDRR), also known as vitamin D-dependent rickets, type II (VDDRII), is a rare genetic disease. Genetic analysis has shown that it is autosomal recessive. Less than 30 kindreds have been reported. The combination of symptoms, i.e., defective bone mineralization, decreased intestinal calcium absorption, hypocalcemia, and increased serum levels of $1\alpha,25(OH)_2D_3$, suggests end-organ resistance to the action of $1\alpha,25(OH)_2D_3$. Patients do not respond to doses of vitamin D, $25(OH)D_3$, or $1\alpha,25(OH)_2D_3$.

The unresponsiveness to $1\alpha,25(OH)_2D_3$ associated with HVDRR has been demonstrated to arise from defects in the gene coding for the VDR_{nuc}. Two types of abnormalities have been defined by binding studies: receptor-negative and receptor-positive phenotypes (see Table IV). Mutations identified in the receptor-negative phenotype involve a mutation that introduces a premature stop codon in the message. The resulting truncated protein is not able to bind ligand. The receptor-positive phenotype arises from one of several missense mutations localized within the zinc finger domains of the DNA-binding domain. Several of these mutant receptors have been demonstrated to be defective in their ability to bind to DNA-cellulose and to be unable to mediate $1\alpha,25(OH)_2D_3$-stimulated gene transcription *in vitro* (Kristjansson *et al.,* 1993; Ritchie *et al.,* 1989; Rut *et al.,* 1994; Sone *et al.,* 1989).

KNOCKOUT OF THE VDR_{NUC}

An animal model of HVDRR was engineered by targeted disruption of DNA encoding the first and the second zinc finger of the DNA-binding domain of the VDR, respectively, by two different groups independently (Li *et al.,* 1997; Yoshizawa *et al.,* 1997). The resultant animals were phenotypically normal at birth. No defects in development and

Table IV Genetic Analysis of the Nuclear Receptor for 1α, 25(OH)$_2$D$_3$: Site of Mutation in the Nuclear Receptor for 1α, 25(OH)$_2$D3

VDR domain	Mutation	Functional consequence	Reference
DNA-binding domain	R30Stop	Premature termination: no DNA binding, no ligand binding	Mechica *et al.* (1997)
	R73Stop		Wiese *et al.* (1993)
	R88Stop		Mechica *et al.* (1997)
	Point mutation intron 4 results in premature stop codon		Hawa *et al.* (1996)
	R30G	Mutations occurring at highly conserved amino acid residue	Sone *et al.* (1989)
	G33D	within the first and second zinc fingers. Mutation interferes	Hughes *et al.* (1988)
	H35Q	with the ability of the receptor to interact normally with DNA	Haussler *et al.* (1997a)
	K421		Rut *et al.* (1994)
	K43E		Rut *et al.* (1994)
	F441		Rut *et al.* (1994)
	G46D		Lin *et al.* (1996)
	R50Q		Saijo *et al.* (1991)
	R70D		Sone *et al.* (1989)
	R73Q		Hughes *et al.* (1988)
	R80Q		Malloy *et al.* (1994)
	K91N/E92Q		Hsieh *et al.* (1995)
Hinge region	148Stop	Premature termination: no ligand binding	Wiese *et al.* (1993)
	Q152Stop		Kristjansson *et al.* (1993)
	Y295Stop	Premature termination no ligand binding	Ritchie *et al.* (1989)
	C190W	HVDRR	Malloy *et al.* (1994)
Ligand-binding domain	S208G	Phosphorylation that modulates transcription.	Jurutka *et al.* (1996)
	S208A	No enhancement of transcription	
	F244G	Impaired transactivation; no RXR dimers	Whitfield *et al.* (1995)
	K246G	Impaired transactivation	Whitfield *et al.* (1995)
	L254G	Impaired transactivation; no RXR dimers	Whitfield *et al.* (1995)
	Q259G		
	L262G		
	R274L	HVDRR	Malloy *et al.* (1994)
	C288G	Impaired ligand binding	Nakajima *et al.* (1996)
	H305Q	Decreased binding (slight); decreased transactivation	Malloy *et al.* (1997)
	1314S	Impaired transactivation and RXR dimerization	Whitfield *et al.* (1996)
	C337G	Impaired ligand binding	Nakajima *et al.* (1996)
	C369G	Impaired transactivation and RXR dimerization	Whitfield *et al.* (1995)
	R391C		Whitfield *et al.* (1996)

growth were observed before weaning, irrespective of reduced expression of vitamin D target genes. After weaning (3 weeks after birth), however, VDR-null mutant mice showed marked growth retardation. No overt abnormalities, however, were found in the heterozygotes even at 6 months. Unexpectedly, all of the VDR-null mutant mice developed alopecia and had few whiskers by 7 weeks. Further, the serum levels of calcium and phosphate were reduced at 4 weeks, with markedly elevated serum alkaline phosphatase activity present in the null-mutant mice, whereas in older VDR-deficient mice, these abnormalities became more prominent. These observations in the VDR-null mutant mice are similar to those in a human vitamin D-dependent rickets type II disease, in which mutations in the VDR gene have been identified in several families, although this disease is not lethal.

In the VDR-null mutant mice at 3 weeks, the serum levels of 1α,25(OH)$_2$D$_3$, 24R,25(OH)$_2$D$_3$, and 25(OH)D$_3$ were the same as those in the heterozygous and wild-type mice. However, a marked 10-fold increase in serum 1α,25(OH)$_2$D$_3$ and a clear reduction (to almost undetectable levels) in serum 24R,25(OH)$_2$D$_3$ developed in the VDR-null mutant mice at 4 weeks and persisted at 7 weeks. Immunoreactive PTH levels were also raised sharply after weaning, and the size of the parathyroid glands in the 70-day-old VDR-ablated mice was increased more than 10-fold. These observations establish that VDR is essential for regulation of the 1α- and 24R-hydroxylases by 1α,25(OH)$_2$D$_3$ after weaning, again supporting the idea that VDR plays a critical role only after weaning. The authors suggest that a functional substitute for VDR is present in milk.

Severe bone malformation was induced by the inactivation of the VDR after weaning. Radiographic analysis of VDR-null mutant mice at 7 weeks revealed growth retardation with loss of bone density. A 40% reduction in bone mineral density was observed in the homozygote mutant mice. In gross appearance and on X-ray analysis of tibia and fibula, typical features of advanced rickets were observed, including widening of epiphyseal growth plates, thinning of the cortex, fraying, cupping, and widening of the metaphysis. In marked contrast, in the VDR-ablated mice in whom normal mineral ion homeostasis had been preserved by feeding of a high-calcium, high-lactose diet,

none of these bone parameters were significantly different from those in wild-type littermates raised under identical conditions. Particularly, the morphology and width of the growth plate were indistinguishable from those in wild-type controls, demonstrating that a calcium/phosphorus/lactose-enriched diet started at 16 days of age in the VDR-null mice permits the development of both normal morphology in the growth cartilage and adjacent metaphysis and normal biomechanical competence of cortical bone. Thus, the remarkable conclusion is that there is no clear contribution of the VDR to normal bone development, skeletal growth, maturation, and remodeling. The major contribution of VDR_{nuc} is its role in intestinal calcium absorption.

The male and female VDR-null mutant mice were infertile. Uterine hypoplasia and impaired folliculogenesis were observed in the female, and decreased sperm count and motility with histological abnormality of the testis were observed in the male. Aromatase activities in these mice were low in the ovary, testis, and epididymis. These results indicated that vitamin D is essential for full gonadal function in both sexes (Kinuta *et al.,* 2000).

KNOCKOUT OF THE 25(OH)D_3–24-Hydroxylase

24R,25$(OH)_2D_3$ is the second major dihydroxylated metabolite of vitamin D_3, which is found in significant concentrations in the serum of humans (Castro-Errecaborde *et al.,* 1991; Jongen *et al.,* 1989; Nguyen *et al.,* 1979), rats (Jarnagin *et al.,* 1985), and chicks (Goff *et al.,* 1995). Although the production of 24R,25$(OH)_2D_3$ by the kidney is tightly regulated (Henry *et al.,* 1984), the biological importance of this compound is still the subject of uncertainty and question (Norman *et al.,* 1982a,b). While several possible biological roles and sites of action have been suggested for 24R,25$(OH)_2D_3$, including the regulation of parathyroid hormone release from the parathyroid gland (Canterbury *et al.,* 1978; Norman *et al.,* 1982a), most studies concerning this vitamin D metabolite have focused on its possible actions on bone biology (Nakamura *et al.,* 1992; Norman *et al.,* 1993b; Seo *et al.,* 1997a). The possible existence of a nuclear or cytosolic-binding protein for 24R,25$(OH)_2D_3$ was reported in the chick parathyroid gland (Merke *et al.,* 1981), the long bone of rat epiphysis (Corvol *et al.,* 1980), and the chick tibial fracture-healing callus (Seo *et al.,* 1996a). However, there has been no general confirmation of these early findings. Also, several more recent reports have described specific actions or accumulation of 24R,25$(OH)_2D_3$ in cartilage (Corvol *et al.,* 1980; Seo *et al.,* 1996b) and bone fracture-healing callus tissue (Lidor *et al.,* 1987; Seo *et al.,* 1997a,b).

A strain of mice deficient for the 25(OH)D-24-hydroxylase enzyme has been generated (St.Arnaud *et al.,* 1997) through homologous recombination in embryonic stem cells in order to address the physiological functions of 24R,25$(OH)_2D_3$. The targeted mutation effectively deleted the heme-binding domain of the cytochrome P450 enzyme, ensuring that the mutated allele could not produce a functional protein. Analysis of the phenotype of the knockout animals revealed fascinating and previously unrecognized roles for 24R,25$(OH)_2D_3$. About half of the mutant homozygote mice born from heterozygote females died before weaning. Bone development of those survivors was abnormal in homozygous mutants born of homozygous females. Histological analyses of the bones from these mice revealed an accumulation of unmineralized matrix at sites of intramembranous ossification, particularly the calvaria and exocortical surface of long bones. However, the growth plates from these mutant animals appeared normal, suggesting that 24R,25$(OH)_2D_3$ is not a major regulator of chondrocyte maturation *in vivo.*

Evidence for a Membrane Receptor (VDR_{mem}) for 1α,25$(OH)_2D_3$

It was originally proposed that some rapid actions of 1α,25$(OH)_2D_3$ may be mediated at the cell membrane, i.e., by a membrane receptor (Nemere *et al.,* 1984). For transcaltachia (the rapid hormonal stimulation of intestinal calcium transport), a candidate membrane receptor [VDR_{mem}] has been identified and partially purified (Nemere *et al.,* 1994). A seven-step purification of the putative VDR_{mem} has been presented where the average enrichment in binding (purification) of [^{3}H]-1,25$(OH)_2D_3$ was ≈ 4500-fold (Nemere *et al.,* 1994). The detergent-solubilized purified basal–lateral VDR_{mem} exhibited a specific and saturable binding for 1α,25$(OH)_2D_3$; the K_D was 0.72×10^{-9} *M* and the B_{max} was 0.24×10^{-12} mol/mg protein. The purified protein migrated on a Superose column with a molecular mass of ≈ 60-kDa. At the present time the VDR_{mem} must be designated as "putative" because it has not yet been cloned so as to reveal its biochemical structure.

Other laboratories have also presented evidence for the existence of a VDR_{mem}. These include presence of the VDR_{mem} in human leukemic NB4 cells (Berry *et al.,* 1999; Bhatia *et al.,* 1995), intestinal enterocytes (Lieberherr *et al.,* 1989), ROS 24/1 cells (Baran *et al.,* 1994), and chondrocyte matrix vesicles (Pedrozo *et al.,* 1999; Schwartz *et al.,* 1988); in some instances, a partial purification has been effected (Baran *et al.,* 1998). Several reviews of rapid responses to 1α,25$(OH)_2D_3$ have appeared (Nemere *et al.,* 1999; Norman, 1997).

Some have questioned what is the true physiological relevance of 1α,25$(OH)_2D_3$-mediated rapid response because no phenotype or disease has yet been described. One hypothesis advanced by Gniadecki (1998b) is that 1α,25$(OH)_2D_3$-initiated rapid responses generate signal transduction pathways, which have the end result of altering gene transcription. Certainly the ability of the VDR_{nuc} operating in the nucleus to activate/suppress gene transcription of appropriate genes is well established. However, the process of genomic signaling lacks two important characteristics. (a) Rapidity: it can take several hours to achieve the transition to a new transcriptional steady state, which requires the integrated change at the transcription level of a gene followed by translational generation of the product protein in adequate amounts and, if necessary, post-translational modifications of the protein. When the production of multiple proteins is required for the desired biological response, the time may be increased even further. (b) Modula-

tion: The fine-tuning and expansion of the initiating signal to change gene transcription. Although the primary stimulus for a biological response is principally dependent on the number of activated liganded VDR$_{nuc}$ heterodimer + coactivator complex bound to the promoter, it may be possible for other signal transduction pathways (MAP kinase activation) to modulate the final outcome. One report clearly demonstrated, via gene array analysis, how activation of MAP kinase altered the expression of 383 genes (Roberts *et al.,* 2000).

In addition, the appearance of visible phenotypes is not necessarily the *sine qua non* for physiological importance. Although it is known that VDR$_{nuc}$ exists in 33 target organs/cell types (Bouillon *et al.,* 1995), it was surprising that a VDR$_{nuc}$KO mouse could be born and, after rescue with a high Ca^{2+} diet, be essentially normal, except for the slow development of alopecia (Amling *et al.,* 1999; Yoshizawa *et al.,* 1997) and, in one strain, uterine hypoplasia (Yoshizawa *et al.,* 1997).

At least three classes of candidate VDR$_{mem}$ can be envisioned. These include receptors with intrinsic tyrosine kinase activity, receptors without tyrosine kinase activity, and receptors coupled to G proteins. An activated G protein-coupled receptor could be linked to several second messenger pathways that could lead to the ultimate activation of Raf, MEK1/MEK2, and the MAP kinases, ERK1/ERK2. It will be essential to identify those upstream second messengers that become activated when the putative VDR$_{mem}$ is occupied by the 6-s-*cis* shape of 1α,25(OH)$_2$D$_3$ and result in the activation of MAP kinase. The prime second messenger candidates that have been shown by a number of laboratories to be modulated by 1α,25(OH)$_2$D$_3$ include Shc (Gniadecki, 1996), Grb2 (Gniadecki, 1996), Src (Gniadecki, 1998a; Khare *et al.,* 1997), Ras.GTP, PLCβ (Le Mellay *et al.,* 1997), PLCγ (Khare *et al.,* 1997), PKCα (Berry *et al.,* 1996; Bissonette *et al.,* 1994), and PKCδ (Berry *et al.,* 1996; Bissonette *et al.,* 1994). This will undoubtedly be an area of intensive investigation in the future.

Preferred Ligand Shape for VDR$_{nuc}$, VDR$_{mem}$, and DBP

Table V summarizes and contrasts the ligand structural preferences of the nuclear receptor, the VDR$_{nuc}$, the putative

Table V Ligand Structural Preferences of the Nuclear Receptor and Putative Membrane Receptor for 1α, 25(OH)$_2$D$_3$ and the Vitamin D-Binding Protein[a]

Property	DBP	VDR$_{nuc}$	VDR$_{mem}$
K_D for 1α,25(OH)$_2$D$_3$	5×10^{-7} *M*	$1-4 \times 10^{-10}$ *M*	$2-7 \times 10^{-10}$ *M*
Number of amino acids (human form)	458	427	Not known
Molecular mass protein	58 kDa	51 kDa	≈ 60 kDa
Agonist B-ring orientation	Neither planar 6-s-*cis* (JN) nor planar 6-s-*trans* (JB) shapes bind well	Bowl-shaped 11 6-s-*trans* shape with A ring 30° above the plane of the C/D rings (from X-ray crystallography; see Fig. 11)	Planar 6-s-*cis* conformer (JN) active Planar-s-*trans* (JB) not functional (see Fig. 4)
A ring hydroxy C-1 C-3	Binding to DBP	Calbindin-D$_{28k}$ induction (genomic response)	Transcaltachia (rapid response)
α β	1α,25(OH)$_2$D$_3$ = 100%	1α,25(OH)$_2$D$_3$ = 100%	1α,25(OH)$_2$D$_3$ = 100%
α α	800% (HJ)	10%	75%
β α	6570% (HH)	1%	25%
β β	450% (HL)	1%	0%; but is an antagonist
Side chain properties	Rigid; binding enhanced with aromatic ring (DF)	Semirigid	Not yet studied
Side chain orientation at C-20	Not known	20S more active (analog IE) than 20R (analog C)	Both 20S and 20R ≅ active
Antagonist analog	Not relevant	Analog MK	Analog HL
Antagonist functional shape	Not relevant	Side chain present as a cyclic lactone, which causes a conformational change in the VDR$_{nuc.}$	Probably planar 6-s-*cis*
General reference citations	Bogaerts *et al.* (2000); Swamy *et al.* (2000)	Norman *et al.* (1999); Van Baelen *et al.* (1980)	Norman *et al.* (1997, 2000a)

[a]In all studies described in this table, the conformationally flexible 1α,25(OH)$_2$D$_3$ was the reference compound where its value in each assay is by definition 100%. The rapid responses studied to define the preferred B-ring orientation for the VDR$_{mem}$ included transcaltachia in the perfused chick intestine and $^{45}Ca^{2+}$ influx in ROS 17/2.8 cells (Norman *et al.,* 1997). The genomic responses used to define the preferred B-ring orientation included transcriptional activation of osteocalcin in MG-63 cells grown in culture (Norman *et al.,* 1997). Additional information on the analogs specified by the two-letter codes (*e.g.,* HL) are provided in Table VI and the structures are presented in Fig. 12.

membrane receptor, the VDR_{mem}, as well as for the plasma transport vitamin D-binding protein, DBP. It is apparent that each of these proteins has a unique ligand-binding domain, which specify that their preferred ligand conformations for the conformationally flexible $1\alpha,25(OH)_2D_3$ are all strikingly different from one another.

It has been generally assumed for receptor–ligand interactions that the ligand is frozen in a single conformation dictated by both the structural constraints of the ligand and the three-dimensional architecture of the peptide chains that create the ligand binding domain of the receptor(s). Ligands for the TR and RAR, as for the VDR_{nuc}, are all conformationally flexible, and the X-ray crystallographic structure for each receptor has revealed that *only one definitive conformer* was present in their ligand-binding domain (Renaud *et al.,* 1995; Wagner *et al.,* 1995). This clearly demonstrates that steroid receptors can capture one ligand conformation from a large population of flexible conformers.

Figure 11 illustrates the preferred conformation of the agonist ligands for the VDR_{nuc} and the VDR_{mem}. It is now known from the X-ray structure of the VDR_{nuc} LBD with $1\alpha,25(OH)_2D_3$ present as a ligand that the preferred agonist

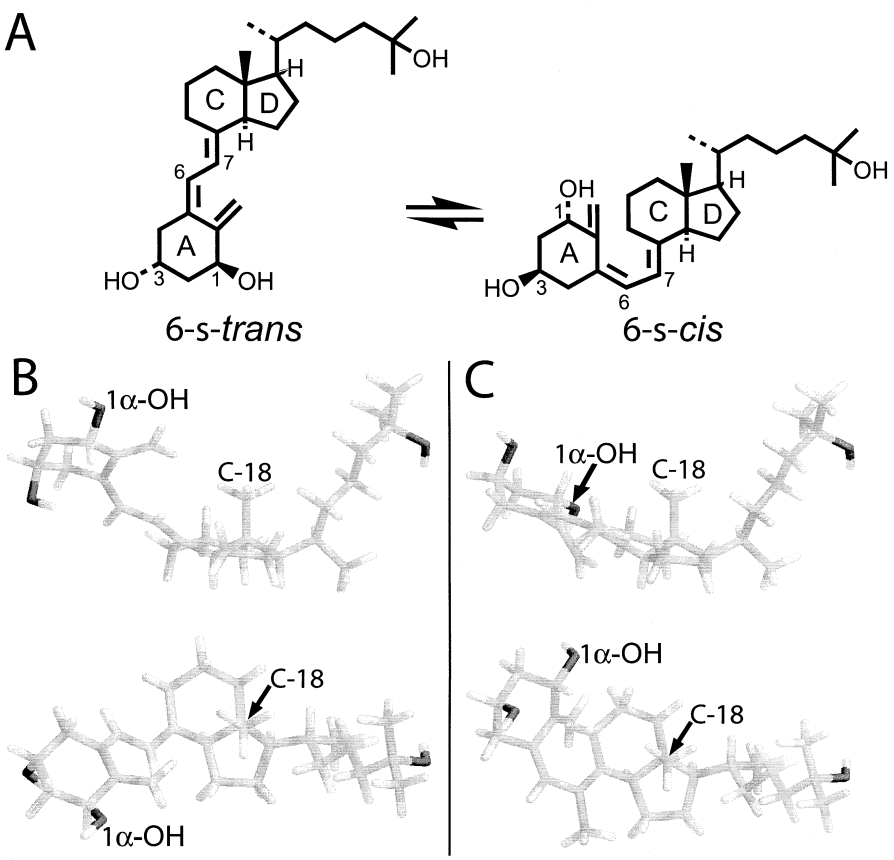


Figure 11 Preferred optimal conformation of agonist ligands for VDR_{nuc} genomic responses (left) and VDR_{mem} rapid responses (right). The three hydroxyl groups in each molecule are shown as black sticks, whereas the remainder of the molecule is shown in gray stick form. For B and C, upper representations show the side view or "edge" representation of the C/D rings + side chain, whereas the lower representations have been rotated 90° toward the viewer. (A) Standard representation of $1\alpha,25(OH)_2D_3$ illustrating the consequences of rotation around the 6,7 carbon bond to generate a population of shapes, including the extremes of 6-s-*trans* and 6-s-*cis* conformers. (B) The stick representation of the shape of $1\alpha,25(OH)_2D_3$ that is present as a ligand in the VDR_{nuc} as revealed by X-ray crystallography (Rochel *et al.,* 2000). The characteristic feature of the ligand in the LBD in the "edge" representation is the bowl-shaped twisted 6-s-*trans* shape with the A ring 30° above the plane of the C/D rings. In addition, the A ring is present as the chair conformer-B, where the 1α-OH is equatorial and the 3β-OH is axial (see Fig. 2C). (C) Shape of the 6-s-*cis* conformer of $1\alpha,25(OH)_2D_3$ is that proposed to be the optimal ligand for the VDR_{mem} as revealed by analog studies (Norman *et al.,* 1993c, 1997). The A ring is present as a chair conformer-B (see Fig. 2C) where the 1α-OH is equatorial and the 3β-OH is axial (see Fig. 2C). The 6-s-*cis*-locked $1\alpha,25(OH)_2$–7-dehydrocholesterol (analog JN; see Fig. 12) is a full agonist of all rapid responses studied thus far (see Tables II and VI). Here the A ring is virtually in the same plane as the C/D rings. The difference in ligand shape for the VDR_{nuc} and VDR_{mem} is particularly evident in the two lower stick representations of B and C. Here the C/D rings + side chain are in the same precise orientation for both representations (they could be superimposed), but the A ring of the VDR_{mem} is planar above the C/D rings, whereas that of the VDR_{nuc} is below the C/D rings.

ligand shape is that represented by a twisted 6-s-*trans* bowl (Rochel *et al.,* 2000). In contrast, an extensive series of studies support the conclusion that the preferred ligand shape for the VDR$_{mem}$ is that represented by a 6-s-*cis*-locked analog such as represented by analogs JM or JN (Norman *et al.,* 1993c, 1997; Zanello *et al.,* 1997). Table VI summarizes the properties of two 6-s-*cis* conformationally restricted analogs, JM and JN, which have achieved prominence in defining the preferred shape for the VDR$_{mem}$.

The preferred ligand for DBP is 25(OH)D$_3$, whereas the preferred ligand of the VDR$_{nuc}$ is 1α,25(OH)$_2$D$_3$. Thus, 25(OH)D$_3$ binds 668-fold more tightly to DBP than 1α,25(OH)$_2$D$_3$. In contrast, 1α,25(OH)$_2$D$_3$ binds 668-fold more tightly to the VDR$_{nuc}$ than 25(OH)D$_3$ (Bishop *et al.,* 1994). As noted in Tables V and VI, some, but not all, of the DBP ligand preferences are somewhat similar to those of the VDR$_{nuc.}$ DBP, like the VDR$_{nuc,}$ prefers neither the precise 6-s-*cis* nor 6-s-*trans* shapes of the B ring, as represented by analogs JN and JB, respectively, but an intermediate shape comparable to analog JW [see Table VI (Norman *et al.,* 1997)]. In JW the plane of the A ring is below the plane of the C/D rings, as dictated by the R orientation of carbon 6; when this is inverted to 6S (as analog JV) so that the A ring is above the plane of the C/D ring, the DBP RCI is reduced ≈20-fold.

Furthermore, some analogs with side chain rigidity have an enhanced binding to both VDR$_{nuc}$ and DBP; the side chain analog LA [(22R)-1α,25(OH)$_2$-16,22,23-triene-D$_3$] with a rigid allene functionality binds 1.54-fold better than 1α,25(OH)$_2$D$_3$ to the VDR$_{nuc}$ (Bouillon *et al.,* 1995), whereas analog DF [22-*p*(-hydroxyphenyl)-23,24,25,26,27-pentanor-1α-(OH)-D$_3$], with an aromatic ring, binds 19.9-fold better than 1α,25(OH)$_2$D$_3$ to DBP (Bishop *et al.,* 1991, 1994). Impressively, analog JX [22-*p*(-hydroxyphenyl)-23,24,25,26,27-pentanor-D$_3$], an analog without a 1α-hydroxyl but with a side chain aromatic ring, binds 2110-fold better than 1α,25(OH)$_2$D$_3$ and 3.15-fold better than 25(OH)D$_3$ to DBP. As summarized in Table V, there are also important differences in ligand affinity between DBP and VDR$_{nuc}$ that are based on the orientation of the carbon 1- and 3-hydroxyls of the A ring. Thus, analog HJ, which has 1α-OH, 3α-OH hydroxyls, binds 8-fold better to DBP than 1α,25(OH)$_2$D$_3$ with 1α-OH, 3β-OH hydroxyls. Similarly, analog HH with a 1β-OH, 3β-OH, binds 65-fold better to DBP than 1α,25(OH)$_2$D$_3$. Thus it is clear that the three-dimensional structure of the DBP LBD imposes unique constraints on the conformation of its preferred ligand, which are different from that of the VDR$_{nuc}$ LBD.

Analogs of 1α, 25(OH)$_2$D$_3$

Extensive efforts in many laboratories and pharmaceutical companies had already generated hundreds of analogs of 1α,25(OH)$_2$D$_3$, which have collectively provided insight into the ligand specificities of the VDR$_{nuc}$ LBD for treatment. Table VI summarizes the properties of several conformationally flexible analogs, which have achieved prominence; all of these conformationally flexible analogs in principal can assume the twisted 6-s-*trans* bowl conformation. The family of 20-epi analogs, represented by ID and IE, has been intriguing; inversion of orientation of the side chain at C-20 results in analogs that are 500- to 200-fold more potent than 1α,25(OH)$_2$D$_3$ with respect to activation of gene expression (Liu *et al.,* 1997; Peleg *et al.,* 1995). Analog KH, which has two eight-carbon side chains, surprisingly is an effective ligand for the VDR$_{nuc}$ (Norman *et al.,* 2000b) (Fig. 12).

An important pair of discoveries was of two analogs of 1α,25(OH)$_2$D$_3$ selectively functioning antagonists either of the VDR$_{mem}$ or the VDR$_{nuc}$. Analog HL is a specific antagonist of rapid responses; these include inhibition of transcaltachia (Norman *et al.,* 1993a), $^{45}Ca^{2+}$ uptake into ROS 17/2.8 cells (Norman *et al.,* 1997), activation of chloride currents in ROS 17/2.8 cells (Zanello *et al.,* 1997), and MAP kinase activation in NB4 cells (Song *et al.,* 1998). In contrast, analog MK is a specific antagonist of the VDR$_{nuc}$ and, when bound to the receptor, blocks the necessary conformation change of the receptor protein LBD helix 12 (see Fig. 9) essential for transactivation. Thus MK blocks gene expression both in whole cells (Miura *et al.,* 1999b) and *in vivo* in the rat (Ishizuka *et al.,* 2000).

Table VI also summarizes the interesting properties of 10 analogs of 1α,25(OH)$_2$D$_3$ that are either approved drugs or are under evaluation for drug development for a variety of vitamin D-related diseases, including osteoporosis, renal osteodystrophy, psoriasis, immunosuppression, and the bone diseases of osteoporosis and renal osteodystrophy (Bouillon *et al.,* 1995). Without exception, these analogs have been identified based on their affinity for the VDR$_{nuc}$ and usually because of a separation of *in vivo* calcemic effects from cell differentiation effects. 1α,25(OH)$_2$D$_3$ is approved for drug use in the diseases of renal osteodystrophy (in 1977), neonatal hypocalcemia, and osteoporosis (in 17 countries of the world, but not in the United States).

Two 1α,25(OH)$_2$D$_3$ analogs are available commercially for the topical treatment of psoriasis. Leo Pharmaceutical's Dovonex (BT) has been approved for clinical use in both Europe (1992) and the United States (1995), whereas Teijin's Bonalfa (CT) was approved for clinical use in Europe (1994). It is anticipated that Chugai's Maxacalcitol (EU) may in the future be approved in Japan for the treatment of psoriasis. All three analogs have structural modifications in their side chain, which tend to improve their antiproliferative and anti-inflammatory effects while diminishing their calcemic effects. Abbott Laboratory's Zemplar (MA) received approval in the United States (1998) for systemic use in the treatment of the secondary hyperparathyroidism associated with chronic renal failure. MA has the side chain of vitamin D$_2$ as well as loss of carbon 19.

Many 1α,25(OH)$_2$D$_3$ analogs are currently under evaluation with regard to their proposed use for treatment of cancers, including acute myeloid leukemia (V, LH), breast (EU, IC, ZHA) or prostate, and colon (LH). These analogs

Table VI Biological Properties of Vitamin D Metabolites and Analogs of $1\alpha,25(OH)_2D_3$ Described in Fig. 12, Table V, and Text

Topic	Analog[a] code	Analog name	Interesting property	RCI[b] DBP	RCI[b] VDR	References
Natural metabolites	C[c]	$1\alpha,25(OH)_2D_3$	Natural hormone; is the reference compound for all analogs in Table V. Approved drug for renal osteodystrophy and osteoporosis; Rocaltrol, Hoffmann-La Roche	100	100	Bouillon *et al.* (1995)
	BO	$25(OH)D_3$	Metabolite produced in liver; no known unique biological properties; binds to DBP exceedingly tightly	66,800	0.15	Bouillon *et al.* (1995)
	BS	(23*S*,25*R*)-$1\alpha,25(OH)_2D_3$-26,23-lactone	Natural metabolite with some biological activity; compare structure with analog MK, which is an antagonist of the VDR_{nuc}	na[d]	0.47	Reichel *et al.* (1987)
Conformationally flexible analogs principally directed toward the nuclear receptor for $1\alpha,25(OH)_2D_3$	V[c]	$1\alpha,25(OH)_2$-16-ene-23-yne-D_3	Possible drug candidate for acute promyelogenous leukemia and retinoblastoma; has low calcemic index	5.4	68	Zhou *et al.* (1989); Zhou *et al.* (1990)
	BT[c]	$1\alpha,24(OH)_2$-22-ene-24-cyclopropyl-D_3	Approved drug for psoriasis; Dovonex, Leo Pharmaceuticals	55	111	Binderup *et al.* (1992)
	CT[c]	$1\alpha,24(OH)_2D_3$	Approved drug for topical application for psoriasis.; Bonalfa, Teijin Pharmaceutical	na	94	Aoki *et al.* (1998)
	EU[c]	$1\alpha,25(OH)_2$-22-oxa-D_3	Proposed for breast cancer and psoriasis; Chugai Co.	22	15	Abe-Hashimoto *et al.* (1993); Matsumoto *et al.* (2000); Van de Kerkhof (1998)
	IC[c]	22a,26a,27a-tri-homo-22,24-diene-$1\alpha,25(OH)_2D_3$	Proposed for breast cancer; Leo Pharmaceuticals	na	17	Danielsson *et al.* (1997); Mathiasen *et al.* (1999)
	ID[c]	20-epi-22-oxa-24a,25a, 26a,27a-tri-homo – 1α, $25(OH)_2D_3$	20-epi orientation and side chain modifications increase antiproliferation potency 500-fold over $1\alpha,25(OH)_2D_3$; proposed as drug for autoimmune graft rejection and psoriasis; Leo Pharmaceuticals	na	25	Binderup *et al.* (1991); Bertolini *et al.* (1999); Peleg *et al.* (1995)
	IE	20-ei-$1\alpha,25(OH)_2D_3$	20-epi orientation increases genomic transactivation potency 1000-fold over $1\alpha,25(OH)_2D_3$	2.6	147	Peleg *et al.* (1995)
	KH	21-(3-hydroxy-3′-methylbutyl) $1\alpha,25(OH)_2D_3$	Analog with two side chains, which surprisingly has a VDR_{nuc} RCI = 38 and is effective at genomic transactivation	2.6	38	Norman *et al.* (2000b)
	LA	(22*R*)-$1\alpha,25(OH)_2$−16,22,23-trans-D_3	Rigidity of side chain increases the VDR_{nuc}RCI to 154	9.1	154	Bishop *et al.* (1994)
	LH[c]	$1\alpha,25(OH)_2$-16-ene-23-yne-26,26-F_6-19-nor-D_3	Proposed for prostate cancer, myeloid leukemia and colon cancer; Hoffmann-La Roche	1	14	Asou *et al.* (1998) Campbell *et al.* (1997)
	MA[c]	19-nor-$1\alpha,25(OH)_2D_2$	Approved drug for treatment of secondary hyperparathyroidism; Zemplar, Abbott Laboratory	163	56	Llach *et al.* (1998); Martin *et al.* (1998)

continues

Table VI *Continued*

Topic	Analog[a] code	Analog name	Interesting property	RCI[b] DBP	RCI[b] VDR	References
	ZHA[c]	1α,25(OH)$_2$-19-nor-14-epi-24-yne-D$_3$	Inhibited *in vitro* MCF-7 cell proliferation and retarded tumor progression in nude mice; has low calcemic properties. Potential drug candidate for breast cancer; Thermex S.A.	20	0.1	Verlinden *et al.* (2000)
Conformationally restricted 6-s-*cis* analogs principally directed toward the membrane receptor for 1α,25(OH)$_2$D$_3$	HF	1α,25(OH)$_2$-d$_5$-pre-D$_3$	A 6-s-*cis* analog, which is a full agonist for rapid responses, but with only weak binding to VDR$_{nuc}$	8.6	10.6	Norman *et al.* (1993c)
	JB	1α,25(OH)-dihydrotachysterol$_3$	A 6-s-*trans* locked analog, which is a very poor agonist of both VDR$_{nuc}$ and VDR$_{mem}$ actions	−0.34	0.12	Norman *et al.* (1997)
	JM	1α,25(OH)$_2$-7-dehydrocholesterol D$_3$	A 6-s-*cis* analog, which is a full agonist for rapid responses,but with only weak binding to VDR$_{nuc}$	−0.68	1.8	Norman *et al.* (1997)
	JN	1α,25(OH)$_2$-lumisterol	A 6-s-*cis* locked analog, which is a full agonist for rapid responses, but with only weak binding to VDR$_{nuc}$	6.6	0.005	Norman *et al.* (1997)
Receptor antagonists	HL	1β,25(OH)$_2$D$_3$	Antagonist of only VDR$_{mem}$-mediated rapid responses	450	1.0	Norman *et al.* (1993a); Zanello *et al.* (1997)
	MK	(23*S*)-25-dehydro-1α-OH-D$_3$-26,23-lactone [TEI-9847; Teijin]	Antagonist of only VDR$_{nuc}$-mediated genomic responses	na	0.57	Miura *et al.* (1999b)
	ML	(23*R*)-25-dehydro-1α-OH-D$_3$-26,23-lactone [TEI-9848; Teijin]	Antagonist of only VDR$_{nuc}$-mediated genomic responses	na	0.3	Miura *et al.* (1999b)
	MU	1β,24(OH)$_2$D$_3$	Antagonist of only VDR$_{mem}$-mediated rapid responses	na	0.5	Norman *et al.* (1993a); Zanello *et al.* (1997)
Analogs that provide insight into the ligand binding domain of the vitamin D-binding protein	DF	22-(*p*-hydroxyphenyl)-1α,25(OH)$_2$D$_3$	Aromatic ring in side chain imposes rigidity and enhances binding to DBP	1990	4.6	Bishop *et al.* (1994); Figadère *et al.* (1991)
	HH	1β,25(OH)$_2$-3-epi-D$_3$	Inversion of orientation of A-ring hydroxyls enhances binding to DBP	6570	0.22	Bishop *et al.* (1994)
	HJ	1α,25(OH)$_2$-3-epi-D$_3$	DBP binding is enhanced	800	24	Bishop *et al.* (1994)
	JW	(1*S*,3*R*,6*R*)-7, 19-Retro-1α,25(OH)$_2$D$_3$	As a quasi 6-s-*trans* locked analog, the DBP binding is enhanced	700	2.6	Bishop *et al.* (1994)
	JX	22-(hydroxyphenyl)-23,24,25,26,27-pentanor-D$_3$	Removal of the 1α-hydroxyl group present in analog DF to generate JX gives a DBP RCI value of 211,000.	211,000	0.002	Bishop *et al.* (1994)

[a] One-, two-, and three-letter analog codes refer to analogs whose structure is presented in Fig. 12; see also Bouillon *et al.* (1995).

[b] Measure of the relative competitive index of an analog in relation to 1α,25(OH)$_2$D$_3$ in binding to either the vitamin D-binding protein (DBP) or the VDR$_{nuc}$. By definition, the RCI for 1α,25(OH)$_2$D$_3$ is set to 100% for both VDR$_{nuc}$ and DBP.

[c] Analogs that are either approved drugs or drug candidates.

[d] Not assayed.

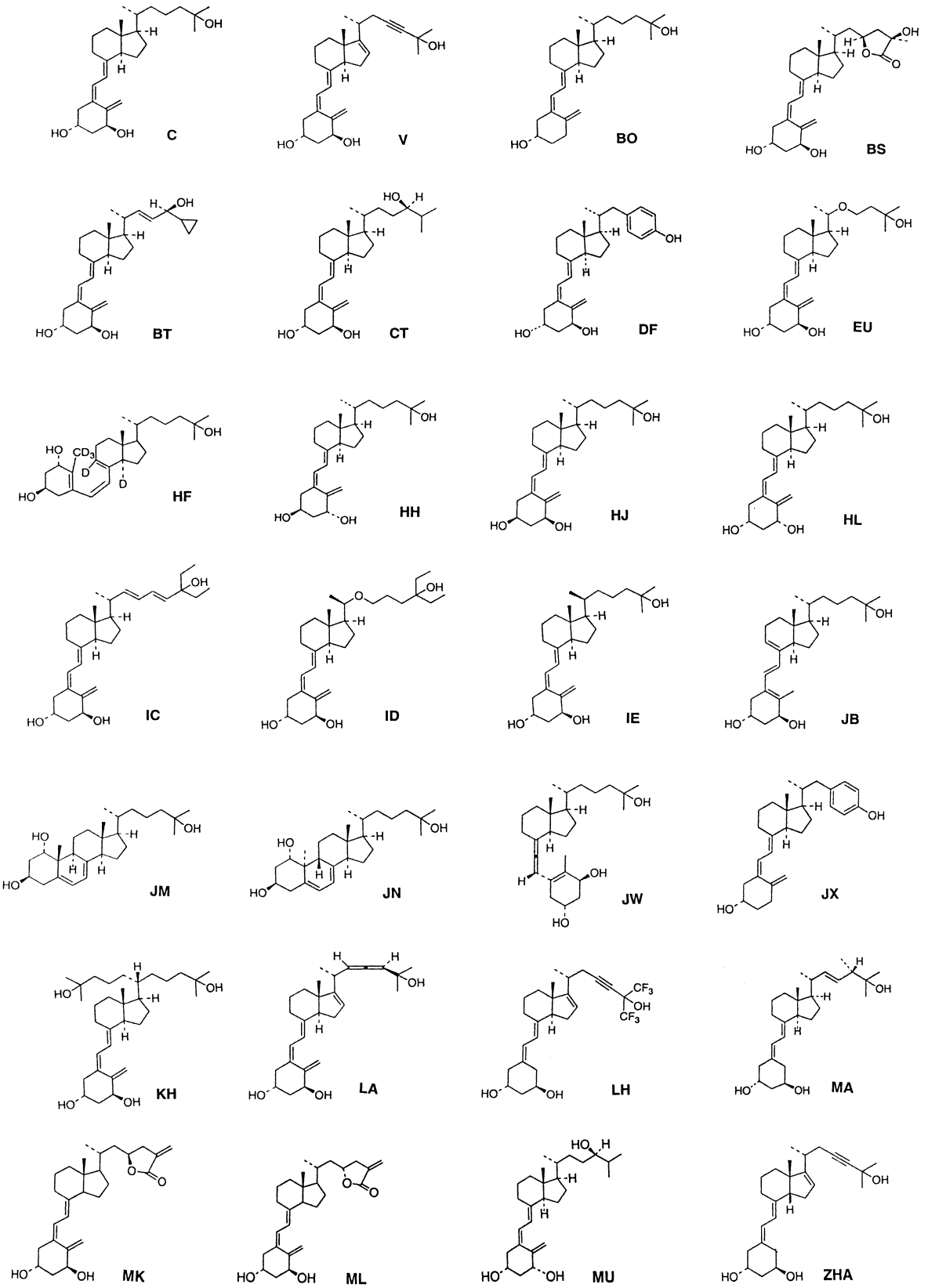


Figure 12 Important $1\alpha,25(OH)_2D_3$ metabolites, agonist analogs, including existing drugs or potential drug candidates, and antagonist analogs. Table VI presents the chemical name and summarizes the important properties of all the vitamin D-related steroids presented here and in the text.

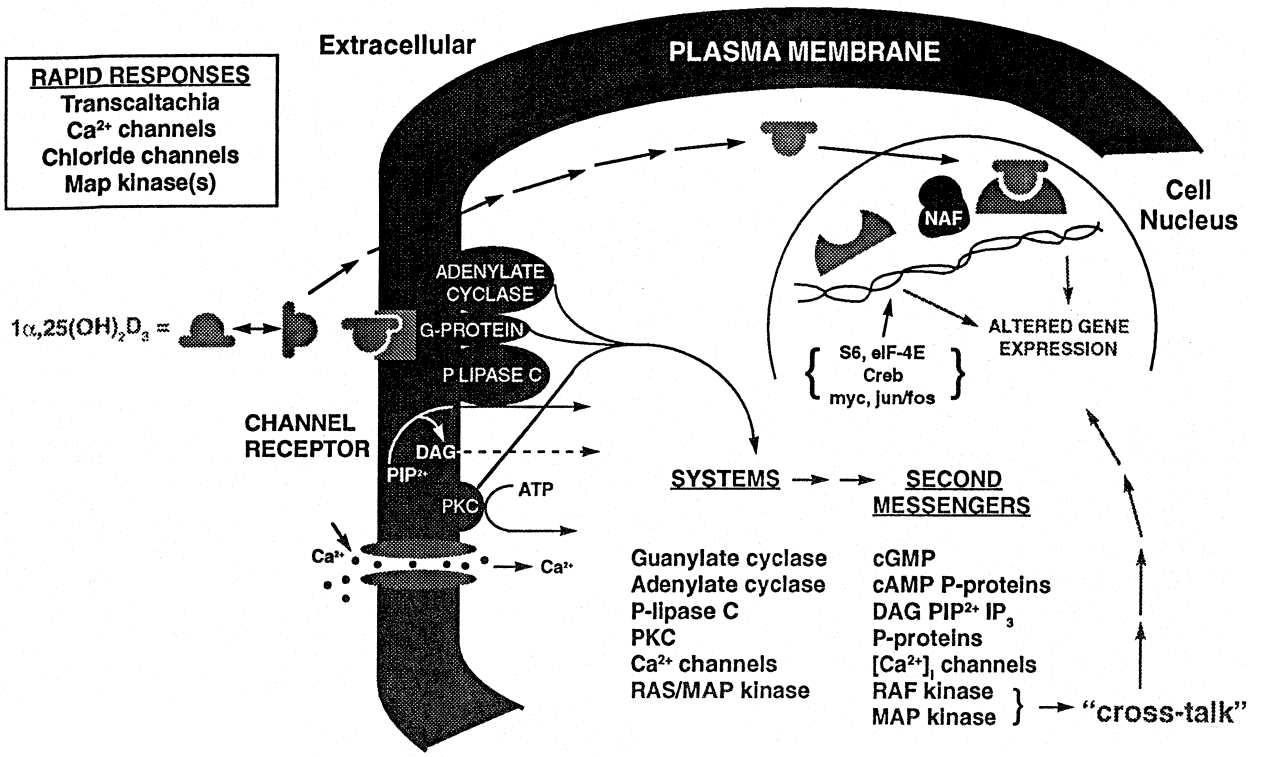


Figure 13 1α,25(OH)$_2$D$_3$ and signal transduction: a working model. The upper left inset lists the rapid responses generated by 1α,25(OH)$_2$D$_3$ whose actions are believed to be described by this schematic model. 1α,25(OH)$_2$D$_3$ can initiate biological responses via both its nuclear receptor and a putative cell membrane receptor (Nemere *et al.,* 1994), which generates rapidly the appearance of second messengers, some of which modulate via cross-talk selective events in the nucleus.

uniformly are significantly more potent than 1α,25(OH)$_2$D$_3$ with respect to their ability to inhibit cell proliferation and promote cell differentiation; all these analogs are currently being studied in animal models of the indicated cancer.

Based on the presence of the VDR$_{nuc}$ in all cells of the immune system, especially antigen-presenting cells (macrophages, and dendritic cells) and activated T lymphocytes, 1α,25(OH)$_2$D$_3$ has been shown to be a potent immunosuppressive agent (Lemire,1997). Thus it is not surprising that 1α,25(OH)$_2$D$_3$ analogs are proposed (ID) for clinical use in autoimmune graft rejection (Bertolini *et al.,* 1999) and the treatment of type I diabetes or prevention of destruction of transplanted islets in type 1 diabetes (Lemire, 1997).

Summary

A working model of a target cell for 1α,25(OH)$_2$D$_3$ is shown in Fig. 13. The signal transduction pathways linked to the VDR$_{nuc}$ and the VDR$_{mem}$ are illustrated

In contrast to classical steroid hormones, like estradiol, which have only one shape, it is apparent that 1α,25(OH)$_2$D$_3$ can generate at least three functionally different shapes, which effectively accommodate the ligand-binding domain requirements of the VDR$_{nuc}$, VDR$_{mem}$, and DBP proteins. With the identification of the target optimal shapes of ligands for the DBP, VDR$_{mem}$ and VDR$_{nuc}$, it is to be anticipated that chemists will continue their synthetic efforts to prepare conformationally restricted analogs that select one target shape for intensive biological studies. These new structures certainly will include nonsteroidal analogs that lack the classic A, seco B, C, D ring structure of 1α,25-(OH)$_2$D$_3$, but which still achieve a significant binding to the appropriate target protein; two examples of reports in this arena are already available (Boehm *et al.,* 1999; Verstuyf *et al.,* 1998). Future structure–function studies are awaited with interest.

It will also be interesting to observe over the next decade to what further extent the manipulation of 1α,25(OH)$_2$D$_3$ structure–function relationships is successful with regard to further development of new drugs that display target organ specificity of action but are devoid of undesirable calcemic effects.

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CHAPTER 32

Vitamin D Gene Regulation

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Vitamin D Metabolism

Vitamin D is a principal factor required for the development and maintenance of bone as well as for maintaining normal calcium and phosphorus homeostasis. In addition, evidence has indicated the involvement of vitamin D in a number of diverse cellular processes, including effects on differentiation and cell proliferation, on hormone secretion, and on the immune system (Darwish and DeLuca, 1993). For vitamin D to affect mineral metabolism as well as numerous other systems, it must first be metabolized to its active form. Vitamin D, which is taken in the diet or is synthesized in the skin from 7-dehydrocholesterol in a reaction catalyzed by ultraviolet irradiation, is transported in the blood by the vitamin D-binding protein to the liver. In the liver, vitamin D is hydroxylated at C-25, resulting in the formation of 25-hydroxyvitamin D_3[25(OH)D_3]. 25-Hydroxy-vitamin D proceeds to the kidney via the serum vitamin D-binding protein. In the proximal convoluted and straight tubules of the kidney nephron, 25(OH)D_3 is hydroxylated at the α position of carbon 1 of the A ring, resulting in the formation of the hormonally active form of vitamin D, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. The kidney can also produce 24,25-dihydroxyvitamin D_3[24,25$(OH)_2D_3$]. 24-Hydroxylase [24 (OH) ase] has been reported to be capable of hydroxylating the 24 position of both 25(OH)D_3 and 1,25$(OH)_2D_3$ (Darwish and DeLuca, 1993; Kumar, 1984) (see Fig. 1). Because the K_m value of 24(OH)ase for 1,25$(OH)_2D_3$ is 1/5 to 1/30 of the K_m value for 25(OH)D_3 (Inaba *et al.,* 1991; Tomon *et al.,* 1990), it has been suggested that the preferred substrate for 24(OH)ase *in vivo* may be 1,25$(OH)_2D_3$ rather than 25(OH)D_3 (Shinki *et al.,* 1992). Studies using mice with a targeted inactivating mutation of the 24(OH)ase gene [24(OH)ase] null-mutant mice] have provided the first direct *in vivo* evidence for a role for 24(OH)ase in the catabolism of 1,25$(OH)_2D_3$ (St-Arnaud *et al.,* 2000). Both chronic and acute treatment with 1,25$(OH)_2D_3$ resulted in an inability of 24(OH)ase-deficient mice to clear 1,25$(OH)_2D_3$ from their bloodstream. Impaired bone formation at specific sites (calvaria, mandible, clavicle, and periosteum of long bones) was also noted in the deficient mice. 24,25-Dihydroxyvitamin D_3 supplementation failed to correct most of the bone abnormalities. Because crossing 24(OH)ase-deficient mice to vitamin D receptor (VDR)-ablated mice totally rescued the bone phenotype, the authors suggested that elevated 1,25$(OH)_2D_3$ levels acting through VDR at specific sites and not the absence of 24,25$(OH)_2D_3$, were responsible for the abnormalities observed in bone development. Whether 24,25$(OH)_2D_3$ is an active metabolite has been a matter of debate. However, most studies indicate that 24,25$(OH)_2D_3$ appears to be relatively inactive when compared with 1,25$(OH)_2D_3$.

The production of 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ is under stringent control. Calcium and phosphorus deprivation results in enhanced production of 1,25$(OH)_2D_3$ (Henry and Norman, 1984). Phosphorus can have a direct effect on the kidney (Fukase *et al.,* 1982) and may also interact with a pituitary factor, which has been suggested to be growth hormone (Gray and Garthwaite, 1985). Elevated PTH resulting from calcium deprivation may be the primary signal mediating the calcium regulation of 1,25$(OH)_2D_3$ synthesis (Boyle *et al.,* 1972; Henry, 1985; Murayama *et al.,* 1999). However, high calcium has been reported to have a direct inhibitory effect on 1-hydroxylation (Fukase *et al.,* 1982). In addition to calcium and phosphorus, 1,25-dihydroxyvitamin D_3 also regulates its own production by inhibiting 1-hydroxylase. The synthesis of 24,25$(OH)_2D_3$ has been reported to be reciprocally regulated when compared with the synthesis of 1,25$(OH)_2D_3$ [stimulated by 1,25$(OH)_2D_3$, and inhibited by low calcium and PTH] (Henry and Norman, 1984). In addition to PTH, phospho-

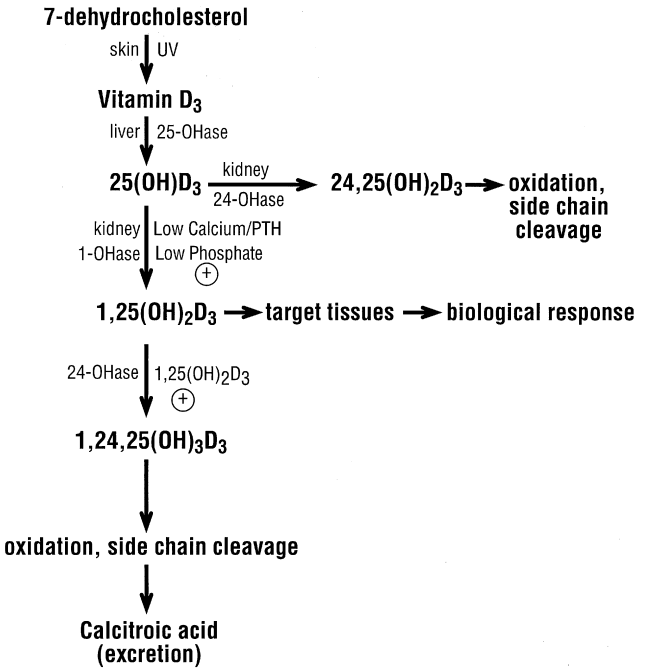

Figure 1 The metabolic pathway for vitamin D.

rus, calcium, and $1,25(OH)_2D_3$, it has been reported that sex hormones can influence production of the renal vitamin D hydroxylases. Estrogens alone or when combined with androgens have been reported to stimulate $1,25(OH)_2D_3$ production, and estradiol has been reported to suppress $24,25(OH)_2D_3$ synthesis in avian species (Pike *et al.,* 1978; Tanaka *et al.,* 1976). It is not clear, however, whether a similar relationship exists between sex steroids and vitamin D hydroxylases in mammalian species (Baski and Kenny, 1978).

Serum $1,25(OH)_2D_3$ levels and the capacity of the kidney to hydroxylate $25(OH)D_3$ to $1,25(OH)_2D_3$ have been reported to decline with age (Armbrecht *et al.,* 1980). In addition, an increase in renal 24-hydroxylase gene expression and an increase in the clearance of $1,25(OH)_2D_3$ with aging have been reported (Matkovits and Christakos, 1995; Wada *et al.,* 1992). These findings have implications concerning the etiology of osteoporosis and suggest that the combined effect of a decline in the ability of the kidney to synthesize $1,25(OH)_2D_3$ and an increase in the renal metabolism of $1,25(OH)_2D_3$ may contribute to age-related bone loss. Whether there is an interrelationship between the decline of sex steroids with age and age-related changes in the 1- and 24-hydroxylase enzymes remains to be determined.

Genomic Mechanism of Action of $1,25(OH)_2D_3$

1,25-Dihydroxyvitamin D_3, similar to other steroid hormones, is known to act by binding stereospecifically to a high-affinity, low-capacity intracellular receptor protein (vitamin D receptor or VDR), resulting in the concentration of the $1,25(OH)_2D_3$ receptor complex in the target cell nucleus and the activation or repression of the transcription of target genes (Darwish and DeLuca, 1993; Haussler *et al.,* 1998). Although the exact mechanisms involved in mediating the effects of $1,25(OH)_2D_3$ in numerous different systems are not clearly defined at this time, the VDR appears to be of major importance (Haussler *et al.,* 1998).

Role of $1,25(OH)_2D_3$ in Classical Target Tissues Bone

Exactly how $1,25(OH)_2D_3$ affects mineral homeostasis is a subject of continuing investigation. It has been suggested that the antirachitic action of $1,25(OH)_2D_3$ is indirect and the result of increased intestinal absorption of calcium and phosphorus by $1,25(OH)_2D_3$, thus resulting in their increased availability for incorporation into bone (Underwood and DeLuca, 1984; Weinstein *et al.,* 1984). Studies using VDR-ablated mice (VDR knockout mice)also suggest that a principal role of the vitamin D receptor in skeletal homeostasis is its role in intestinal calcium absorption (Li *et al.,* 1997; Yoshizawa *et al.,* 1997; Amling *et al.,* 1999). VDR knockout mice were found to be phenotypically normal at birth, but developed hypocalcemia, hyperparathyroidism, and alopecia within the first month of life. Rickets and osteomalacia were seen by day 35. When VDR knockout mice were fed a calcium/phosphorus/lactose-enriched diet, serum-ionized calcium levels were normalized, the development of hyperparathyroidism was prevented, and the animals did not develop rickets or osteomalacia, although alopecia was still observed. Thus, it was suggested that skeletal consequences of VDR ablation are due primarily to impaired intestinal calcium absorption. *In vitro* studies, however, have shown that $1,25(OH)_2D_3$ can resorb bone (Raisz *et al.,* 1972). Although $1,25(OH)_2D_3$ stimulates the formation of bone-resorbing osteoclasts, receptors for $1,25(OH)_2D_3$ are not present in osteoclasts but rather in osteoprogenitor cells, osteoblast precursors, and mature osteoblasts. Stimulation of osteoclast formation by $1,25(OH)_2D_3$ requires cell-to-cell contact between osteoblastic cells and osteoclast precursors and involves upregulation by $1,25(OH)_2D_3$ in osteoblastic cells of osteoclast differentiating factor (or osteoprotegerin ligand; see Chapter 7; Takeda *et al.,* 1999; Yasuda *et al.,* 1998a,b). Osteoclast differentiating factor/osteoprotegerin ligand, induced by $1,25(OH)_2D_3$, as well as by PTH, interleukin 11, and prostaglandin E_2 in osteoblasts/stromal cells, is a member of the membrane-associated tumor necrosis factor ligand family that enhances osteoclast formation by mediating direct interactions between osteoblast/stromal cells and osteoclast precursor cells. Osteoclastogenesis inhibitory factor/osteoprotegerin, a member of the tumor necrosis factor receptor family, is a soluble decoy receptor for osteoprotegerin ligand that antagonizes osteoprotegerin ligand function, thus blocking osteoclastogenesis. Osteoprotegerin is downregulated by $1,25(OH)_2D_3$ (Yasuda *et al.,* 1998a).

In addition to increasing the availability of calcium and phosphorus for incorporation into bone and stimulating

osteoclast formation, $1,25(OH)_2D_3$ also has a direct effect on osteoblast-related functions. For example, $1,25(OH)_2D_3$ has been reported to stimulate the production of osteocalcin (Price and Baukol, 1980) and osteopontin (Prince and Butler, 1987) in osteoblastic cells. The exact function of osteocalcin, a noncollagenous protein associated with the mineralized matrix, is not known. However, increased synthesis of osteocalcin has been positively correlated with new bone formation (Hauschka *et al.,* 1989; also see Chapter 5). Concerning osteopontin, also a major noncollagenous bone protein, it has been suggested that this secreted, glycosylated phosphoprotein is important for resorption of the bone matrix (the OPN-$\alpha_v\beta_3$ integrin interaction has been reported to be important for the adherence of the osteoclast to bone), as well as for mineralization (Denhardt and Guo, 1993; also see Chapter 15). Studies in OPN-deficient mice indicate significantly less osteoclasts in the deficient mice, confirming the *in vitro* findings of a role for OPN in osteoclast recruitment (Asou *et al.,* 1999). The Osf2/Cbfa1 transcription factor, identified as a transcriptional regulator of osteoblast differentiation, is involved in regulation of the expression of OPN and OC and has also been reported to be regulated by $1,25(OH)_2D_3$ (Ducy *et al.,* 1997; Javed *et al.,* 1999). Although the regulation of OPN, osteocalcin, and Osf2/Cbfa1 by $1,25(OH)_2D_3$ provides evidence for a role of $1,25(OH)_2D_3$ in osteoblast function, further studies are needed to define the interrelationship between the regulation by $1,25(OH)_2D_3$ of these proteins and the process of bone remodeling mediated by the osteoblast.

Intestine

In addition to its effect on bone, another extensively studied action of $1,25(OH)_2D_3$ is the stimulation of intestinal calcium absorption (Wasserman and Fullmer, 1995). Although the exact mechanisms involved in this process have still not been defined, it has been suggested that the calcium absorptive process occurs in three phases. The first phase, which may occur by nongenomic mechanisms, involves calcium transfer into the cell. The second phase occurs more slowly and involves the movement of calcium through the cell interior. It has been suggested that the interaction of $1,25(OH)_2D_3$ with the intestinal VDR and the genomic-mediated upregulation of a calcium-binding protein, known as calbindin, occurs during this phase. One of the most pronounced effects of $1,25(OH)_2D_3$ is increased synthesis of calbindin [for reviews see Christakos (1995) and Christakos *et al.* (1989)]. Two major subclasses of calbindin have been described: a protein of a molecular weight of ~9000 (calbindin-D_{9k}) and a protein with a molecular weight of ~ 28,000 (calbindin-D_{28k}). Calbindin-D_{9k} has two calcium-binding domains, has been observed only in mammals, and is present in highest concentration in mammalian intestine. Calbindin- D_{28k}, unlike calbindin-D_{9k}, is highly conserved in evolution and has four functional high-affinity calcium-binding sites. Calbindin-D_{28k} is present in highest concentrations in avian intestine and in avian and mammalian kidney, brain, and pancreas. There is no amino acid sequence homology between calbindin-D_{9k} and calbindin-D_{28k}. In VDR-ablated mice, which demonstrate impaired intestinal calcium absorption, calbindin-D_{9k} mRNA is reduced dramatically (Li *et al.,* 1998). It has been suggested that the role of intestinal calbindin in the second phase of the intestinal calcium absorptive process is to facilitate transcellular calcium diffusion (Wasserman and Fulllmer, 1995). Studies using analogs of $1,25(OH)_2D_3$ suggest that there need not be a direct correlation between calbindin induction and stimulation of intestinal calcium transport. Intestinal calbindin-D_{9k} mRNA but not intestinal calcium transport has been reported to be induced by $1,25(OH)_2D_3$ 24-homologues (Krisinger *et al.,* 1991). In addition, 1,25,28trihydroxyvitamin D_2 has been found to have no effect on intestinal calcium absorption but to result in a significant induction in rat intestinal calbindin-D_{9k} mRNA and protein (Wang *et al.,* 1993). These findings provide evidence that calbindin alone is not responsible for the $1,25(OH)_2D_3$-mediated intestinal transport of calcium. Although immunocytochemical evidence has indicated that calbindin is localized predominantly in the cytoplasm and is not associated with cellular membranes, evidence has suggested that some calbindin can be localized inside small vesicles and lysosome structures of the chick intestinal cell (Nemere *et al.,* 1991). Calbindin was also found to be associated with filamentous elements that can be isolated with tubules and microtubules of the chick small intestine (Nemere *et al.,* 1991). It was suggested that changes in the cellular localization of calbindin are involved dynamically in $1,25(OH)_2D_3$-dependent calcium transport in the intestine. In addition to acting as a facilitator of intestinal calcium diffusion, it is also possible that calbindin in the intestine may act as an intracellular buffer to prevent toxic levels of calcium from accumulating in the intestinal cell during $1,25(OH)_2D_3$-dependent transcellular calcium transport (Christakos *et al.,* 1989).

The third phase of $1,25(OH)_2D_3$-dependent intestinal calcium transport is calcium extrusion from the intestinal cell, which involves calcium transport against a concentration gradient. The intestinal plasma membrane calcium pump (PMCA-1) and PMCA-1mRNA have been shown to be stimulated by $1,25(OH)_2D_3$ in vitamin D-deficient rats and chicks, suggesting for the first time that the intestinal calcium absorptive process may involve a direct effect of $1,25(OH)_2D_3$ on calcium pump expression (Cai *et al.,* 1993; Wasserman *et al.,* 1992; Zelinski *et al.,* 1991). Although $1,25(OH)_2D_3$ has been reported to affect transcription of the intestinal PMCA gene, the mechanisms involved in this regulation remain to be determined. Further studies concerning the interrelationship between the vitamin D endocrine system and the intestinal calcium pump should result in a better understanding of additional factors involved in $1,25(OH)_2D_3$-mediated intestinal calcium transport.

Kidney

In addition to bone and intestine, a third target tissue involved in the regulation by $1,25(OH)_2D_3$ of mineral homeostasis is the kidney. Although there is some controversy

concerning the role of $1,25(OH)_2D_3$ in renal calcium transport, micropuncture data, as well as studies using a mouse distal-convoluted tubule cell line, have indicated that vitamin D metabolites can enhance the stimulatory effect of PTH on calcium transport in the distal nephron (Friedman and Gesek, 1993; Winaver *et al.,* 1980).Most recent studies have provided evidence that $1,25(OH)_2D_3$ increases PTH receptor mRNA and binding activity in distal tubule cells, providing a mechanism whereby $1,25(OH)_2D_3$ enhances the action of PTH (Sneddon *et al.,* 1998). In the mouse, both vitamin D-dependent calcium-binding proteins (calbindin-D_{9k} and calbindin- D_{28k}) have been reported to be localized in the distal nephron (distal convoluted tubule, connecting tubule, and cortical collecting tubule) (Rhoten *et al.,* 1985). Kinetic analysis has suggested that the two proteins affect renal calcium reabsorption by different mechanisms. Calbindin-D_{28k} stimulates the high-affinity system in the distal luminal membrane (Bouhtiauy *et al.,* 1994a), whereas calbindin-D_{9k} was found to enhance the ATP-dependent calcium transport of the basolateral membrane (Bouhtiauy *et al.,* 1994b). These findings provide evidence for a role for calbindins in vitamin D-dependent calcium transport processes in the kidney. Studies related to the cloning of a putative apical calcium channel in $1,25(OH)_2D_3$ responsive epithelia (the proximal duodenum as well as the distal tubule and placenta) have suggested a mechanism of calcium entry into $1,25(OH)_2D_3$ responsive epithelia (Hoenderop *et al.,* 1999; Vennekens *et al.,* 2000). It will be of interest in future studies to examine whether calbindin-D_{28k} affects calcium entry by interacting directly with an apical calcium entry channel or whether calbindin can affect other proteins involved in regulating the channel. Thus, $1,25(OH)_2D_3$ may affect calcium transport in the distal tubule by enhancing the action of PTH and by inducing the calbindins. In addition to calbindins, the plasma membrane calcium pump has also been localized immunocytochemically exclusively to the distal tubule and to the collecting duct (Borke *et al.,* 1988). However, the interrelationship between the renal calcium pump and $1,25(OH)_2D_3$ is not clear at this time. In addition to the suggested role of $1,25(OH)_2D_3$ in the tubular reabsorption of calcium, another important effect of $1,25(OH)_2D_3$ in the kidney is inhibition of the $25(OH)D_3$ 1α-hydroxylase enzyme and stimulation of the 24-hydroxylase enzyme. Both the 1α-hydroxylase and 24(OH)ase genes have been cloned, and studies indicate that they are regulated through liganded VDR (Takeyama *et al.,* 1997; Shinki *et al.,* 1997; Murayama *et al.,* 1999; Kerry *et al.,* 1996). Megalin, a member of the LDL receptor superfamily expressed in the neuroepithelium and on the apical surface of the proximal tubular epithelium, has been reported to play an important role in the renal uptake of $25(OH)D_3$ (Nykjaer *et al.,* 1999). In megalin knockout mice there was abnormal urinary calcium excretion of $25(OH)D_3$ that resulted in vitamin D deficiency and bone disease. These results suggest that megalin is essential to deliver the precursor for the generation of $1,25(OH)_2D_3$. Effects of vitamin D on phosphate reabsorption in the proximal tubule have also been suggested. Vitamin D has been reported to increase or decrease renal phosphate reabsorption depending on the parathyroid status and on experimental conditions. The gene for X-linked hypophosphatemic vitamin D-resistant rickets (PEX or HYP), a disease whose main feature is renal phosphate leak, has been identified (Rowe *et al.,* 1996). A putative vitamin D responsive element was noted in this gene, suggesting that $1,25(OH)_2D_3$ may play a role in regulating the HYP gene (Rowe *et al.,* 1996). The presence of a putative vitamin D responsive element in the promoter of the Na^+/phosphate cotransporter and regulation of this promoter by VDR and $1,25(OH)_2D_3$ also suggest a role for $1,25(OH)_2D_3$ in renal phosphate transport (Taketani *et al.,* 1997). In addition to modulation of the $25(OH)D_3$ hydroxylases and a few reports concerning effects on phosphate transport in the proximal tubule and enhancement of calcium transport in the distal nephron, in general, the effects of $1,25(OH)_2D_3$ in the kidney are not well understood. Although autoradiography indicated that the VDR was localized exclusively in the distal nephron (Stumpf *et al.,* 1980), studies using immunocytochemistry and reverse transcription polymerase chain reaction (RT PCR) have shown that VDR and VDR mRNA are also localized in glomeruli, proximal tubules and the collecting duct (Liu, *et al.,* 1996a), thus suggesting multiple genomically mediated actions of $1,25(OH)_2D_3$ within the kidney. Further research is needed in order to provide new insight concerning the renal effects of $1,25(OH)_2D_3$.

Parathyroid Glands

In the regulation of mineral homeostasis, the parathyroid glands are also an important target of $1,25(OH)_2D_3$ action. 1,25-Dihydroxyvitamin D_3 inhibits the secretion and synthesis of PTH. A direct action of $1,25(OH)_2D_3$ on the preproparathyroid hormone gene has been reported (Demay *et al.,* 1992; Mackey *et al.,* 1996).

Nonclassical Actions of $1,25(OH)_2D_3$

Effects of $1,25(OH)_2D_3$ on Differentiation and Proliferation

In addition to affecting tissues involved in mineral homeostasis, $1,25(OH)_2D_3$ has been reported to affect numerous other systems. Because $1,25(OH)_2D_3$ receptors have been identified in tissues not involved in calcium homeostasis, it has been suggested that the actions of $1,25(OH)_2D_3$ in these nonclassical target tissues are mediated, at least in part, by genomic mechanisms. One of the best characterized actions of $1,25(OH)_2D_3$ in a number of different normal and malignant cells is the ability of $1,25(OH)_2D_3$ to inhibit proliferation and to stimulate differentiation (Suda, 1989; Suda *et al.,* 1990). The effect of $1,25(OH)_2D_3$ on the inhibition of proliferation and the stimulation of differentiation is of interest because it has been related to the treatment of skin lesions found in psoriasis with $1,25(OH)_2D_3$ or analogs of $1,25(OH)_2D_3$ (this topic is discussed in detail in

Chapter 33). In addition to keratinocytes, $1,25(OH)_2D_3$ has been reported to inhibit the proliferation and induce the differentiation of leukemia cells (Suda, 1989) and to inhibit the proliferation of a number of malignant cells, including colon, breast, and prostate cancer cells (Kane *et al.,* 1996; James *et al.,* 1998; Wang *et al.,* 2000; Zhao *et al.,* 1997). A very active area of current investigation is the development of analogs of $1,25(OH)_2D_3$, which can inhibit cell growth and promote differentiation but do not affect serum calcium and the testing of their therapeutic potential in the treatment of leukemia and other malignancies.

Effects of $1,25(OH)_2D_3$ on Hormone Secretion

In addition to affecting the secretion of PTH, $1,25(OH)_2D_3$ has been reported to affect the secretion of other hormones that do not have a primary role in the regulation of mineral homeostasis. In the pituitary, $1,25(OH)_2D_3$ has been reported to enhance the secretion of both thyroid stimulating hormone (TSH) and prolactin (d'Emden and Wark, 1989; Murdoch and Rosenfeld, 1981). An induction of prolactin mRNA and transcription by $1,25(OH)_2D_3$ have also been reported in pituitary GH3 cells (Castillo *et al.,* 1999). Studies using VDR knockout mice have indicated that vitamin D is essential for full gonadal function in both sexes (Kinuta *et al.,* 2000). Although vitamin D plays a role in estrogen biosynthesis partially by maintaining calcium heomeostasis, direct regulation by $1,25(OH)_2D_3$ of the aromatase gene was suggested (Kinuta *et al.,* 2000). 1,25-Dihydroxyvitamin D_3 treatment can also enhance the secretion of insulin from the pancreas (Chertow *et al.,* 1983; Clark *et al.,* 1981; Norman *et al.,* 1980), the first nonclassical target tissue in which $1,25(OH)_2D_3$ receptors were identified (Christakos and Norman, 1979). Although normalization of insulin secretion by vitamin D has been observed in vitamin-deficient rats, it has been argued that this effect of vitamin D may be secondary to a primary effect of vitamin D on serum calcium or to other beneficial effects of vitamin D on growth and nutrition. This is still a matter of debate, however. It is indeed possible that $1,25(OH)_2D_3$ may act together with calcium to control insulin secretion. One of the earliest indications that the β cell may be a target for $1,25(OH)_2D_3$ came from immunocytochemical studies that localized calbindin-D_{28k} to the islet (Morrissey *et al.,* 1975). Studies using pancreatic islets from calbindin-D28k null-mutant mice (knockout mice) and β cell lines stably transfected and overexpressing calbindin have provided evidence for a role for calbindin in the modulation of depolarization-stimulated insulin release and suggest that calbindin can control the rate of insulin release via the regulation of intracellular calcium (Sooy *et al.,* 1999).

Effects of $1,25(OH)_2D_3$ on the Immune System

1,25-Dihydroxyvitamin D_3 has also been reported to affect the differentiation and function of cells of the immune system (Manolagas *et al.,* 1994).

Effects of $1,25(OH)_2D_3$ include inhibition of T-cell proliferation and regulation of the interleukins 1–3 and 6, regulation of interferon-γ, tumor necrosis factor, and granulocyte/macrophage colony-stimulating factor (6M-CSF). The effects of $1,25(OH)_2D_3$ on the suppression of T-cell proliferation and on the expression of certain cytokines are in contrast to the reported effect of $1,25(OH)_2D_3$ on the enhancement of macrophage phagocytic activity. Due to its immunosuppressive actions, it has been suggested that $1,25(OH)_2D_3$ or analogs of $1,25(OH)_2D_3$ may prevent the induction of certain experimental autoimmune disorders and may have beneficial effects when given in combination with immunosuppressive drugs such as cyclosporin A.

Transcriptional Regulation by $1,25(OH)_2D_3$

Vitamin D-Regulated Genes

The genomic mechanism of $1,25(OH)_2D_3$ action involves binding to specific DNA sequences in the promoter region of target genes. To date, more than 50 vitamin D-dependent genes have been identified in different target tissues in a number of species [see Hannah and Norman (1994) and Segaert and Bouillon (1998) for lists of vitamin D-dependent genes]. However, only a limited number of vitamin D responsive elements (VDREs) have been defined (Table I). On the basis of this small number of natural VDREs, in general, the VDRE consensus consists of two direct imperfect repeats of the hexanucleotide sequence GGGTGA separated by three nucleotide pairs.

CALBINDIN-$D_{9K,}$ CALBINDIN-D_{28K}

As mentioned previously, one of the most pronounced effects of $1,25(OH)_2D_3$ is increased synthesis of calbindin. Although sequence elements in the mouse calbindin-D_{28k} promoter (−200/−169) and in the rat calbindin-D_{9k} promoter (−489/−445) that respond to $1,25(OH)_2D_3$ have been identified, the response observed using the calbindin-D_{9k} or the calbindin-D_{28k} responsive sequences is modest (Darwish and DeLuca, 1992; Gill and Christakos, 1993). This modest response reflects previous *in vivo* findings that indicated that

Table I Vitamin D Responsive Elements Present in Vitamin-Regulated Genes

Rat 24-hydroxylase	AGGTGA gtg AGGGCG (−151/−137)
	CGCACC cgc TGAACC (−259/−245)
Mouse osteopontin	GGTTCA cga GGTTCA (−757/−743)
Human osteocalcin	GGGTGA acg GGGGCA (−499/−485)
Rat osteocalcin	GGGTGA atg AGGACA (−455/−441)
Mouse calbindin-D_{28k}	GGGGGA tgtg AGGAGA (−198/−183)
Mouse Calbindin-D_{9k}	GGGTGT cgg AAGCCC (−489/−475)
Avian integrin β_3	GAGGCA gaa GGGAGA (−770/−756)
Human p21	AGGGAG att GGTTCA (−779/−765)

$1,25(OH)_2D_3$ induces the expression of the calbindin-D_{9k} or the calbindin-D_{28k} gene by a small, rapid transcriptional stimulation followed by a large accumulation of calbindin mRNA long after $1,25(OH)_2D_3$ treatment (Christakos *et al.,* 1989). These findings suggest that the large induction of calbindin mRNA by $1,25(OH)_2D_3$ may be due primarily to posttranscriptional mechanisms. More recent studies noted the requirement of the homeodomain protein Cdx2, a transcription factor active only in intestinal epithelium, for calbindin-D_{9k} expression and that cooperation between the proximal calbindin-D_{9k} promoter and a distal element located in an open chromatin structure (−3600/−3400) is needed for vitamin D responsiveness (Colnot *et al.,* 1998). These studies suggest that the mechanism of action of $1,25(OH)_2D_3$ on calbindin regulation is more complicated than the conventional hormone-receptor transcriptional activation model. Calbindin-D_{9k} and calbindin-D_{28k} are regulated by a number of hormones in addition to $1,25(OH)_2D_3$. Calbindin-D_{9k} in rat uterus and calbindin-D_{28k} in mouse uterus, oviduct, and ovary have been reported to be regulated by estradiol [see Christakos (1995) and Christakos *et al.,* (1989) for review]. 1,25-Dihydroxyvitamin D_3 has no effect on calbindins in these female reproductive tissues. Glucocorticoid administration has been reported to inhibit the expression of intestinal calbindin-D_{9k} mRNA (Huang *et al.,* 1989; Li and Christakos, 1991). The inhibition of calbindin may be related to the decrease in intestinal calcium absorption, which has been observed with glucocorticoid treatment. Regulation of calbindin-D_{28k} by retinoic acid has also been reported (Wang and Christakos, 1995). In addition, neurotrophin 3, brain-derived neurotrophic factor, fibroblast growth factor, and tumor necrosis factor have all been observed to increase the expression of calbindin-D_{28k} in brain, suggesting regulation of calbindin by signal transduction, as well as by steroids [see Christakos (1995) for review]. Thus, it has become evident that calbindin is no longer considered a calcium-binding protein whose synthesis is dependent solely on vitamin D. Calbindin is present in a number of different tissues, may have multiple functions, and can be regulated by different ligands as well as by signal transduction.

OSTEOCALCIN, OSTEOPONTIN

Studies concerning the regulation by vitamin D of two other calcium-binding proteins, osteocalcin (OC) and osteopontin (OP), which are secreted by the osteoblasts, have resulted in the most information concerning transcriptional activation by $1,25(OH)_2D_3$. VDREs in both the human and the rat OC promoter (Demay *et al.,* 1990; Kerner *et al.,* 1989; Morrison *et al.,* 1989; Owen *et al.,* 1990; Ozono *et al.,* 1990) and in the mouse OP promoter (Noda *et al.,* 1990) have been well characterized. An AP1 site, which binds members of the Jun/Fos protooncogene family, has been reported to be closely juxtaposed to the VDRE in the human OC promoter (Ozono *et al.,* 1990). It has also been proposed that the rat OC VDRE contains an AP1 element within the VDRE (not juxtaposed as has been reported for the hOC VDRE) (Owen *et al.,* 1990). An AP1 site is not found within or juxtaposed to the mouse OP VDRE. It has been reported that the AP1 element can be involved in the synergistic enhancement of the $1,25(OH)_2D_3$-dependent transcriptional activation of the OC gene. However, expression of cjun and cfos in ROS 17/2.8 cells has been reported to suppress basal activity as well as the $1,25(OH)_2D_3$-induced response (Schule *et al.,* 1990). Thus it has been proposed that the AP1 site and AP1 proteins may play an important role in activating or suppressing both basal and $1,25(OH)_2D_3$-dependent OC transcription depending on the state of differentiation of the cell. In addition to an AP1 element, a glucocorticoid responsive element has also been identified overlapping the TATA box in both the rat and the human osteocalcin genes (Morrison *et al.,* 1989; Stromstedt *et al.,* 1991). Glucocorticoids, which dampen osteoblast activity, were reported to have a modest suppressive effect on the basal activity of the OC promoter but completely blocked the induction of OC promoter CAT expression by $1,25(OH)_2D_3$, providing a mechanism of glucocorticoid repression of the osteocalcin gene. More recent studies noted the importance of Cbfa transcriptional activators for basal and vitamin D responsive transcription of the OC gene (Javed *et al.,* 1999) and that the multifunctional transcriptional regulator YY1 represses $1,25(OH)_2D_3$ induced transcription of the OC gene (Guo *et al.,* 1997).

24-HYDROXYLASE

Most recently, vitamin D responsive elements have been identified in the rat 24-hydroxylase [24(OH)ase] gene (Kerry *et al.,* 1996; Ozono *et al.,* 1995; Zierold *et al.,* 1994). The 24(OH)ase gene is the first vitamin D-dependent gene reported to be controlled by two independent VDREs (at −259/−245 and at −151/−137). It has been suggested that the proximal VDRE is more responsive to $1,25(OH)_2D_3$ than the distal VDRE (Ozono *et al.,* 1995). A binding site for the Ras-activated Ets transcription factor has been identified downstream from the proximal VDRE, and this site was found to be critical for $1,25(OH)_2D_3$ mediated 24(OH)ase transcription (Dwivedi *et al.,* 2000). The most pronounced effects of $1,25(OH)_2D_3$ in intestine and kidney are increased synthesis of 24(OH)ase and calbindin (Matkovits and Christakos, 1995). However, unlike calbindin, which is only modestly transcriptionally responsive to $1,25(OH)_2D_3$, 24(OH)ase is strongly responsive to $1,25(OH)_2D_3$ at the level of transcription.

INTEGRIN $\alpha_v\beta_3$

1,25-Dihydroxyvitamin D_3 has also been reported to transcriptionally activate α_v and β_3 integrin genes. Integrin $\alpha_v\beta_3$ is expressed in the osteoclast plasma membrane, has been reported to bind to osteopontin through the amino acid sequence RGD, and has an important role in bone resorption (see Chapter 17). A vitamin D responsive element has been reported in the avian β_3 integrin gene (at −770/−756) (Cao *et al.,* 1993). The magnitude of the transcriptional response to $1,25(OH)_2D_3$ of the avian β_3 integrin gene is modest, similar to the transcriptional response of the calbindin genes to $1,25(OH)_2D_3$.

PARATHYROID HORMONE

The first demonstration of a negative VDRE was by Demay *et al.* (1992), who indicated that sequences in the human parathyroid hormone (PTH) gene (−125/−101) mediate transcriptional repression by $1,25(OH)_2D_3$. Only a single copy motif (AGGTCA) is identified within this region, and vitamin D receptor binding to this element does not require the retinoid X receptor (Mackey *et al.,* 1996). The response is tissue specific because the 25-bp oligonucleotide was reported to mediate transcriptional repression in GH4C1 cells but not in ROS 17/2.8 cells.

INTERLEUKIN-2 AND GRANULOCYTE/MACROPHAGE COLONY-STIMULATING FACTOR

Mechanisms involved in mediating the effects of $1,25(OH)_2D_3$ in systems other than those involved in maintaining mineral homeostasis have only recently begun to be explored. A decrease in the proliferation of T lymphocytes in the presence of $1,25(OH)_2D_3$ is correlated with a decrease in interleukin-2 (IL-2) mRNA and GM-CSF mRNA. Transcriptional repression of these genes contributes to the overall immunosuppressive effects of $1,25(OH)_2D_3$ (Alroy *et al.* 1995; and Towers *et al.,* 1999). Mechanisms involved in the repression by $1,25(OH)_2D_3$ of IL-2 and GM-CSF have been provided (Alroy *et al.,* 1995; Towers *et al.,* 1999). VDR can block the positive transcription factors NFATp and Jun/Fos, which bind to a composite site containing a consensus NFAT1-binding site. These findings provide novel insight concerning how $1,25(OH)_2D_3$ can act as an immunosuppressive agent and may provide a general mechanistic basis for how steroid receptors elicit immunosuppressive responses.

To provide insight concerning the mechanisms involved in the effect of $1,25(OH)_2D_3$ on the differentiation of leukemic cells into monocyte/macrophages, a cDNA library was prepared from the myelomonocytic U937 cell line and screened with probes generated from either $1,25(OH)_2D_3$-treated or untreated cells. The cyclin D-dependent kinase inhibitor p21 was found to be induced transcriptionally by $1,25(OH)_2D_3$, and a functional VDRE was identified in the p21 promoter (see Table I) (Liu, *et al.,* 1996b). Transient overexpression of p21 in U937 cells in the absence of $1,25(OH)_2D_3$ resulted in the expression of monocyte/macrophage-specific markers, suggesting that p21, which is involved in blocking cell cycle progression, may be a key factor involved in $1,25(OH)_2D_3$-mediated differentiation of leukemic cells.

Factors Involved in Vitamin D-Mediated Transcriptional Regulation

VITAMIN D RECEPTOR (VDR)

VDR Regulation Due to the importance of VDR in the molecular mechanism of vitamin D action, the regulation of VDR has been a focus of a number of studies. Upregulation of VDR by $1,25(OH)_2D_3$ has been shown in several different systems, including rat intestine (Strom *et al.,* 1989), pig kidney LLCPK-1 cells (Costa *et al.,* 1985), and HL-60 leukemia cells (Lee *et al.,* 1989). Whether the homologous upregulation of VDR involves an induction of VDR mRNA has been a controversial topic. However, it is not likely that an increase in VDR mRNA in response to $1,25(OH)_2D_3$ plays a major role in the regulation of the VDR. It has been suggested that homologous upregulation of the VDR is most probably due to increased stability of the occupied receptor (Lee *et al.,* 1989; Wiese *et al.,* 1992; Arbour *et al.,* 1993). VDR has also been reported to be regulated by a number of other factors, including activation of protein kinase A and protein kinase C. Treatment of NIHT3 mouse fibroblasts with $(Bu)_2$cAMP or forskolin (Krishnan and Feldman, 1992) and treatment of mouse osteoblasts (MC3T3-E1 cells) or rat osteosarcoma cells (UMR-106-01) with forskolin or PTH (Krishnan *et al.,* 1995) was reported to result in an induction in VDR abundance. Treatment of these cells with the phorbol ester, phorbol myristate acetate (PMA), whose actions are mediated by protein kinase C, resulted in a downregulation of VDR (Krishnan and Feldman, 1991; Krishnan *et al.,* 1995). Up- or downregulation of VDR in NIHT3 cells by forskolin or PMA, respectively, resulted in a corresponding induction or attenuation of reporter activity in cells transfected with the human OC VDRE fused to the reporter gene chloramphenicol acetyltransferase (Krishnan and Feldman, 1992). Treatment of UMR cells with PTH enhanced the $1,25(OH)_2D_3$-mediated induction of 24(OH)ase mRNA (Krishnan *et al.,* 1995; Armbrecht *et al.,* 1998). Thus the functional response corresponded to the change in VDR. However, opposite findings concerning the effect of activation of protein kinase A or protein kinase C on VDR have been reported by others (Reinhardt and Horst, 1990, 1994), suggesting that proliferation state, cell type, and stage of differentiation affect the interaction between $1,25(OH)_2D_3$ and signal transduction pathways. In general, studies suggest cooperativity between signal transduction pathways and $1,25(OH)_2D_3$. Effects of second messenger systems may be on the VDR. However effects on the promoter of the target gene or on other transcription factors that may be interacting with the VDR also need to be considered. Further studies related to the regulation of VDR will be facilitated by the preliminary analysis of the hVDR gene structure that spans more than 60 kb, consists of at least 14 exons, and is directed by two distinct promoters (Miyamoto *et al.,* 1997)

Phosphorylation of the VDR Although the VDR, similar to other steroid receptors, is phosphorylated, the exact functional role of phosphorylation of the VDR remains to be further elucidated. It has been suggested that phosphorylation may play a role in the binding of the receptor to DNA or in the interaction of VDR with other transcription factors. Phosphorylation of VDR was shown to involve serine residues. Serine-208 in the ligand-binding domain has been identified as a site of phosphorylation that accounts for at least 60% of the phosphorylation of the receptor (Hilliard *et al.,* 1994; Jurutka *et al.,* 1993). Casein kinase II has been reported to mediate VDR phosphorylation at serine-208 (Jurutka *et al.,* 1996). Although transcriptional activation by $1,25(OH)_2D_3$ is not dependent on serine-208 phosphorylation, studies have

shown that VDR phosphorylation by casein kinase II at serine-208 plays an important role in potentiating transcriptional activation (Jurutka *et al.,* 1996). These studies provide the first evidence of a functional role of phosphorylation of the VDR. Knowledge of the crystal structure of the VDR (Rochel *et al.,* 2000) will facilitate the definitive identification of other possible phosphorylation sites.

VDR Receptor Homodimerization vs Heterodimerization

Earlier reports indicated that the VDR functions primarily as a heterodimer with RXR for activation of gene transcription. Because VDR derived from Baculovirus (MacDonald *et al.,* 1991) or yeast (Sone *et al.,* 1990) systems or *in vitro*-translated VDR (Liao *et al.,* 1990) was not reported to interact directly with VDREs, it was suggested that VDR is unable to form natural homodimers. More recently, studies have indicated that purified VDR can bind as a homodimer to certain VDREs but that RXR was required for binding to other VDREs (Freedman *et al.,* 1994; Nishikawa *et al.,* 1994). However, the physiological relevance of VDR homodimers has been questioned. Cheskis and Freedman (1994) reported that VDR exists as a monomer in solution and homodimerization occurs upon binding to the OPN VDRE. 1,25-Dihydroxyvitamin D_3 was reported to destabilize homodimerization, resulting in VDR/RXR heterodimer formation (Fig. 2). Thompson *et al.* (1998) also reported that formation of the VDRE-complexed VDR-RXR heterodimer is strikingly dependent on the presence of $1,25(OH)_2D_3$. These studies suggest that although the VDR homodimer can exist in solution, the heterodimer is the functional transactivating species. 9-*cis*-Retinoic acid has been reported to decrease heterodimer formation by driving the equilibrium from the VDR heterodimer to the RXR homodimer or to the interaction of RXR with other receptors (Cheskis and Freedman, 1994; MacDonald *et al.,* 1993). Further studies using VDR mutants also suggest the importance of heterodimerization, as none of the mutants without the capability to form heterodimers showed $1,25(OH)_2D_3$-dependent transcriptional activation (Nakajima *et al.,* 1994). Although at this time the VDR homodimer does not appear to have a role in transcriptional activation, it is possible in future studies that VDR homodimers may be shown to have a functional role in enhancing the transcription of $1,25(OH)_2D_3$-dependent target genes yet to be identified.

Regions of VDR within the ligand-binding domain that may be crucial for heterodimerization have been suggested. Functional studies have indicated that the region in the C-terminal between amino acids 317 and 395 may have an important role in heterodimerization (Nakajima *et al.,* 1994; Jin *et al.,* 1996). A second interaction domain was reported between amino acids 244 and 263 (Rosen *et al.,* 1993; Whitfield *et al.,* 1995; Jin *et al.,* 1996). The two regions of VDR correspond to portions of helices 7–10 and 3–4, respectively. Although these studies are suggestive, a more complete understanding of the three-dimensional contacts between VDR and RXR will be obtained now that the crystal structure of the VDR ligand domain bound to its ligand has been published (Rochel *et al.,* 2000).

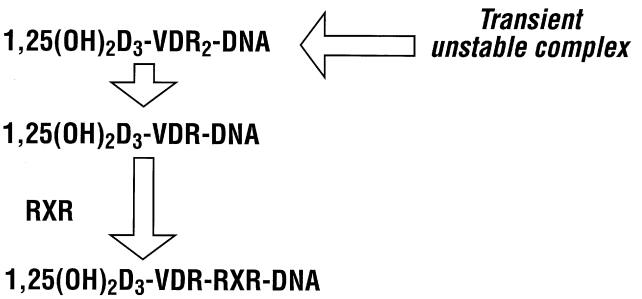

Figure 2 Proposed model of VDR homodimerization and heterodimerization with RXR. It has been reported that VDR exists as a monomer in solution and homodimerization occurs upon binding to the OPN VDRE. 1,25-Dihydroxyvitamin D_3 can destabilize homodimerization, resulting in VDR/RXR heterodimer formation (see Cheskis and Freedman, 1994).

INTERACTION OF VDR WITH TRANSCRIPTION MACHINERY

The mechanisms involved in VDR-mediated transcription following binding of the VDR-RXR heterodimer to DNA have begun to be investigated. Initiation of basal transcription involves binding of TFIID, which is composed of the TATA box-binding protein and associated TAFs, to the TATA element. After the binding of TFIID to the TATA element, other factors, including TFIIA, TFIIB, RNA polymerase II, TFIIE, TFIIF, and TFIIH, are recruited and associated with the complex. Studies have suggested that VDR can interact physically and functionally with TFIIB (Blanco *et al.,* 1995; Masuyama *et al.,* 1997).The interaction of TFIIB is with unliganded VDR, and the $1,25(OH)_2D_3$ ligand disrupts the VDR-TFIIB complex (Masuyama *et al.,* 1997). These findings suggest that VDR prerecruits TFIIB and, in the presence of ligand, TFIIB is released for assembly into the preinitiation complex to facilitate activated transcription.

Several TAFs have been suggested to be involved in VDR-mediated transcriptional activity. The TFIID subunit $TAF_{II}135$ potentiates the transcriptional activity of VDR (Mengus *et al.,* 1997). In addition, $TAF_{II}28$ (Mengus *et al.,* 2000) and $TAF_{II}55$ (Lavigne *et al.,* 1999) interact with two regions of the VDR (one region spanning α helices H3 to H5 of the VDR and a second region corresponding to α helix 8).Determinants for interaction with $TAF_{II}28$ or $TAF_{II}135$ are not identical. A mutation in the H3–H5 region that determines interaction with $TAF_{II}28$ was reported to abolish VDR-mediated transactivation (Mengus *et al.,* 2000), thus indicating that the specific amino acids within that region of the VDR ligand-binding domain are needed for transactivation.

SRC/P160 COACTIVATORS

Over the past few years a number of proteins known as p160 coactivators (based on one of the first identified members, the 160-kDa protein, steroid receptor coactivator-1;SRC-1) that bind to steroid receptors and enhance their activity have been identified. Three related family members, based on homologies, include SRC-1/NcoA1, GRIP-1/TIF-2, and ACTR/pCIP (for reviews, see McKenna *et al.,* 1999; Xu

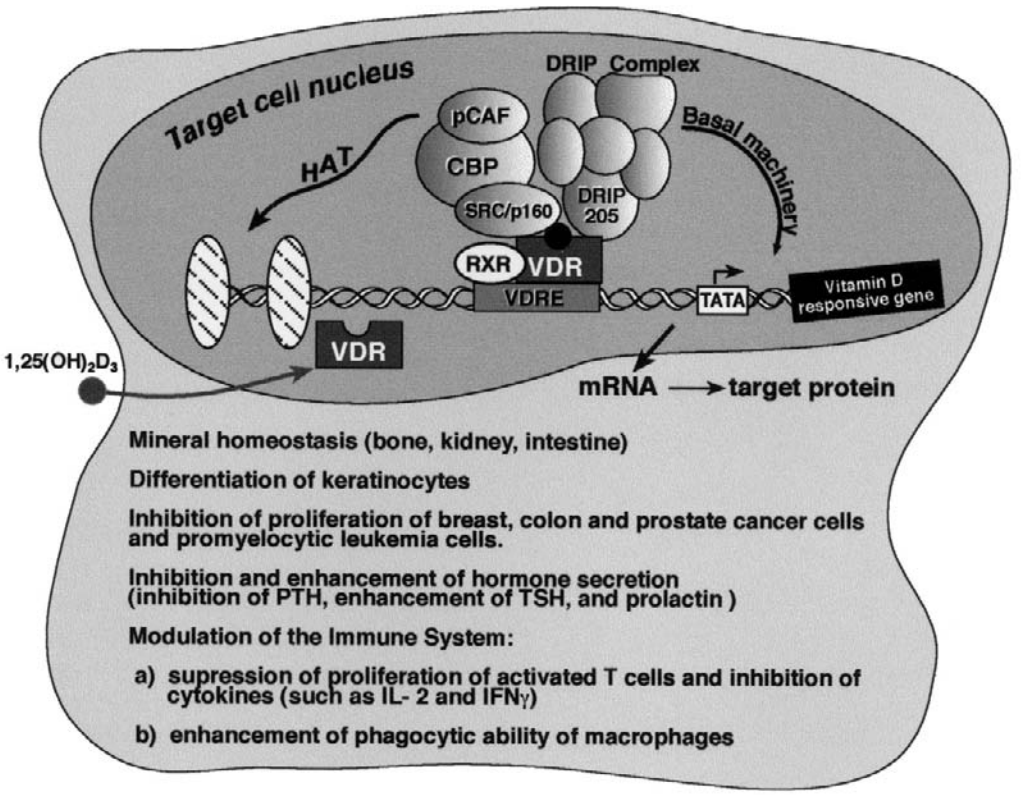

Figure 3 Genomic mechanism of action of $1,25(OH)_2D_3$ in target cells. 1,25-Dihydroxyvitamin enters the target cell and interacts with the nuclear VDR, which heterodimerizes with RXR. After interaction with the VDRE, transcriptional activation or repression proceeds through the interaction of VDR with coactivators and with the transcription machinery. Two models for the genomic action of $1,25(OH)_2D_3$ are shown. The histone acetyltransferase (HAT) activity-containing complex of SRC/p160 and CBP may be recruited first by VDR in response to the ligand. This would lead to DNA accessibility that would allow as a second step the binding of the DRIP complex (DRIP 205 subunit binds directly to VDR) and recruitment of RNA polymerase II (sequential model). Alternatively, there could be simultaneous chromatin remodeling and basal machinery recruitment (cooperative model). 1,25-Dihydroxyvitamin D_3 is known to affect mineral homeostasis, to differentiate keratinocytes, to inhibit the proliferation of cancer cells, to affect hormone secretion, and to modulate the immune system. Adapted with permission from Christakos *et al.* (1996).

et al., 1999). They interact with the AF2 domain of steroid receptors, including VDR (C-terminal helix 12 contains the core AF2) in a ligand-dependent manner. Studies indicate that helix 3 of the VDR is also important for interaction with p160 coactivators (Jimenez-Lara *et al.,* 1999; Kraichely *et al.,* 1999). These coactivators have histone acetylase (HAT) activity. This modification of histones is thought to destabilize the interaction between DNA and the histone core, liberating DNA for transcription. These coactivators can also form complexes with CBP (CREB-binding protein). CBP also has HAT activity. Thus the SRC/p160 family of coactivators can recruit CBP to the nuclear receptor, resulting in a multisubunit complex.

VITAMIN D RECEPTOR INTERACTING PROTEINS (DRIP) COMPLEX

In addition to the SRC/p160 family of coactivators, VDR-mediated transcription is also mediated by a coactivator complex, DRIP (Rachez *et al.,* 1998, 1999). These proteins are also called TRAP and ARC, depending on the transcription factor initially identified as the target (TR or androgen receptor, respectively), but are now thought to have broader target specificity due to their close identity. Studies have indicated that the complex does not have HAT activity but rather functions, at least in part, through recruitment of RNA polymerase II (Rachez *et al.,* 1999). The CBP/SRC coactivator complex may be needed first for chromatin remodeling followed by the recruitment of the transcription machinery by the DRIP/TRAP/ARC complex (sequential model) or there may be simultaneous chromatin remodeling and basal machinery recruitment (cooperative model) (Fig. 3).

Future Directions

New target genes, novel vitamin D responsive elements, and new factors involved in vitamin D-mediated transcription will undoubtedly be identified in numerous different systems, which are currently known to be affected by $1,25(OH)_2D_3$. Sequences divergent from the

current consensus VDRE may be observed, which should expand our understanding of the sequences involved in $1,25(OH)_2D_3$-mediated genomic responses. In addition to transcriptional regulation, it is likely that posttranscriptional mechanisms will be an important mechanism of control of a number of newly identified target genes. In addition to studies concerning the mechanisms involved in mediating the genomic actions of $1,25(OH)_2D_3$, further studies related to the physiological significance of target proteins are needed in the future. Finally, in the next few years, with the elucidation of the crystal structure of VDR, we will obtain an increased understanding of the structure of VDR in the presence and absence of ligand and/or protein partners. Based on the structural information, synthetic analogs of $1,25(OH)_2D_3$ may be designed that would selectively modulate specific $1,25(OH)_2D_3$ responses. Thus, new insight into the multiple roles of $1,25(OH)_2D_3$ will be obtained and selective modulation of $1,25(OH)_2D_3$ responses in bone and other target tissues may indeed be possible.

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CHAPTER 33

Photobiology and Noncalcemic Actions of Vitamin D

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Photobiology of Vitamin D

Photosynthesis of Previtamin D_3 and Its Conversion to Vitamin D_3

When human skin is exposed to sunlight, a photochemical process occurs that is essential for the maintenance of calcium homeostasis and a healthy skeleton. During exposure to sunlight, the ultraviolet B (UV-B; 290–315 nm) portion of the solar spectrum is responsible for photolyzing 7-dehydrocholesterol (the precursor cholesterol; provitamin D_3) to previtamin D_3(Holick, 1994). Once formed, previtamin D_3 undergoes an internal isomerization of its three double bonds to form a more thermodynamically stable, 5,6-cis-triene and is transformed in vitamin D_3 (Fig. 1). For warm-blooded animals, such as humans, this process would, under normal circumstances, take approximately 24 hr for 50% of previtamin D_3 to convert to vitamin D_3. However, in cold-blooded animals, this process could take several days (Holick *et al.,* 1980). It has now been found that there is membrane enhancement for the conversion of previtamin D_3 to vitamin D_3 in both cold-blooded and warm-blooded animals, including humans (Holick *et al.,* 1995). 7-Dehydrocholesterol is found principally in the cell membrane. Within the membrane, the hydrophobic side chain of 7-dehydrocholesterol is aligned with the hydrophobic chains of the fatty acids and cholesterol, thereby restraining the confirmation of previtamin D_3 when it is formed (Fig. 2). Thus, when 7-dehydrocholesterol is exposed to sunlight, 7-dehydrocholesterol is photolyzed to the s-*cis*, s-*cis* conformer of previtamin D_3. In an organic solvent, the s-*cis*,s-*cis*-previtamin D_3 conformer is thermodynamically unstable and immediately isomerizes to the s-*cis*, s-*trans* form. Because only the s-*cis*, s-*cis* conformer is able to isomerize to vitamin D_3, the entrapment of previtamin D_3 in its s-*cis*, s-*cis* form within the plasma membrane promotes a more than 10-fold increase in its rate of isomerization to vitamin D_3 when compared to the same reaction in an organic solvent (Tian *et al.,* 1994; Holick *et al.,* 1995). This process guarantees that the precious previtamin D_3 that is made in the skin is converted efficiently to vitamin D_3. In addition, as vitamin D_3 is being formed from previtamin D_3, its conformational change probably permits it to selectively exit from the membrane into the extracellular space.

Factors That Regulate Photosynthesis of Previtamin D_3 in Skin

SUNLIGHT-MEDIATED PHOTOLYSIS

It is well known that intense prolonged exposure to sunlight will not cause vitamin D intoxication. The reason for this is that during the initial exposure to sunlight, 7-dehydrocholesterol is converted to previtamin D_3. However, because previtamin D_3 is photolabile, when exposed to sunlight it is converted to lumisterol and tachysterol, which are thought to be biologically inert on calcium metabolism (Holick *et al.,* 1981) (Fig. 1). Once previtamin D_3 is isomerized to vitamin D_3, vitamin D_3 is also extremely photosensitive and is isomerized rapidly by sunlight to supersterol 1, suprasterol 2, and 5,6-transvitamin D_3 (Fig. 1), which are also thought to be either biologically inert or have less activity on calcium metabolism than vitamin D_3 (Webb *et al.,* 1989).

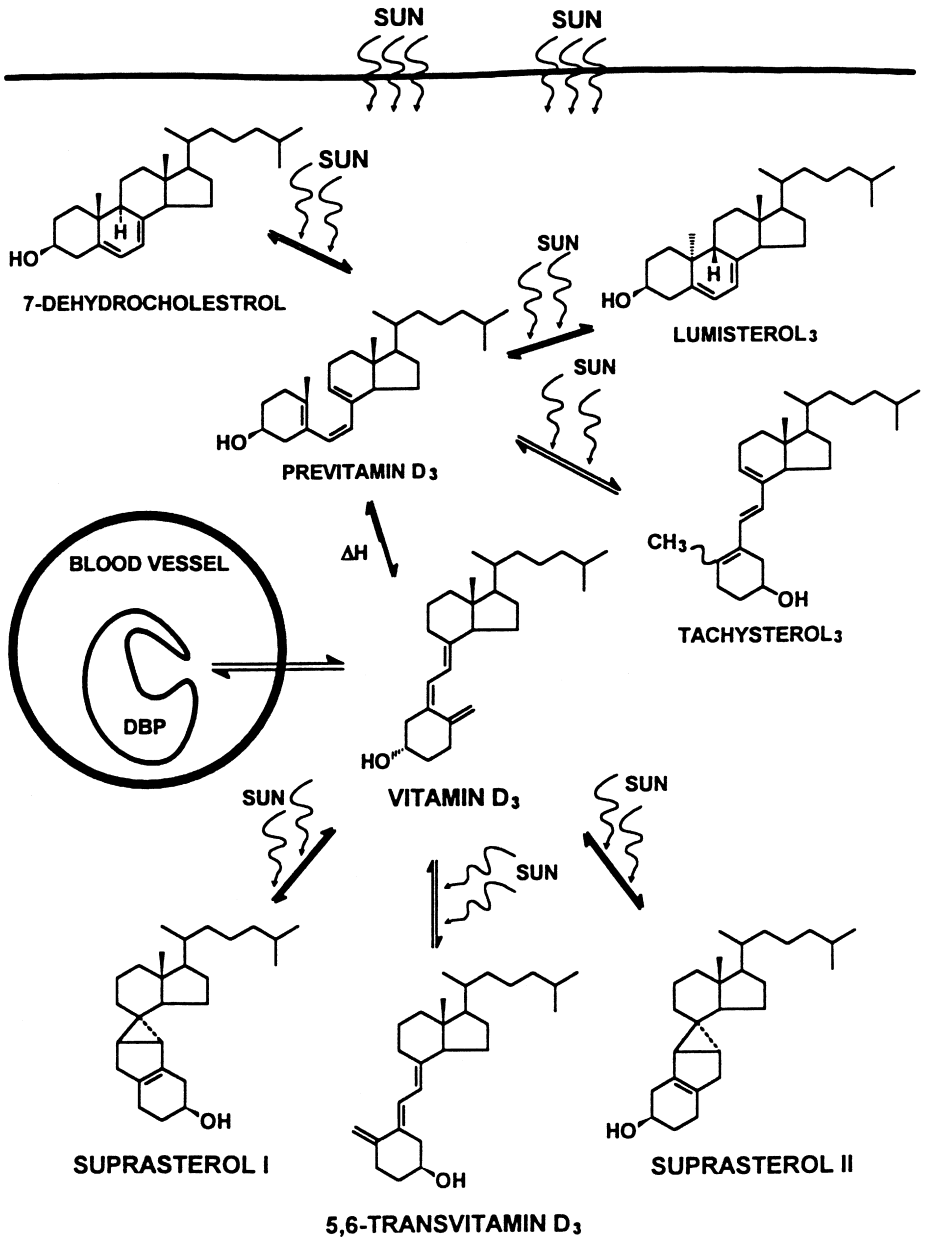

Figure 1 Photochemical events in the skin that lead to the production of vitamin D_3 and the regulation of vitamin D_3 in the skin. Reproduced with permission from Holick (1994).

MELANIN, SUNSCREENS, CLOTHING, GLASS, AND PLASTICS

Melanin is a natural sunscreen that effectively absorbs ultraviolet B radiation, thereby competing with 7-dehydrocholesterol for these photons. As a result, increased skin pigmentation requires longer exposure to sunlight to produce the same amount of previtamin D_3 as in a lighter-skinned individual (Clemens *et al.,* 1982).

Sunscreen use is highly recommended, especially for individuals who are prone to sunburning. Sunscreens such as melanin absorb ultraviolet B radiation. Therefore, the topical application of a sunscreen will substantially diminish or completely prevent the cutaneous production of previtamin D_3. When young adults were covered with a sunscreen preparation with a sun protection factor of 8 (SPF 8) followed by a whole body exposure to one minimal erythemal dose of simulated sunlight, they were unable to elevate their circulating concentrations of vitamin D above baseline values (Matsuoko *et al.,* 1987) (Fig. 3). Similarly, clothing absorbs most ultraviolet radiation and therefore prevents the cutaneous production of vitamin D_3 (Matsuoko *et al.,* 1994). Chronic use of a sunscreen will diminish circulating concentrations of 25-hydroxyvitamin D_3 as a measure of vitamin D status (Matsuoko *et al.,* 1992). In addition to sunscreens, exposure of the skin to sunlight that has passed through windowpane glass or Plexiglas will not permit any significant synthesis of vitamin D_3 in the skin because most glass and plastics absorb ultraviolet B radiation efficiently (Holick, 1994).

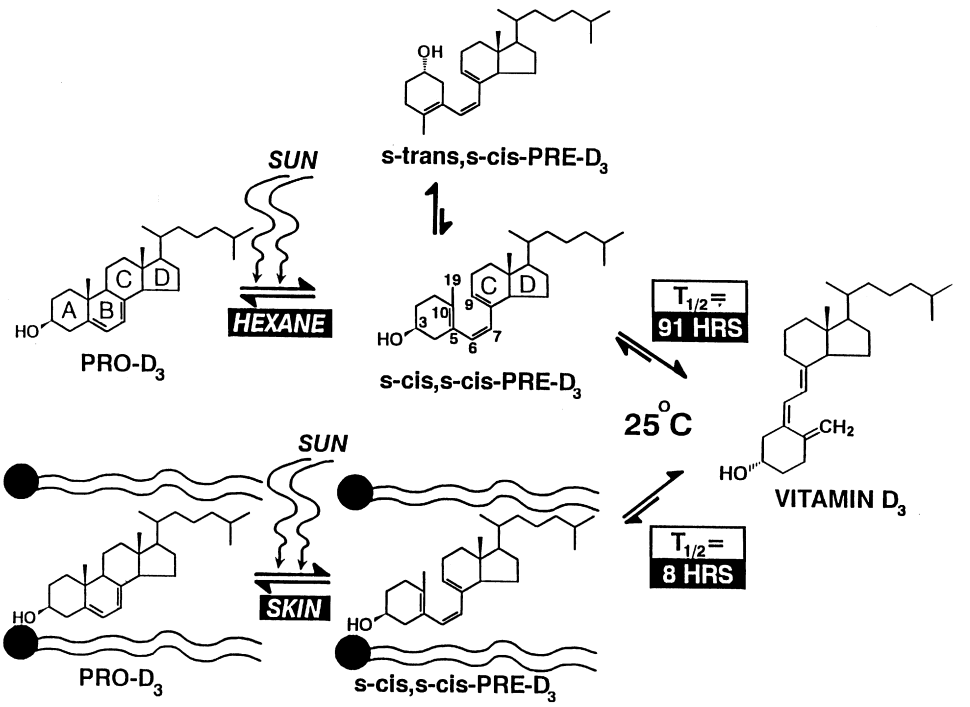

Figure 2 Photolysis of provitamin D_3 (pro-D_3) into previtamin D_3 (pre-D_3) and its thermal isomerization to vitamin D_3 in hexane and in skin. In hexane, pro-D_3 is photolyzed to s-*cis*,s-*cis*-pre-D_3. Once formed, this energetically unstable conformation undergoes a conformational change to the s-*trans*,s-*cis*-pre-D_3. Only the s-*cis*,s-*cis*-pre-D_3 can undergo thermal isomerization to vitamin D_3. The s-*cis*,s-*cis* conformer of pre-D_3 is stabilized in the phospholipid bilayer by hydrophilic interactions between the 3β-hydroxyl group and the polar head of the lipids, as well as by van der Waals interactions between the steroid ring and side chain structure and the hydrophobic tail of the membrane lipids. This "entrapment" significantly decreases its conversion to the s-*trans*,s-*cis* conformer, thereby facilitating the thermal isomerization of s-*cis*,s-*cis*-pre-D_3 to vitamin D_3. Reproduced with permission from Holick *et al.* (1995).

AGING

Aging influences a variety of metabolic processes. Therefore, it is not surprising that aging also markedly decreases the free concentrations of 7-dehydrocholesterol in the epidermis (MacLaughlin and Holick, 1985). When healthy young and elderly volunteers were exposed to the same amount of simulated sunlight, the circulating concentrations of vitamin D in the young volunteers (aged 22–30 years) increased to a maximum of 30 ng/ml within 24 hr after exposure, whereas the older subjects (aged 62–80 years) were only able to achieve a maximum concentration of 8 ng/ml (Holick *et al.,* 1989) (Fig. 4).

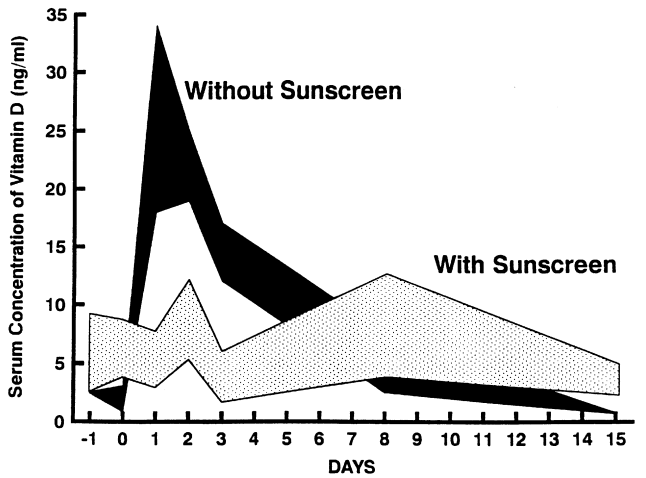

Figure 3 Circulating concentrations of vitamin D in healthy volunteers who applied an oil that contained a sunscreen with SPF-8 or no sunscreen over their entire bodies after a single exposure to one minimal erythemal dose of simulated sunlight. Adapted from Matsuoka *et al.,* 1987. Reproduced with permission from Holick, (1994).

SEASON, LATITUDE, AND TIME OF DAY

Season, latitude, and time of day can greatly influence the cutaneous production of vitamin D_3. As the zenith angle of the sun becomes more oblique, the ultraviolet B photons have to pass through the stratospheric ozone layer at a more oblique angle. This results in the ozone layer absorbing an increasing number of ultraviolet B photons. This can have a dramatic effect on the cutaneous production of previtamin D_3 (Webb *et al.,* 1988) (Fig. 5). In Boston, exposure to sunlight between the months of March and October is capable of producing previtamin D_3 in the skin. However, between the months of November and February, little if any cutaneous vitamin D_3 production can occur no matter how long one stays outdoors. The time of day also greatly influences the cutaneous production of vitamin D_3 (Fig. 6). During the summer in Boston, exposure of the skin to sunlight from

Figure 4 Circulating concentrations of vitamin D in response to a whole body exposure to one minimal erythemal dose in healthy young and elderly subjects. Adapted from Holick *et al.* (1989). Reproduced with permission from Holick (1994).

07:00 to as late as 17:00 hr eastern standard time (EST) resulted in previtamin D_3 production in human skin. However, in the spring and autumn, previtamin D_3 synthesis began at approximately 09:00 EST and ceased at approximately 15:00 EST.

Perspective on Utilization of Sunlight for Vitamin D

It is not well appreciated that casual exposure to sunlight provides most of us with our vitamin D requirement. With the exception of cod liver oil, fatty fish, and other fish liver oils, there are very few foods that have naturally occurring vitamin D. Although some foods are fortified with vitamin D, most notably milk, a recent survey of the vitamin D content in milk suggests that more than 50% of milk samples in the United States contained less than 80% of the vitamin D content stated on the label and approximately 15% contained no detectable vitamin D (Tanner *et al.,* 1988; Holick *et al.,* 1992; Chen *et al.,* 1993). The alarming increase in the incidence of skin cancer that has been directly related to an increased exposure to sunlight has prompted widespread use of sunscreening agents for preventing the damaging effects of sunlight on the skin. Because children and young adults will not routinely cover all sun-exposed areas with a sunscreen all of the time, there is no need for concern about the topical use of sunscreens in causing vitamin D

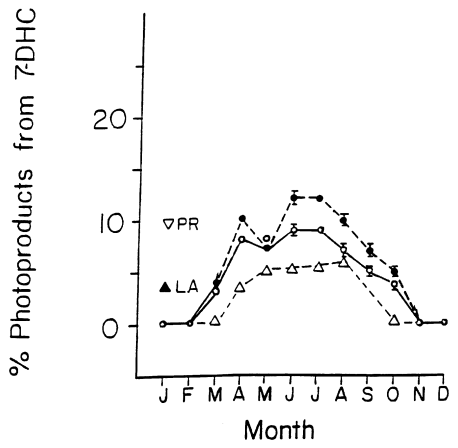

Figure 5 Photosynthesis of previtamin D_3 lumisterol, and tachysterol (photoproducts) after exposure of 7-dehydrocholesterol to sunlight in Boston (42°N) for 1 (○) and 3 (●) hr, Edmonton, Canada (52°N) after 1 hr (△) each month for 1 year, Los Angeles (34°N) (▲) and Puerto Rico (18°) in January (▽) Adapted from Webb *et al.* (1988).

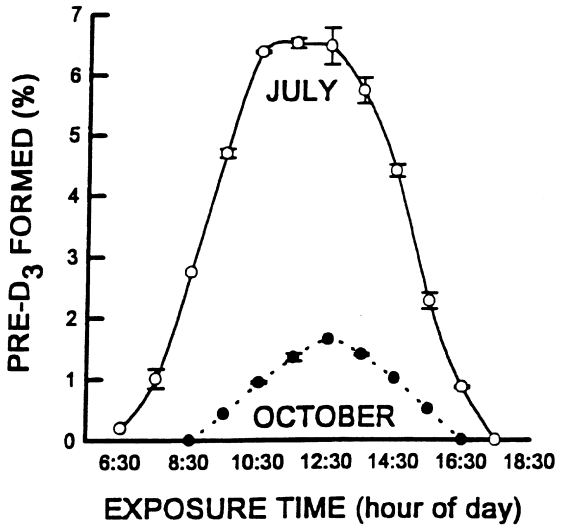

Figure 6 Photosynthesis of previtamin D_3 at various times on cloudless days in Boston in October and July. Adapted from Lu *et al.* (1992). Reproduced with permission from Holick. (1994).

deficiency in this population. However, elderly people, who have decreased capacity to produce vitamin D_3 in their skin, are concerned about developing wrinkles and skin cancer and will religiously topically apply a sunscreen on all sun-exposed areas before going outdoors. This can result in vitamin D insufficiency or overt vitamin D deficiency (Matsuoko *et al.,* 1988). People over 65 years of age who are not taking a vitamin D supplement can satisfy their bodies' vitamin D requirement by exposing their hands, arms, and face to suberythemal doses of sunlight (usually 5 to 15 min, depending on location and time of day) two to three times a week. For those who wish to stay outdoors for longer than the initial suberythemal exposure, it is recommended that they apply a sunscreen with a sun protection factor of equal to or greater than 15 to sun-exposed areas. Therefore, the intelligent use of sunlight to promote the cutaneous synthesis of vitamin D_3 and the topical use of sunscreening agents after the initial exposure prevent the damaging effects due to excessive chronic exposure to sunlight while providing it beneficial effect: vitamin D_3 (Holick, 1994).

Vitamin D Metabolism

Once vitamin D is made in the skin or ingested in the diet, it is bound to the vitamin D-binding protein (DBP). It travels to the liver where it is metabolized to 25-hydroxyvitamin D [25(OH)D]. Once formed, it leaves the liver bound to the DBP and is filtered in the gomerulus into the ultrafiltrate. 25(OH)D-DBP is reabsorbed from the ultrafiltrate by the tubules by the endocytitic receptor megalin (Nykjaer *et al.,* 1999). This endocytotic process is required to preserve 25(OH)D and to deliver it to the renal tubular cells for cytochrome P_{450} 25(OH)D-1α-hydroxylase (1α-OHase). This mitochondrial enzyme hydroxylates 25(OH)D on carbon 1 to form the biologically active form of vitamin D: 1,25-dihydroxyvitamin D [1,25$(OH)_2$D](Holick, 1999).

Originally, it was believed that the kidney was the sole source of 1α-OHase. This was based on the observation that anephric rats could not metabolize 25$(OH)D_3$ to 1,25$(OH)_2D_3$ (DeLuca, 1998; Holick, 1989b). This was also confirmed by many observations that low and undetectable concentrations of 1,25$(OH)_2D_3$ are present in patients who have no kidneys or no kidney function (Holick, 1989b). There is, however, compelling evidence that a wide variety of tissues also possess 1α-OHase activity. The first tissue demonstrated to have 1α-OHase was the skin (Bikle *et al.,* 1986). It has now been demonstrated that normal prostate and prostate cancer cells express 1α-OHase activity (Schwartz *et al.,* 1998). Using *in situ* hybridization and antibodies to 1α-OHase, it was found that 1α-OHase was present in the basal keratinocytes, hair follicles, lymph nodes, parasympathetic ganglion, pancreas, islet cells, adrenal medulla, brain (cerebellum and cerebral cortex), and placenta (Zehnder *et al.,* 2001).

Although the exact function of the extrarenal 1α-OHase is not well understood, it appears that this enzyme may be important for producing 1,25$(OH)_2D_3$ locally to act as a cellular growth modulator (Holick, 2001). To determine the effect of this enzyme on cellular growth and differentiation, we made a plasmid construct containing the 1α-OHase gene that was tagged with the green fluorescent protein gene (Flanagan *et al.,* 1999). A prostate cell line LnCaP that has a vitamin D receptor (VDR) but no 1α-OHase activity was transfected with the 1α-OHase plasmid. It was observed that cells expressed in their mitochondria a protein that had green fluorescence. These cells also had the capability of converting 25$(OH)D_3$ to 1,25$(OH)_2D_3$, whereas cells transfected with on empty vector were unable to produce any 1,25$(OH)_2D_3$. Cells transfected with the 1α-OHase construct were exposed to 25$(OH)D_3$, as were cells transfected with the empty vector construct. Cells transfected with 1α-OHase gene had decreased proliferative activity in the presence of 10^{-8} and 10^{-7} *M* 25$(OH)D_3$ whereas there was no effect in cells transfected with the empty vector (Flanagan *et al.,* 1999). These results suggest that 1,25$(OH)_2D_3$ may be produced locally in a wide variety of cells and that the function of 1,25$(OH)_2D_3$ is to regulate cell growth (Holick, 2001). This could be the explanation for why people who live at higher latitudes, and therefore make less vitamin D_3, are more likely to die of colon, breast, prostate, and ovarian cancer (Garland *et al.,* 1989, 1991; Ahonen *et al.,* 2000; Schwartz *et al.,* 1998). It may be that higher circulating concentrations of 25(OH)D are required in order for the extrarenal 1α-OHase to maximally function to produce 1,25$(OH)_2D_3$ locally to regulate cell growth and prevent metastatic activity of cells that become cancerous (Holick, 2000; Holick 2001).

Noncalcemic Actions of 1,25-Dihydroxyvitamin D_3

Nuclear Localization of ^{3}H-1,25$(OH)_2D_3$ in Noncalcemic Tissues

In 1979, Stumpf and colleagues reported that autoradiographic analysis of frozen sections of tissues from vitamin D-deficient rats that received an intravenous injection of [^{3}H-]1,25$(OH)_2D_3$ showed nuclear localization of [^{3}H-]1,25$(OH)_2D_3$ in a multitude of tissues that were not associated with calcium metabolism, including pituitary gland, thymus, gonads, stomach, breast, pancreas, and skin. Since this initial observation, a variety of investigators have reported that these tissues, as well as transformed cells and cancer cells, possess a vitamin D receptor (VDR) (Table I) (Eisman *et al.,* 1981; Colston *et al.,* 1981; Abe *et al.,* 1981; Tanaka *et al.,* 1982; Simpson *et al.,* 1985; Holick, 1995).

Noncalcemic Functions of 1,25-Dihydroxyvitamin D_3

CANCER CELLS

Initially, when normal tissues and cells such as the skin and immune cells were found to have receptors for 1,25$(OH)_2$D, it was thought that this was either an artifact or

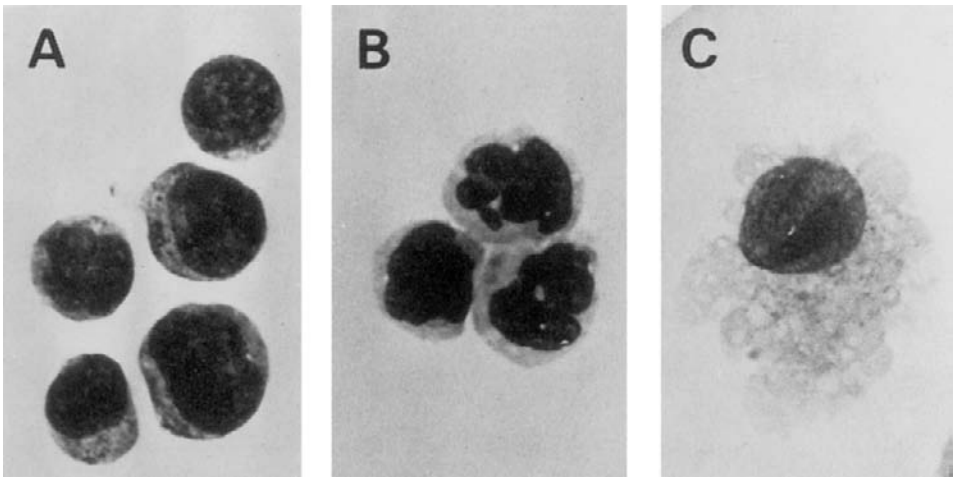

Figure 7 Morphologic changes in HL-60 cells treated with vehicle (A), 1.2×10^{-8} *M* of $1\alpha,25(OH)_2D_3$ (B), or 1.0×10^{-9} *M* of TPA (C) for 3 days. Stained by Wright–Giemsa procedures. From Suda *et al.* (1984), with permission.

was of little physiologic significance. In 1981, Eisman and co-workers reported that 80% of 54 breast cancer tissues possessed VDR activity. During the same year, Abe *et al.* (1981) and Feldman *et al.* (1981) reported that a mouse myeloid leukemic cell line (M-1) and melanoma cells, respectively, possessed a VDR (VDR+). Abe *et al.* (1981) showed a dose-dependent induction of differentiation of these myeloid leukemic cells by $1,25(OH)_2D_3$, and Colston *et al.* (1981) found that $1,25(OH)_2D_3$ inhibited melanoma cell proliferation. Cultured human promyleocytic leukemic cells (HL-60), which were VDR+, responded in a similar fashion (Fig. 7) (Tanaka *et al.,* 1982; Suda *et al.,* 1984). $1,25(OH)_2D_3$ was found to decrease cellular proliferative activity, reduce c-myc-mRNA, and induce the expression of monocyte-specific cell surface antigen 63D3 (Tanaka *et al.,* 1982). Of great interest was the *in vivo* observation that when M-1 leukemic mice were treated with $1,25(OH)_2D_3$ or 1α-hydroxyvitamin D_3 (1α-OH-D_3), their survival was enhanced substantially compared to the control group (Fig. 8) (Honma *et al.,* 1982). This suggested the possibility of using $1,25(OH)_2D_3$ or one of its analogs as an antiproliferative agent for the treatment of some leukemias and other malignant disorders.

IMMUNE SYSTEM

In the early 1980s, with the revelation that many tissues possessed a VDR, it was of great interest to determine whether cells of the immune system also possessed a VDR. Initial studies showed that resting T lymphocytes from the circulation did not possess VDR activity. However, upon stimulation with phytohemoglutinin or concanavalin A (Con A), these cells were induced to produce a VDR (Bhalla *et al.,* 1983; Tsoukas *et al.,* 1984). Once activated T lymphocytes developed VDR activity, they responded to $1,25(OH)_2D_3$ in a variety of ways, including decreased interleukin (IL)-2, interferon-γ, and GM-CSF production (Tsoukas *et al.,* 1984; Bhalla *et al.,* 1986; Binderup, 1992). Like resting T lymphocytes, resting B lymphocytes do not possess a VDR. When B cells were stimulated, a VDR was induced, which resulted in decreased DNA synthesis and

Table I Vitamin D Receptor Activity

Calcemic tissues	
Small intestine	
Bone	
Kidney	
Noncalcemic tissues	
Pituitary	Epidermis
Prostate	Melanocytes
Gonads	Hair follicles
Thymus	Dermis
Parathyroids	Monocytes
Pancreas	Lymphocytes
Breast	Myocytes
Stomach	Cardiac muscle
Placenta	

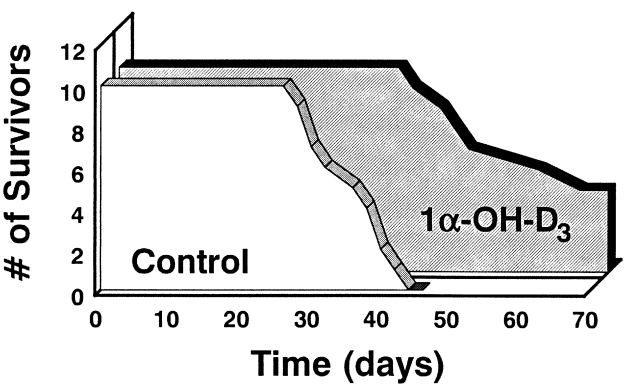

Figure 8 Mice injected with M-1 cell leukemia had a prolongation in their survival after receiving 1α-OH-D_3. Adapted from Honma *et al.* (1982).

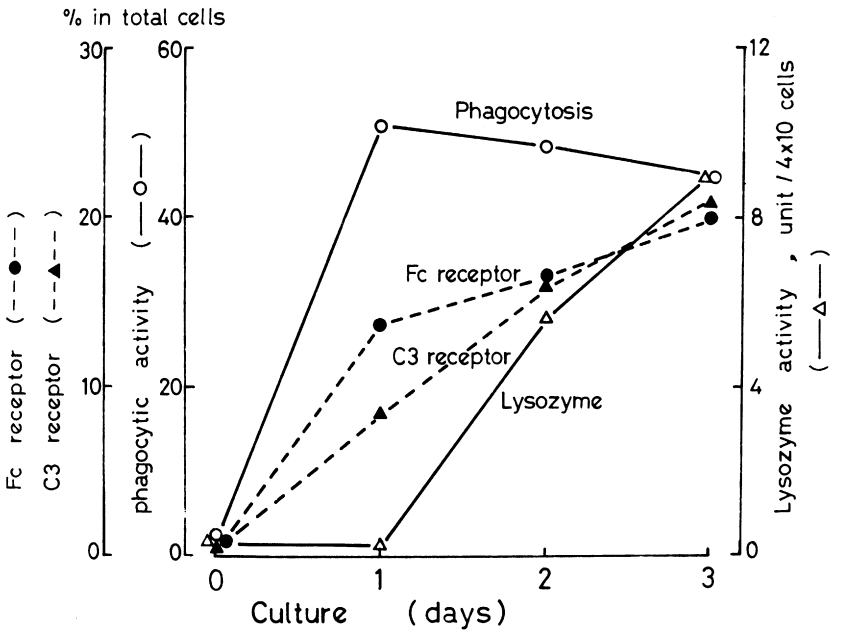

Figure 9 Time course of change in differentiation-associated properties of MI cells induced by $1\alpha,25(OH)_2D_3$. Cells were incubated with 1.2×10^{-8} *M* of $1\alpha,25(OH)_2D_3$ for 3 days. At indicated times, cells were harvested and their lysozyme activity was determined. In addition, percentages of phagocytic cells and of cells with Fc and C3 rosettes within the treated culture were determined. From Suda *et al.* (1984), with permission.

immunoglobulin production in response to $1,25(OH)_2D_3$ (Lemire *et al.,* 1984; Provvedine *et al.,* 1986). Circulating monocytes also possessed a VDR. In transformed and normal monocytes, $1,25(OH)_2D_3$ induced phagocytic activity in a time-and dose-dependent manner, increased OKII binding, augmented IL-1 production, enhanced lysosomal activity, and increased expression of cell surface antigens, including Fc and C_3 (Fig. 9) (Gray and Cohen, 1985; Amento, 1987; Suda *et al.,* 1984). When normal human monocytes were incubated with $1,25(OH)_2D_3$, cells developed morphologic and enzymatic changes consistent with their differentiation into macrophages (Provvedini *et al.,* 1986).

Therefore, it would appear that the immune system is potentially very sensitive to the modulating activities of $1,25(OH)_2D_3$. However, the exact physiological role of $1,25(OH)_2D_3$ on regulating the immune system is not well understood. An insight into the potential physiologic action of $1,25(OH)_2D_3$ on the immune system can best be seen in animals and patients with vitamin D deficiency and in patients with an inborn error in the metabolism of 25-OH-D to $1,25(OH)_2D$ or a defective VDR. Patients with vitamin D-deficient rickets have been noted to have recurrent infections, mainly of the respiratory tract (Lorente *et al.,* 1976). Vitamin D-deficient patients also have a depressed inflammatory and phagocytic response that is corrected by vitamin D replacement (Lorente *et al.,* 1976). A more subtle defect in the immune system is seen in patients with vitamin D receptor defects [vitamin D-dependent rickets type II (DDR II)]. Circulating mononuclear cells from these patients that had been stimulated previously with Con A did not respond to the same degree as normal monocytic cells to the antiproliferative activity of $1,25(OH)_2D_3$(Koren *et al.,* 1985). Furthermore, $1,25(OH)_2D_3$ and 1α-OH-D_3 treatment restored deficient macrophage and lymphocyte activities in vitamin D-deficient rats, in patients with vitamin D resistance, and in renal failure patients (Weintraub *et al.,* 1989; Binderup, 1992; Kitajima, 1989; Tabata *et al.,* 1988).

If $1,25(OH)_2D_3$ played a critical role in maintaining the immune system, then one might expect that patients with vitamin D deficiency or patients unable to either produce $1,25(OH)_2D$ (vitamin D-dependent rickets type I) or respond to $1,25(OH)_2D_3$ (DDR II) would be overcome by bacterial and viral infections. However, with the exception of some subtle recurrent infections in the respiratory tract, this is not so. Therefore, there is little evidence that $1,25(OH)_2D_3$ plays a critical role in maintaining a competent immune system.

$1,25(OH)_2D_3$ has a variety of *in vitro* and *in vivo* effects on the immune system. However, the *in vitro* observations do not necessarily predict *in vivo* outcomes. This may be due to the multitude of effects $1,25(OH)_2D$ has on T and B lymphocytes and monocytes. *In vivo,* the combination of these effects manifest themselves in numerous ways. In mice, $1,25(OH)_2D_3$ substantially reduces the development of autoimmune thyroiditis (Fournier *et al.,* 1990), encephalomyelitis (Lemire and Archer, 1991), and multiple sclerosis (Hayes *et al.,* 1997). $1,25(OH)_2D_3$ prolongs the survival of transplanted skin allografts in mice (Chiocchia *et al.,* 1991) and prevents the incidence of autoimmune diabetes in NOD mice (Mathieu *et al.,* 1994) (Fig. 10). Whereas *in vitro* $1,25(OH)_2D_3$ decreases immunoglobulin

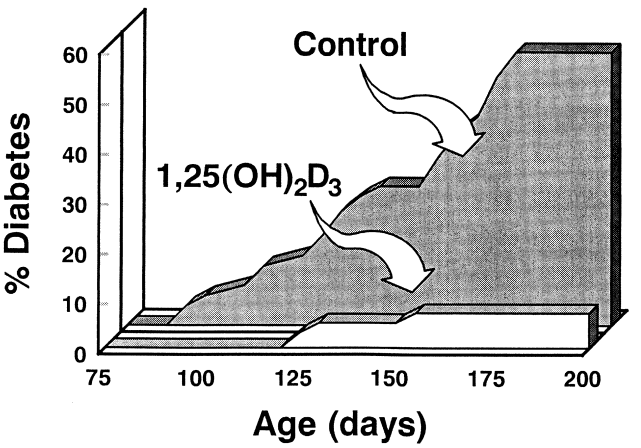

Figure 10 The effect of $1,25(OH)_2D_3$ on reducing the incidence of diabetes mellitus type I in NOD mice. Adapted from Mathieu *et al.* (1994).

synthesis in B lymphocytes, *in vivo,* its precursor analog, 1-α-OH-D_3, leads to an increase in primary antibody response (Komori *et al.,* 1985).

SKIN

The original observation that $[^3H]1,25(OH)_2D_3$ localized in the nuclei of cells in the basal layer of the epidermis has now been extended to include nuclei of cells in the outer root sheath of the hair follicle and in the stratum granulosum and stratum spinosum of the epidermis (Stumpf *et al.,* 1979, 1984). The presence of and amounts of VDR in keratinocytes appear to be related to the proliferative and differentiation activity of the cells; more VDR activity is observed in preconfluent proliferating cells than in postconfluent cells (Pillai *et al.,* 1987). VDR immunoreactivity has been detected in nuclei of dermal papilla cells and outer root sheath keratinocytes of the hair follicle. During hair follicle proliferation, the VDR immunoreactivity was enhanced significantly in both cell types, suggesting a potential role of $1,25(OH)_2D_3$ in regulating the hair cycle (Reichrath *et al.,* 1994). Although the physiologic function of $1,25(OH)_2D_3$ in these skin cells is not well understood, in cultured human and murine keratinocytes, $1,25(OH)_2D_3$ inhibited their proliferation in a dose-dependent fashion and caused them to terminally differentiate (Fig. 11) (Hosomi *et al.,* 1983; Smith *et al.,* 1986; Pillai *et al.,* 1987). Human skin fibroblasts also have VDR and respond to the hormone in a similar manner (Feldman *et al.,* 1982; Clemens *et al.,* 1983; Holick, 1995).

When cultured melanoma cells with VDR+ were incubated with $1,25(OH)_2D_3$, this hormone inhibited their proliferation and induced them to differentiate (Colston *et al.,* 1981). These data suggest that melanocytes may also be a target cell for $1,25(OH)_2D_3$. There is also immunohistochemical evidence for the presence of VDR in melanocytes from skin biopsies of patients with psoriasis (Milde *et al.,* 1991). However, there is no direct evidence that normal human melanocytes either possess a VDR or respond to $1,25(OH)_2D_3$ (Mansur *et al.,* 1988).

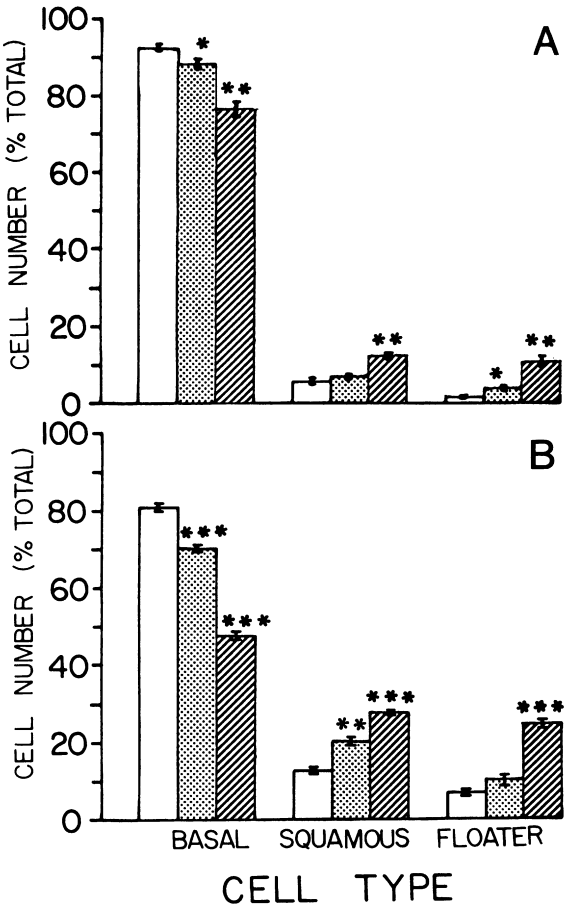

Figure 11 Effect of $1\alpha,25(OH)_2D_3$ on the morphologic differentiation of cultured human keratinocytes. The proportion of different keratinocyte cell types after 1 (A) or 2 (B) weeks of incubation with vehicle alone (open bar), $1\alpha,25(OH)_2D_3$ at 10^{-10} *M* (dotted bar), or $1\alpha,25(OH)_2D_3$ at 10^{-8} *M* (striped bar). Each bar represents the mean of triplicate determinations ± SEM. Student's *t* test was used to assess the level of significance ($^*p < 0.05$; $^{**}p < 0.001$). Reproduced with permission from Smith *et al.* (1986).

OTHER TISSUES

A wide variety of other cells and tissues from the brain to the gonads possess VDR (Clemens *et al.,* 1988). Cultured chick embryo skeletal myoblasts have receptor binding for $1,25(OH)_2D_3$ (Boland *et al.,* 1985). Furthermore, when cultured VDR+ myoblast cells (G-8 and H9c2) were incubated with $1,25(OH)_2D_3$, there was a dose-dependent decrease in cell proliferation and induction of terminal differentiation. When the cells became fused microtubules, VDR activity decreased (Simpson *et al.,* 1985). VDR is also present in rodent heart tissue, and when isolated cardiac muscle cells were exposed to $1,25(OH)_2D_3$, the hormone increased calcium uptake in a time - and dose-dependent fashion (Weishaar and Simpson, 1989).

Ovaries and testes have VDR activity. Sertoli cells in culture increase rapid uptake of calcium when exposed to $1,25(OH)_2D_3$ (Akerson and Walters, 1992). Of great interest was the observation that primary cultured prostate cells derived from normal, benign prostatic hyperplasia and

prostate cancer tissues possess VDR (Skowronski *et al.,* 1995). Prostate cancer cell lines and primary cultures of stromal and epithelial cells derived from normal and malignant prostate tissues respond in a dose-dependent fashion to the antiproliferative activity of $1,25(OH)_2D_3$.

β islet cells of the pancreas possess a VDR. There is some evidence that $1,25(OH)_2D$ may alter insulin secretion (Cade and Norman, 1986). The parathyroid glands possess VDR, and there is strong evidence that $1,25(OH)_2D_3$ suppresses preproparathyroid hormone mRNA levels (Naveh-Many and Silver, 1990).

Relevance of VDR and $1,25(OH)_2D_3$ in Noncalcemic Cells and Tissues

PHYSIOLOGIC ACTIONS

It is remarkable that most cells and tissues in the human body possess VDR and are therefore potential target tissues for $1,25(OH)_2D_3$ (Table I). Although there is very strong evidence *in vitro* and *in vivo* that $1,25(OH)_2D_3$ can have a wide range of noncalcemic activities that have an impact on the function of the immune system, skin, gonads, prostate gland, brain, skeletal and smooth muscle, and pancreas, the true physiologic function of $1,25(OH)_2D_3$ is not well understood. To put this into perspective, patients who are vitamin D deficient or patients who suffer from DDR II and are therefore totally resistant to the action of $1,25(OH)_2D_3$ do not seem to have major deficits in the physiologic function of most of the tissues described. There is subtle evidence that vitamin D deficiency causes muscle weakness and alters the immune system to make these patients more prone to some infections, and in the case of DDR II, the patients often suffer from alopecia (DeMay, 1995; Holick, 1995). They, however, do not have a higher incidence of cancer such as leukemia, they do not suffer from diabetes mellitus, and their skin appears to be normal with no evidence of hyperproliferation, such as psoriasis or pigmentation disorders.

PHARMACOLOGIC ACTIONS

The recognition in the early 1980s that $1,25(OH)_2D_3$ inhibited proliferation and induced differentiation of normal and tumor cells that possessed VDR was greeted with great excitement. The observation that mice with an M-1 cell leukemia had a marked prolongation in their survival when they received 1-α-OH-D_3 or $1,25(OH)_2D_3$ suggested that the antiproliferative activity of $1,25(OH)_2D_3$ and its analogs could be used to treat a variety of cancers (Honma *et al.,* 1982). Eighteen patients with myelodysplasia (preleukemia) were treated with 2μg of $1,25(OH)_2D_3$ for 12 weeks. A majority of the patients initially had a significant increase in their granulocyte, monocyte, and platelet counts, suggesting that $1,25(OH)_2D_3$ was inhibiting the proliferation and inducing terminal differentiation of the myelodysplastic cells. After 12 weeks of the study, however, there was no significant difference in the blood count for granulocytes, monocytes, and platelets compared to baseline and most patients progressed to acute myelocytic leukemia. In addition, most of the patients had developed hypercalcemia, limiting the amount of drug that could be used (Koeffler *et al.,* 1985). Three patients with myelofibrosis who received $1,25(OH)_2D_3$ (0.5 μg daily) had some improvement in their blood count indices after therapy (Arlet *et al.,* 1984).

When HL-60 cells are incubated with $1,25(OH)_2D_3$, most of the cells that have VDR respond to its antiproliferative and prodifferentiation activities. However, when $1,25(OH)_2D_3$ is removed from the culture, cells that did not commit to full differentiation reverted back to their original (anaplastic) state of activity (Bar-Shavit *et al.,* 1986). It is likely that in the population of leukemia cells, there are cells that have either defective or absent VDR. As a result, these clones of cells become the predominant cell type that may also be more blastic. There continues to be interest in developing potent analogs of $1,25(OH)_2D_3$ that have little calcemic activity and potent antiproliferative activity. These analogs could potentially be used as part of combination therapy for some cancers, such as colon, breast, prostate, and some leukemias.

Clinical Utility of Noncalcemic Actions of $1,25(OH)_2D_3$ and Its Analogs

Use of $1,25(OH)_2D_3$ and Its Analogs for Treatment of Skin Diseases

RATIONALE FOR THEIR USE

In the mid-1980s, there was mounting evidence that epidermal skin cells were very sensitive to the antiproliferative activity of $1,25(OH)_2D_3$ (Hosomi *et al.,* 1983; Smith *et al.,* 1986). Because psoriasis is a nonmalignant hyperproliferative disorder of the epidermis, it was reasoned that if psoriatic skin cells possessed a VDR, then it might be possible to use $1,25(OH)_2D_3$ or one of its analogs to decrease psoriatic keratinocyte proliferation, thereby treating this disorder. Before initiating a clinical trial to evaluate the therapeutic efficacy of $1,25(OH)_2D_3$, McLaughlin *et al.* (1985) obtained skin biopsies from six patients with psoriasis to determine whether cultured psoriatic fibroblasts responded to the antiproliferative activity of $1,25(OH)_2D_3$. It was found that psoriatic fibroblasts had a partial resistance to the antiproliferative activity of $1,25(OH)_2D_3$, and it was concluded that pharmacologic rather than physiologic amounts of $1,25(OH)_2D_3$ and its analogs could be used for the treatment of psoriasis (McLaughlin *et al.,* 1985) (Fig. 12). At the same time, Morimoto *et al.* (1985) treated an osteoporosis patient with 1α-OH-D and observed that this patient, who also suffered from psoriasis, had significant improvement in her disease while on therapy. There are now numerous reports that topical $1,25(OH)_2D_3$, as well as topical application of analogs of $1,25(OH)_2D_3$, including 1,24-dihydroxyvitamin D_3 and calcipotriene (Dovonex; Bristol-Meyers Squibb, Buffalo, NY), is safe and effective for the treatment of psoriasis

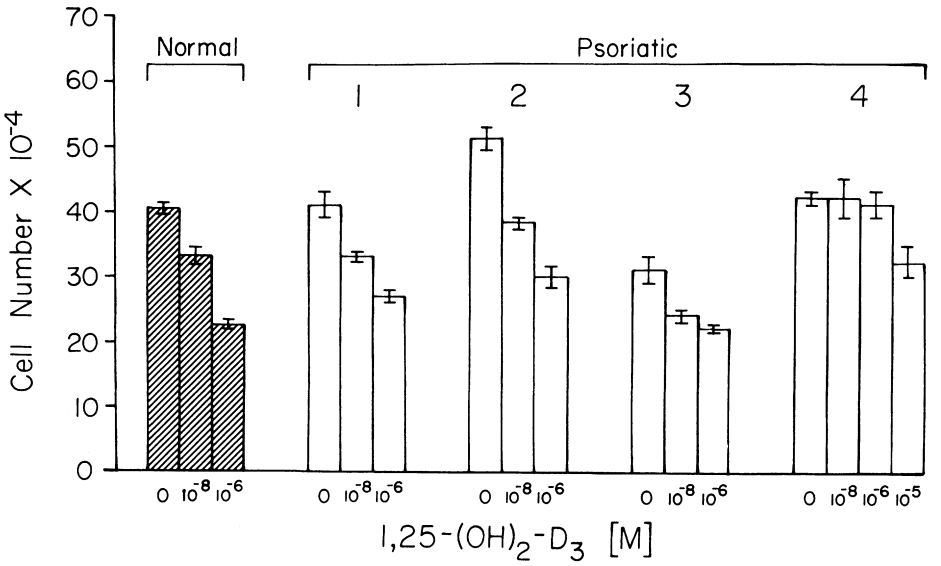

Figure 12 Effect of $1,25(OH)_2D_3$ on the growth of cultured dermal fibroblasts obtained from a normal adult or from four different patients with psoriasis. Fibroblasts were incubated in medium-containing vehicle alone or with $1,25(OH)_2D_3$ at 10^{-8}, 10^{-6}, or 10^{-5} mol/liter. After 7 days the cells were harvested and counted. Each bar represents the mean ± SEM cell number of triplicate cultures. Reproduced with permission from Smith *et al.* (1988).

(Kato *et al.,* 1986; van DeKerkhof *et al.,* 1989; Kragballe *et al.,* 1989; Staberg *et al.,* 1989; Langer *et al.,* 1992; Bourke *et al.,* 1993; Langer *et al.,* 1993; Holick, 1993; Bruce *et al.,* 1994; Perez *et al.,* 1996, 2001).

There has been great concern that in light of the earlier studies using $1,25(OH)_2D_3$ for treating preleukemia that caused severe hypercalcemia (Koeffler *et al.,* 1985) that $1,25(OH)_2D_3$ would not be a safe medication for treating psoriasis. However, for the most part, these concerns have not proven to be correct (Holick, 1993; Perez, *et al.,* 1996a,b).

Clinical Trials with Topical $1,25(OH)_2D_3$ and Its Analogs

The topical application of $1,25(OH)_2D_3$ (15 μg/g petrolatum) caused significant improvement by reducing scale, erythema, and plaque after 2 months of therapy (Fig. 13). After observing no untoward side effects from the therapy, 22 patients topically applied the $1,25(OH)_2D_3$ ointment over all of their lesions (2000–5000 cm^2), using up to 10 g of $1,25(OH)_2D_3$ ointment per day or 150 μg of the $1,25(OH)_2D_3$ daily. Scaling, plaque thickness, and erythema of psoriatic lesions showed excellent or moderate improvement in 90.9% of all cases with marked reduction in the psoriasis area severity index (PASI) score (Perez *et al.,* 1996). There was no change in serum $1,25(OH)_2D_3$ levels, which was also reflected in no significant change in either the 24-hr urinary excretion of calcium or the serum calcium concentration. This is quite remarkable when one considers that the kidney produces only about 2 μg of $1,25(OH)_2D_3$ each day. With the topical application of 70 times the total daily renal production of $1,25(OH)_2D_3$, one would have expected that some of the $1,25(OH)_2D_3$ would be transported across the skin into the circulation, causing hypercalciuria and hypercalcemia. This is especially true, as psoriasis causes a defect in the barrier function of the skin, thereby potentially enhancing the penetration of the drug. Although it is not known why there are no untoward side effects when using topical $1,25(OH)_2D_3$, it is likely that several mechanisms help prevent untoward toxicity. $1,25(OH)_2D_3$ probably partitions itself well between the petrolatum and the epidermis. Once the epidermal cells are exposed to $1,25(OH)_2D_3$, they turn on a cascade of metabolic processes to degrade $1,25(OH)_2D_3$ to a biologically inactive water-soluble calcitroic acid (DeLuca, 1988). It is also reasonable to consider that since the cellular components of the epidermis and dermis possess VDR, they act as an effective sponge to bind most of the $1,25(OH)_2D_3$, thereby preventing its entrance into the dermal capillary bed (Holick, 1987; Holick, 1993).

Several other analogs have been developed for the treatment of psoriasis. The most commonly used analog is calcipotriene (Dovonex). The strategy for developing this analog was to alter the side chain so that it would be metabolized rapidly and, therefore, less prone to developing hypercalciuria and hypercalcemia (Binderup and Braum, 1988; Kragballe *et al.,* 1989; Kragballe, 1991). Indeed, calcipotriene is metabolized and degraded rapidly (Binderup *et al.,* 1988; Sorensen *et al.,* 1990). Calcipotriene at 50 μg/g of ointment or cream is used worldwide for the treatment of psoriasis. Other analogs, including 1,24-hydroxyvitamin D_3 (Kato *et al.,* 1986) and hexafluoro-1,25-dihydroxyvitamin D_3 (Durakovic *et al.,* 2001), have been shown to be effective for treating psoriasis. However, calcipotriene can cause a dermatitis that occurs on very sensitive skin areas, such as

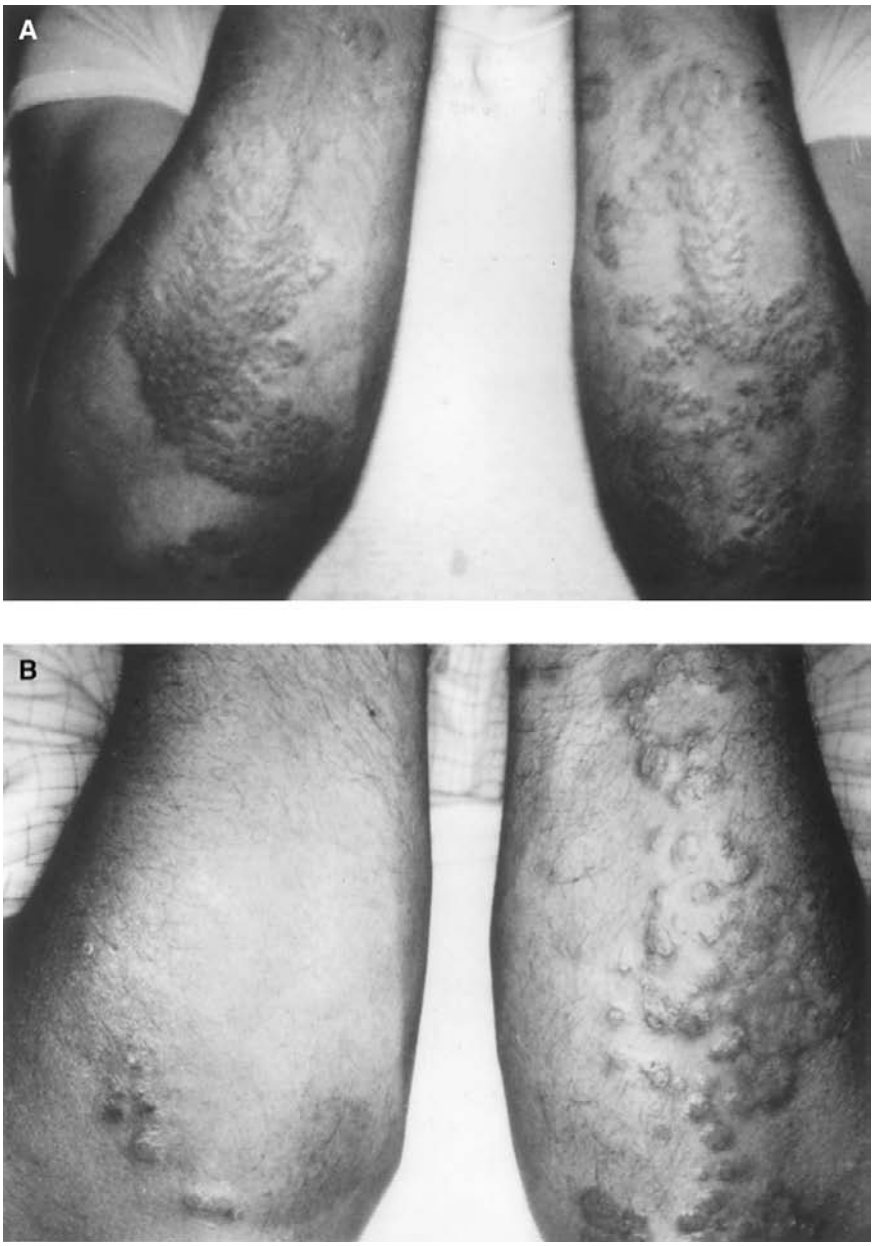

Figure 13 (A) Arms of a patient with a long history of plaque psoriasis before treatment with the topical form of 1,25-dihydroxyvitamin D_3. (B) The same patient who applied only petroleum jelly on the left forearm (at right) and petroleum jelly containing 15 μg/g of $1,25(OH)_2D_3$ on the right forearm (at left) for 3 months. Reproduced with permission from Holick (1994).

the face and genital regions (Yipp *et al.,* 1999), and very rarely hypercalcemia (Hoek *et al.,* 1994).

CLINICAL TRIAL WITH ORAL $1,25(OH)_2D_3$

For patients with more than 10% of their bodies affected with psoriasis, the topical application of $1,25(OH)_2D_3$ and other vitamin D analogs can be inconvenient. Eighty-four patients with psoriasis vulgaris or erythrodermal psoriasis were treated with 0.5 μg of $1,25(OH)_2D_3$ given at night and increased by 0.5 μg every 2 weeks as long as serum and urine calcium levels were normal. The usual therapeutic dose was between 1 and 3 μg each night. The treatment varied from 6 months to 3 years. Overall clinical assessment showed that 88% of all patients taking oral $1,25(OH)_2D_3$ had some improvement in their disease (Perez *et al.,* 1996b). Serum calcium concentrations did not increase outside of the normal range in most of the patients. Twenty-Four-hour urinary excretion increased, but was also not outside the normal range in most patients. Bone mineral density measurements and renal ultrasound scans for kidney stones at 6-month intervals for up to 4 years were unchanged from baseline (Perez *et al.,* 2001; Holick, 1996).

TREATMENT OF PSORIATIC ARTHRITIS WITH $1,25(OH)_2D_3$

It has been estimated that approximately 10% of patients with psoriasis suffer from psoriatic arthritis. In an open-label trial, we found that 10 patients with active psoriatic arthritis who received up to 2.5 μg of oral $1,25(OH)_2D_3$ each night had a statistically significant improvement in mean tender joint count and physician global assessment (Huckins *et al.,* 1991). Forty percent of patients had greater than 50% improvement in their disease and an additional 30% had greater than 25% improvement.

Conclusion

Casual exposure to sunlight provides most humans with their vitamin D requirement. Because vitamin D plays an essential role in the maintenance of a healthy skeleton, it is important that all vertebrates, including humans, have a steady supply of vitamin D. The skin is not only the site for the synthesis of this important calciotropic hormone, but is also a major target tissue for $1,25(OH)_2D_3$. The skin may also be a site for the metabolism of 25-OH-D to $1,25(OH)_2D$ (Bikle *et al.,* 1986). It is remarkable that $1,25(OH)_2D_3$ has so many potential biologic actions. As a result, $1,25(OH)_2D_3$ and its analogs have been developed for the treatment of a wide variety of clinical disorders. $1,25(OH)_2D_3$ and its analogs have been very effective in the treatment of hypocalcemic disorders and for the treatment of metabolic bone diseases associated with acquired and inherited disorders of 25(OH)D metabolism and VDR defects (DeMay, 1995; Holick, 1999). $1,25(OH)_2D_3$ and its analogs have also been shown to be of value for the treatment of osteoporosis (Tilyard *et al.,* 1992). What has been most intriguing about $1,25(OH)_2D_3$ is its potent antiproliferative properties. One might assume that because $1,25(OH)_2D_3$ is such a potent antiproliferative agent that its chronic use for the treatment of a hyperproliferative disorder would ultimately result in an atrophy of the treated tissues. For example, for the treatment of psoriasis, would the chronic use of $1,25(OH)_2D_3$ and its analogs cause senescence of the skin similar to topical steroids? All of the experience has suggested that $1,25(OH)_2D_3$ will not cause any thinning of the skin, unlike topical steroids. This suggests that $1,25(OH)_2D_3$ is able to sense the antiproliferative state and return the activity of the cell to normal. It is for this reason that $1,25(OH)_2D_3$ and its analogs hold such promise for the treatment of a wide variety of proliferative disorders, most notably some cancers.

The observations that many nonrenal tissues, including the skin, colon, and prostate, have 1α-OHase activity opens a new chapter in the vitamin D story (Holick, 2001). Why $1,25(OH)_2D$ would be produced locally in the skin, prostate, colon, and so on remains unknown but may be important in the regulation of cell growth (Fig. 14). The observation that the product of the Wilm's tumor gene modulates cellular proliferative activity of renal and hemopoetic cells and also regulates the expression of the VDR provides insight into the complexity of the function of $1,25(OH)_2D$ in cell growth (Mauer *et al.,* 2001).

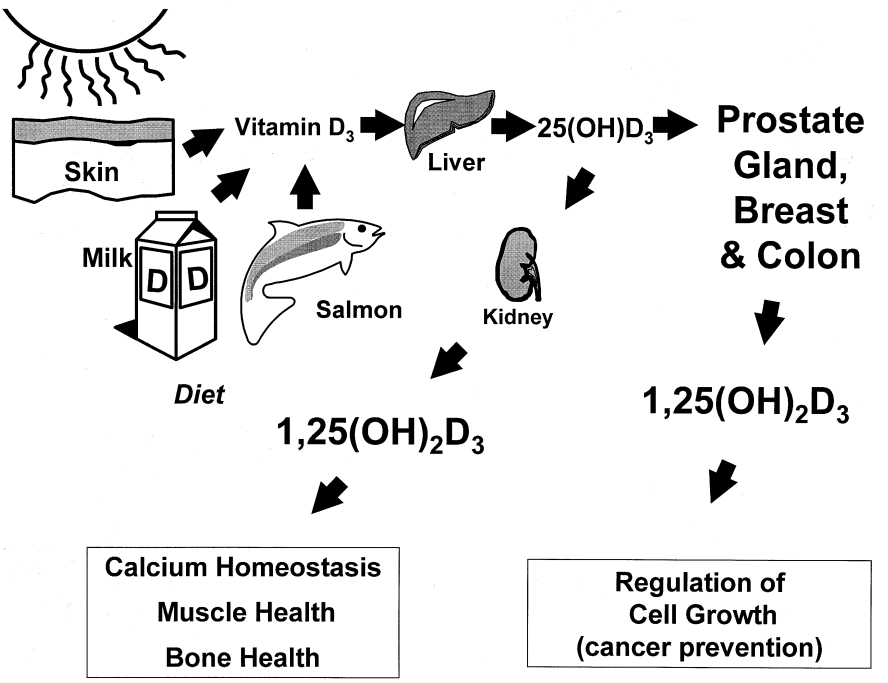

Figure 14 Metabolism of $25(OH)D_3$ to $1,25(OH)_2D_3$ in the kidney that is responsible for maintaining circulating levels of $1,25(OH)_2D_3$ on bone and muscle health and in tissues not related to calcium metabolism. This extrarenal metabolism may be important for regulating cell growth and decreasing malignant cell growth. Reproduced from Holick (2001), with permission.

$1,25(OH)_2D_3$ and its analog 1α-OH-D_3 have been used successfully during the past two decades for treating a variety of acquired and inborn errors in the metabolism of 25(OH)D to $1,25(OH)_2D$, as well as other hypocalcemic disorders (DeMay, 1995; Holick, 1995). The revelation that $1,25(OH)_2D_3$ has noncalcemic activities, including regulating proliferation and differentiation of cells and altering the immune function, sparked great interest in developing selective analogs of $1,25(OH)_2D$ that had the desirable noncalcemic actions of $1,25(OH)_2D$ without the potential toxic side effect on calcium metabolism. There are a very large number of analogs that have been synthesized and well reviewed by Bouillon *et al.* (1995). At the present time, there are very few analogs of $1,25(OH)_2D_3$ that are available commercially and have limited calcemic activity.

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CHAPTER 34

Structure and Molecular Biology of the Calcitonin Receptor

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Introduction

Calcitonin (CT) is a 32 amino acid peptide hormone that was originally identified as a hypocalcemic factor (Copp *et al.,* 1962). In mammals, these effects are attributed to inhibition of bone resorption and enhanced renal calcium excretion (Friedman and Raisz, 1965; Raisz *et al.,* 1967; Raisz and Niemann, 1967; Warshawsky *et al.,* 1980). The application of autoradiographic and radioligand-binding techniques with iodinated CT and, more recently, the use of reagents derived from cloned CT receptors (CTR) have permitted definitive identification and localization of the tissue and cellular distribution of CTRs. These results demonstrate that, in addition to receptors on osteoclasts and renal cells (Warshawsky *et al.,* 1980; Nicholson *et al.,* 1986), CTRs are widely distributed in diverse tissues and cell types, many of which are not involved in the regulation of mineral ion homeostasis. These include the central nervous system (Fischer *et al.,* 1981; Goltzman, 1985), placenta (Nicholson *et al.,* 1988), ovary (Azria, 1989), testis (Chausmer *et al.,* 1980), spermatozoa (Silvestroni *et al.,* 1987), lymphocytes (Marx *et al.,* 1974), and breast (Tverberg *et al.,* 2000). In addition, immunoreactive CT and CT mRNA expression have been colocalized in the mammary gland (Tverberg *et al.,* 2000), intestine, thymus, bladder, lung, testis, ovary, stomach, central nervous system, pituitary, and adrenal glands (Azria, 1989). The CTR is also expressed in a number of human cancer cell lines, including lung (Findlay *et al.,* 1980), prostate (Shah *et al.,* 1994), and breast origin (Findlay *et al.,* 1981) and was found in primary breast cancer cells (Gillespie *et al.,* 1997). This diverse tissue distribution of the ligand, as well as the receptor, indicates that CT may have a more complex function in addition to its activities in regulating calcium homeostasis.

There is good evidence that CT may function as a regulatory hormone in development. For example, in *Xenopus* embryos, addition of CT to the ambient water of the developing eggs produces larvae with multiple defects in oral–facial architecture and in the central nervous system (Burgess, 1982, 1985). These findings are consistent with faulty neural induction, perhaps resulting from effects of CT on migrating cells during gastrulation. It is interesting to speculate that these effects may be analogous to the inhibition of formation of polykaryons by CT during the generation of osteoclasts from mononuclear hematopoietic progenitors (Takahashi *et al.,* 1988b; Vaes, 1988). Further support for the role of CT in early vertebrate development is provided by the observations of Gorn *et al.* (1995a), who demonstrated that overexpression of procalcitonin in the two-cell stage of zebrafish embryos resulted in a variable axis duplication. Of interest, they were able to detect both CTR and CT mRNA at these tissue sites, consistent with an autocrine regulatory feedback system in the zebrafish embryo similar to that suggested by studies in mammalian species.

There are also observations that support a role for CT in mammalian development. For example, CT interaction with CTR (which is upregulated 25-fold between the one-cell and the eight-cell stage blastocyst) increases intracellular levels of calcium in preimplantation embryos and accelerates their development (Wang *et al.,* 1998). In addition, the murine embryonic teratocarcinoma cell line F-9 has receptors for CT that are functionally coupled to adenylate

cyclase (Evain *et al.,* 1981; Binet *et al.,* 1985). Treatment of these cells with dibutryl cAMP induces the cells to assume a neural-like morphology, which is accompanied by the loss of CTRs and a marked decrease in CT-induced cAMP responses (Evain *et al.,* 1981). Under these conditions, F-9 cells are induced to synthesize and secrete CT, suggesting that CT may act as an autocrine regulatory hormone during the differentiation process (Binet *et al.,* 1985).

Of particular interest, evidence shows that CT may have a role in early stages of embryonic bone formation or in the production of ectopic bone. For instance, CT has been shown to stimulate adenylate cyclase activity during the period of palatal fusion and oral–cranial development in golden hamsters (Waterman *et al.,* 1977). Farley and co-workers (1988) have shown that CT directly stimulates bone cell proliferation and bone formation in embryonic chicken skeletal tissues. These findings are consistent with an inductive effect of CT on mesodermal cells of osteoblast lineage or on osteoblasts themselves. It is still not clear, however, whether CT acts directly on bone-forming cells and their progenitors or indirectly through other target cells. CT has also been shown to produce effects in a model of ectopic bone formation. In these studies, administration of CT during early stages of endochondral bone formation induced by demineralized bone matrix results in increased bone formation that appears to be secondary to the enhanced proliferation of cartilage and bone progenitor cells (Weiss *et al.,* 1981). It is of interest that when CT was administered after bone formation was initiated, subsequent bone formation was suppressed, suggesting a differential response to this hormone, depending on the stage of cellular differentiation and the presence of different target cell populations.

CTR Protein Structure and Signaling

The CTR was initially cloned from the porcine renal epithelial cell line LLC-PK_1 (Lin *et al.,* 1991a,b). Analysis of the predicted 482 amino acid sequence of this cDNA demonstrated seven hydrophobic regions that could generate transmembrane (TM)-spanning domains. It has since been found that CTR is a member of a subfamily of the seven transmembrane domain G protein-coupled receptor superfamily termed $GPCR_{II}$. Members of this receptor family include receptors for parathyroid hormone/parathyroid hormone-related peptide (PTH/PTHrP) (Jüppner *et al.,* 1991; Abou-Samra *et al.,* 1992), corticotropin-releasing factor (Chen *et al.,* 1993), and, in addition, receptors for the glucagon family of peptides, glucagon (Jelinek *et al.,* 1993), secretin (Ishihara *et al.,* 1991), vasoactive intestinal peptide (Ishihara *et al.,* 1992), glucagon-like peptide 1 (Thorens, 1992), growth hormone-releasing hormone (GHRH) (Mayo, 1992), and pituitary adenylate cyclase-activating peptide (PACAP) (Pisegna and Wank, 1993). The most recent addition to this family is the so-called insect diuretic hormone receptor from adult *Manduca sexta* that stimulates fluid secretion and cAMP synthesis in the Malphighian tubules (Reagan, 1994). The peptide that activates this receptor belongs to the corticotropin-releasing factor peptide family (Chen *et al.,* 1993).

Analysis of the protein structure and amino acid sequences of the CTR (see Fig. 1) and related members of the family demonstrates that they share several common features. All of the predicted receptor proteins contain an extended extracellular amino (N)-terminal region that contains multiple potential glycosylation sites and conserved cysteine residues (Segre and Goldring, 1993). Glycosylation, particularly at Asn 78 and Asn 83 of the archetypal hCTR, is important for high-affinity binding of CT (Ho *et al.,* 1999). While the TM domain sequences are conserved (40–60% identical), the N-terminal domains are generally less than 25% identical. These observations suggest that the TM domain regions may have a more generic function, whereas the N-terminal domain fulfills specialized functions, such as ligand binding and receptor specificity. This speculation is supported by the functional consequences of site-directed mutagenesis of the receptors and the evaluation of both chimeric PTH/PTHrP-CT receptors and chimeric CT and PTH ligands (Jüppner *et al.,* 1993; Bergwitz *et al.,* 1996). Results indicate that the C-terminal portions of the ligands bind to the N-terminal extracellular domains of the receptors, whereas the N-terminal regions of the ligands interact with membrane-embedded domains to trigger receptor activation and signal transduction. With respect to the C-terminal intracellular domain of the receptors, except for the amino acid sequences immediately adjacent to the seventh TM domain, which are highly homologous and have been implicated in coupling to G proteins, the C-terminal regions are not conserved among $GPCR_{II}$ family members. One possibility is that the C terminus has different functions in the individual receptors. Alternatively, this region may not have a specific or critical function and is thus less constrained by selection and is more susceptible to sequence drift (Lok *et al.,* 1994).

The CTR is coupled to multiple signal transduction pathways through interaction with members of the heterotrimeric G protein family. Binding of CT to the CTR can stimulate activation of the adenylate cyclase/cAMP/ protein kinase A pathway (Chabre *et al.,* 1992; Force *et al.,* 1992) through the G protein G_s; the phosphoinositide-dependent phospholipase C pathway, which results in both Ca^{2+} mobilization (Teti *et al.,* 1995) and protein kinase C activation (Chakraborty *et al.,* 1994) via G proteins of the G_q family; and the phosphatidylcholine-dependent phospholipase D pathway, which also results in protein kinase C activation (Naro *et al.,* 1998) and can, under certain conditions, inhibit adenylate cyclase via the G_i subclass of G proteins (Shyu *et al.,* 1999). Of interest, the coupling of the CTR to specific G proteins and activation of individual signal pathways are affected by the stage of the cell cycle (Chakraborty *et al.,* 1991; Shyu *et al.,* 1999). In synchronized LLC-PK_1 cells, it has been reported that CTR-coupled G_i modulates the adenylyl cyclase activity stimulated by CTR-coupled G_s in the S

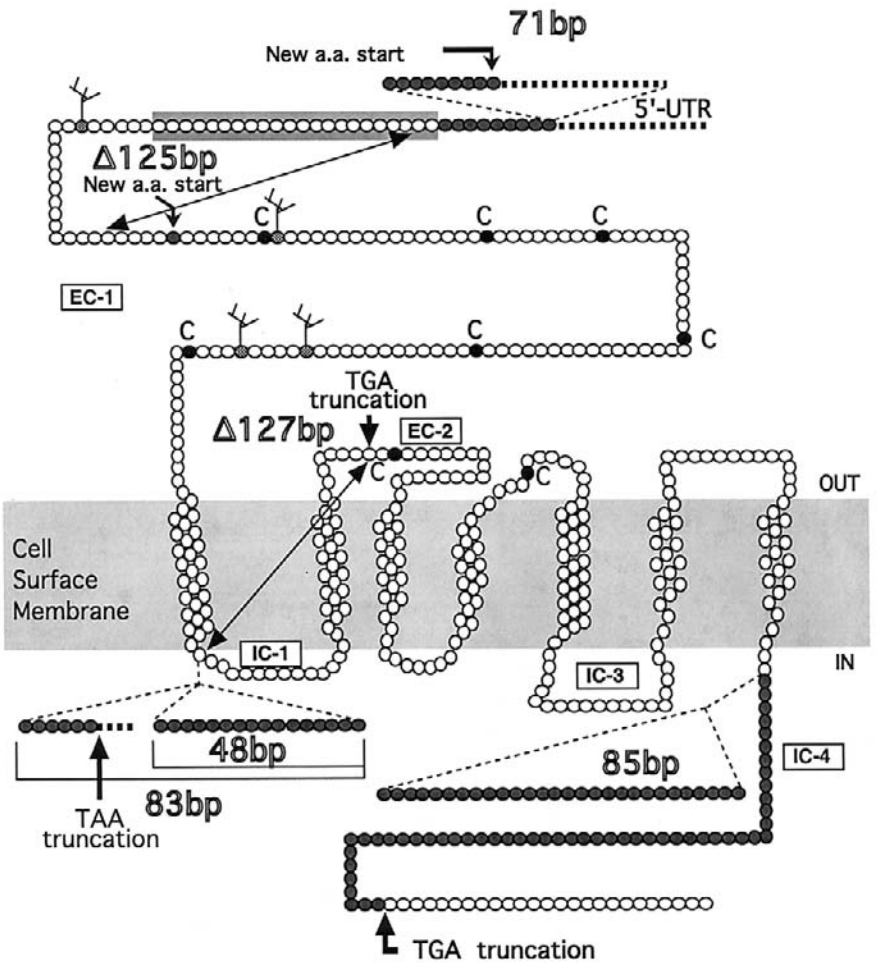

Figure 1 mRNA splice variants of human CTR depicted as expected protein translation products. White circles represent the amino acids (a.a.) of the archetypal human CTR isoform. The a.a. positions denoted as black circles with a C nearby are the conserved cysteines and shaded circles with the branch structure are the putative sites for N-glycosylation. Boxed circles denote the putative signal sequence. Gray circles represent amino acids added or changed by the addition or deletion of variably spliced mRNA regions as indicated. The domains of interest are noted as follows: IC-1, IC-3, and IC-4, first, third, and fourth intracellular domains, respectively; EC-1 and EC-2, first and second extracellular domain, respectively.

phase, but not in the G_2 phase of the cell cycle, and that this could be regulated by PKC-dependent negative regulation of the CTR-G_i-adenylyl cyclase coupling (Shyu *et al.,* 1999).

The CTR-mediated increases in cytosolic-free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and inositol phosphate production (Force *et al.,* 1992) are transduced by the activation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-biphosphate to generate two second messengers: inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Rhee and Bae, 1997). The IP_3 generated binds to IP_3 receptors (ligand-gated ion channels) on the endoplasmic reticulum (ER), resulting in a release of the ER Ca^{2+} stores into the cytoplasm (Berridge, 1993). Prolonged elevation of $[Ca^{2+}]_i$ levels, beyond the initial transient resulting from the emptying of ER calcium stores, leads secondarily to an influx of extracellular Ca^{2+}. This sustained plateau phase of calcium mobilization is the outcome of capacitative calcium entry, a process that couples the depletion of intracellular stores to the influx of extracellular calcium through the specialized calcium channels (Findlay *et al.,* 1995; Teti *et al.,* 1995). Thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase needed for repletion of calcium stores, induces a transient $[Ca^{2+}]_i$ increase by blocking reuptake, whereas ionomycin, a calcium ionophore, depletes the calcium stores directly. Both cause capacitative calcium entry in T cells. In HEK-293 cells stably transfected with rat or porcine CTRs (Findlay *et al.,* 1995) and the porcine renal tubular cell line LLC-PK_1 (Teti *et al.,* 1995), pretreatment with thapsigargin induced a transient increase and a sustained plateau, and further treatment with CT did not increase $[Ca^{2+}]_i$ over the thapsigargin-induced plateau. In HEK-293 cells transfected with a rat CTR, the CT-induced calcium influx was not inhibited by pertussis toxin, suggesting that although a G protein(s) may transduce the

CT signal, it is not a substrate for pertussis toxin ADP ribosylation (Findlay *et al.,* 1995).

The mechanism by which CT increases cAMP levels involves activation of adenylyl cyclase, a process that is dependent on interaction with the G protein G_s. In order to establish the molecular basis for G_s activation by CTR, a chimeric CTR/IGF II-R has been used to identify at least two distinct segments of the CTR, which have the capacity to interact with G_s proteins and activate cAMP (Orcel *et al.,* 2000). One segment is localized to residues 327–344 in the third intracellular loop (IC3) and the other to residues 404–418 in the membrane-proximal portion of the fourth intracellular domain (IC4). Similar regions of the predicted intracellular domains have been associated with G protein interactions in other members of the $GPCR_{II}$ family of receptors.

Signal pathways regulating the effects of CT on specific osteoclast activities have been studied extensively. However, interpretation of the role of the individual pathways responsible for the specific effects on osteclast function has been difficult because the results have often been dependent on the specific cell culture model employed and the species from which the cells were derived. In general, data indicate that activation of the adenylyl cyclase signal cascade induces the arrest of cell motility, in part, mediated by stimulation of the sodium pump. In contrast, a CT-induced elevation of $[Ca^{2+}]_i$ levels has been shown to be responsible for osteoclast retraction (Chambers *et al.,* 1985; Su *et al.,* 1992). We have observed that CT treatment of a chicken osteoclast-like cell line (HD-11EM) stably transfected with the archetypal human CTR induced changes in proliferation and cell shape (from cuboidal to stellate) and a loss of adhesion (Galson *et al.,* 1998). CT had a complex effect on cell growth with an early (within 4 hr) increase in proliferation followed by a block at G_2 (D. L. Galson, M. R. Flannery, and S. R. Goldring, unpublished results). All of these cellular responses to CT were independent of G_s signaling and adenylyl cyclase activation. PMA treatment was able to mimic much of the effects of CT treatment, implying that PKC activation regulated all of the CT-induced cellular responses observed. However, the PKC inhibitor Ro-31-8220 could only completely block the CT-induced loss of adhesion and partially block the CT-induced early proliferative response and had no effect on CT-induced cell morphology. These studies provide further evidence that CT effects on osteoclast function involve complex and independent signal pathways.

There is growing evidence of a role for CT and CTR signaling in modulating cell growth and for the general involvement of GPCR in regulating mitogen-activated protein kinase (MAPK) signaling networks (Gutkind, 1998). MAPKs, including Erk1/2, JNK, and SAPK, lie at the end of parallel protein kinase cascades and play important roles in many biological processes, including differentiation and normal and aberrant cell growth. Analysis of the effects of CT on cell proliferation has demonstrated that CT, paradoxically, can both contribute to mitogenesis and mediate growth suppression depending on the cellular context. For example, CT suppresses cellular proliferation of the human breast cancer cells lines T47D and MCF7 (Ng *et al.,* 1983), whereas it has a mitogenic action in primary human prostate cancer cells and a cell line LnCaP derived from prostate cancer (Shah *et al.,* 1994). The CT-induced suppression of T47D is thought to be mediated by the specific activation of the type II isoenzyme of the cAMP-dependent protein kinase (Ng *et al.,* 1983). However, CT increases both cAMP and $[Ca^{2+}]_i$ in the prostate cells (Shah *et al.,* 1994).

Cell lines stably transfected with CTRs have been used to gain further insights into the mechanisms by which CT regulates cell proliferation. Evdokiou *et al.* (1999) showed that CT suppressed the cellular proliferation of HEK-293 cells transfected with the rat or human CTR. No evidence of cell necrosis or apoptosis was detected, and growth inhibition appeared to be associated with an accumulation of cells in the G_2 phase of the cell cycle. The CT-induced G_2 block was associated with a rapid and sustained induction of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ mRNA and protein and reduction in p53 mRNA and protein.

In HEK-293 cells transfected with the rabbit CTR, CT induced Shc tyrosine phosphorylation, Shc-Grb2 association and phosphorylation, and activation of the MAPKs Erk1 and Erk2 (Chen *et al.,* 1998). Erk1/2 activation occurred through both a G_i pathway leading to activation of Ras and a G_q pathway, which raises $[Ca^{2+}]_i$ levels and activates PKC via a Ras-independent pathway. While the CT-induced increase in $[Ca^{2+}]_i$ levels was necessary, it was not sufficient for full activation of Erk1/2. CT-induced activation of the Erk1/2 MAPK pathway appears to be involved in CTR-mediated growth suppression. Raggatt *et al.* (2000) reported that inhibition of the CT-induced phosphorylation of Erk1/2 by the MEK inhibitor PD98059 in HEK-293 cells expressing the archetypal hCTR partially blocked the reported growth inhibitory effects of CT on these cells (Evdokiou *et al.,* 1999). These included blocking the associated accumulation of cells in G_2 and the CT induction of $p21^{WAF1/CIP1}$. These data suggest that activation of Erk1/2 by CT-liganded CTR is an important downstream effector in modulating cell cycle progression.

CT may exert its effects on cell shape and attachment via interactions of the CTR with the signal pathways linked to modulating the focal adhesion complex. CT stimulates tyrosine phosphorylation of the focal adhesion-associated protein HEF1, paxillin, and focal adhesion kinase (FAK) and their consequent complex formation by a mechanism dependent on both increased $[Ca^{2+}]_i$ levels and the activation of PKC (Zhang *et al.,* 1999). However, unlike the Erk1/2 response, CT-induced phosphorylation of HEF1 was completely pertussis toxin insensitive, suggesting that only activation of G_q is involved. Although the G_q pathway appears to be involved in the phosphorylations of Erk1/2 and of HEF1 and paxillin, the mechanisms by which these responses are transduced are largely independent (Zhang *et al.,* 2000). For instance, inhibition of MEK activity by

PD98059 reduced the CT-induced phosphorylation of Erk1/2 but not of HEF1 and paxillin. Also, unlike the CT-induced phosphorylation and activation of Erk1/2, the CT-induced phosphorylation of HEF1 was inhibited by cytochalasin D, suggesting that the actin cytoskeleton has a role in transducing the signal from the CTR to the focal adhesion-associated proteins. In addition to an intact cytoskeleton, cell attachment with engagement of integrins with extracellular matrix proteins and catalytically active Src are also required for CT-induced phosphorylation of HEF1 and paxillin. Regulation of the activity of these adhesion-related proteins may play a role in mediating CT induction of changes in cell shape and motility.

It has been demonstrated that the archetypal human CTR, when coexpressed with any of the three receptor activity-modifying proteins (RAMP1, 2, or 3), is also a receptor for the 37 amino acid peptide hormone amylin (Christopoulos *et al.,* 1999; Foord and Marshall, 1999; Muff *et al.,* 1999; Zumpe *et al.,* 2000). This peptide has effects on insulin release, glucose uptake, and glycogen synthesis in skeletal musculature (Wimalawansa, 1997). The RAMPs are a three member family of single TM proteins that act to modify the affinity of the CTR for the different peptide ligands of the CT family (CT, CGRP, amylin, and adrenomedullin). For instance, hCTR coexpressed with RAMP1 in COS-7 cells has higher affinity for human CGRPα and amylin and lower affinity for human CT than hCTR coexpressed with RAMP2 (Zumpe *et al.,* 2000). The interaction of the CTR with RAMPs represents an additional mechanism for modulating the function of the CTR and its response to ligands.

CTR Protein Isoforms Derived by Alternative mRNA Splicing

The CTR gene has a complex structural organization with several CTR protein isoforms derived from alternative splicing of transcripts from a single gene (Goldring, 1996). These isoforms, which are functionally distinct in terms of ligand-binding specificity and/or signal transduction pathway utilization, are distributed both in a tissue- and in a species-specific pattern (Lin *et al.,* 1991a; Gorn *et al.,* 1992, 1995b; Sexton *et al.,* 1993; Houssami *et al.,* 1994; Kuestner *et al.,* 1994; Yamin *et al.,* 1994; Zolnierowicz *et al.,* 1994; Albrandt *et al.,* 1995; Ikegame *et al.,* 1995; Shyu *et al.,* 1996; Galson *et al.,* 1996, 1997; Anusaksathien *et al.,* 2001). At least six different isoforms involving coding sequence exons of the human CTR (hCTR) have been described (Fig. 1). Each of these cDNAs would generate proteins with different predicted structural features.

Much of the understanding of the structure–function relationships in the hCTR have been gleaned from the analysis of two isoforms of the hCTR that differ by the presence or absence of a 48-bp exon (exon 7b) encoding 16 amino acids in intracellular domain 1 (IC1) (Fig. 1). These two isoforms exhibit significant differences in their pattern of coupling to signal transduction pathways and ligand-binding affinity (Gorn *et al.,* 1992, 1995b; Frendo *et al.,* 1994; Kuestner *et al.,* 1994). The hCTR isoform without the 48-bp exon (hCTR-48$^-$; "archetypal" hCTR) has lower binding affinity for salmon CT (sCT) (K_d 15 n*M*) than the 48-bp exon containing hCTR (hCTR-48$^+$) isoform (K_d 1.5 n*M*). In contrast, the hCTR-48$^-$ isoform demonstrates a much more significant ligand-mediated cAMP response to sCT and human CT (hCT) compared to the hCTR-48$^+$ isoform (Gorn *et al.,* 1995b). However, the ability of the hCTR-48$^+$ isoform to couple to adenylyl cyclase is affected differentially in different cell types (Nussenzveig *et al.,* 1994; Albrandt *et al.,* 1995; Gorn *et al.,* 1995b; Moore *et al.,* 1995). In contrast to the hCTR-48$^-$ isoform, the hCTR-48$^+$ isoform does not couple to PLC (Gorn *et al.,* 1992; Nussenzveig *et al.,* 1994; Moore *et al.,* 1995) nor to PLD (Naro *et al.,* 1998) and therefore does not induce PKC nor trigger an increase in $[Ca^{2+}]_i$ levels. When transfected into HEK-293 cells, the hCTR-48$^+$ isoform fails to transduce the CT-induced growth suppression signal mediated by the activation of Erk1/2 that was observed with the hCTR-48$^-$ isoform (Raggatt *et al.,* 2000). However, this group also reported that both the hCTR-48$^+$ and the hCTR-48$^-$ isoforms in HEK-293 could mediate CT-induced acidification of the extracellular medium by an unknown mechanism, suggesting that the hCTR-48$^+$ isoform may activate previously unrecognized pathways. The presence of the 16 amino acids in IC1 also inhibits ligand–receptor complex internalization (Moore *et al.,* 1995). Although in many tissues, the hCTR-48$^-$ isoform mRNA appears to be the predominant form, there are significant levels of the hCTR-48$^+$ mRNA in the osteoclast-like cells of giant cell tumor, CD34$^+$ cells from cord blood, placenta, and ovary (Gorn *et al.,* 1992, 1995b; Frendo *et al.,* 1994; Kuestner *et al.,* 1994; Albrandt *et al.,* 1995; Galson *et al.,* 1996). The porcine CTR (pCTR) also contains a variably spliced 48-bp exon encoding an additional 16 amino acids in IC1 (Zolnierowicz *et al.,* 1994). While the position of the variably utilized 16 amino acids in IC1 is identical with the hCTR, only two of the amino acids are conserved. The pCTR-1b (48+) is ~1000-fold less abundant in LLC-PK$_1$ cell mRNA than pCTR-1a (48−).

Both human (Gorn *et al.,* 1992, 1995b) (Fig. 1) and mouse CTRs (Anusaksathien *et al.,* 2001) (Fig. 2) contain a variably spliced exon that adds an in-frame upstream ATG with the potential to generate receptors with an additional 18 or 17 amino acids at the N terminus, respectively. Additionally, a hCTR mRNA has been identified that has a 125-bp deletion (hCTR-Δ125) from within exon 3 to mid exon 4, which includes the initiator Met (Albrandt *et al.,* 1995). Therefore, translation starts at an internal Met (M48), resulting in an N-terminal deletion of 48 amino acids. This N-terminally truncated hCTR isoform, which exhibits high binding affinity to sCT and a strong dose-dependent cAMP response to sCT, hCT, and human amylin, is expressed in mammary carcinoma cell lines (T47D and MCF-7), as well

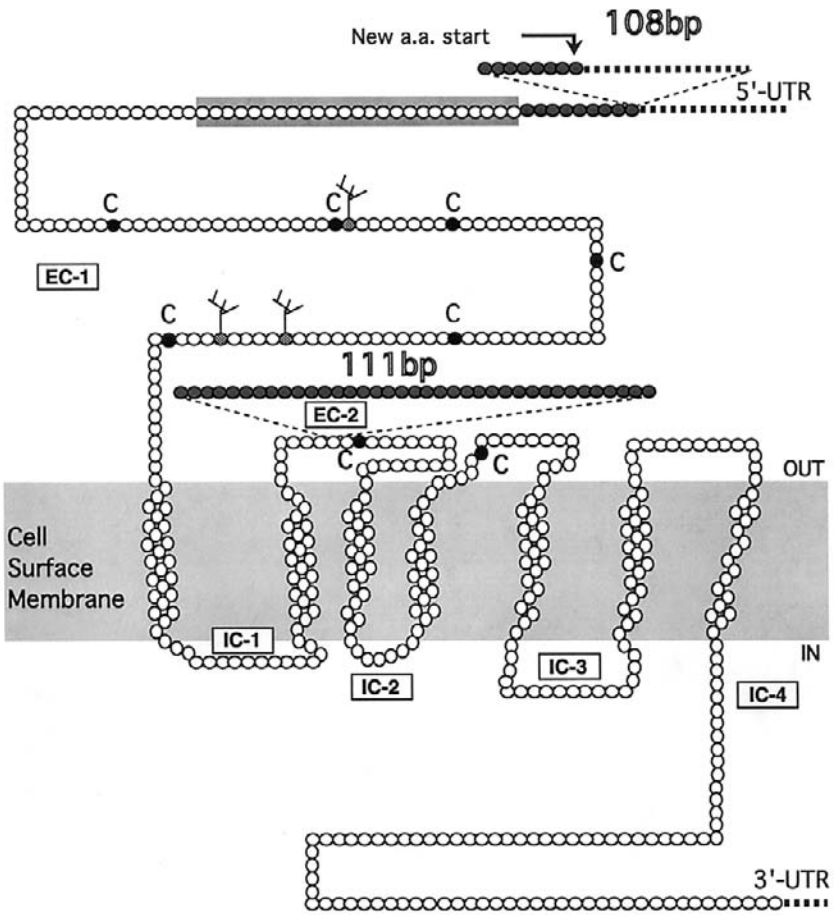

Figure 2 mRNA splice variants of murine CTR depicted as expected protein translation products. White circles represent the amino acids (a.a.) of the mCTR C1a isoform. The a.a. positions denoted as black circles with a C nearby are the conserved cysteines and shaded circles with the branch structure are the putative sites for N-glycosylation. Boxed circles denote the putative signal sequence. Gray circles represent amino acids added by the addition of variably spliced mRNA regions as indicated. The domains of interest are noted as follows: IC-1, IC-2, IC-3, and IC-4, first, second, third, and fourth intracellular domains, respectively; EC-1 and EC-2, first and second extracellular domain, respectively.

as in kidney, skeletal muscle, lung, and brain. The Δ125-bp and 71^{+}-bp isoforms of the hCTR exhibited modest alteration in ligand-binding affinity compared to the archetypal CTR isoform (hCTR-48^{-}), but the selectivity of ligand binding and activation of phospholipase C have not yet been tested.

Interestingly, two types of variably spliced mRNA products have been identified in hCTR that generate translation terminations shortly after transmembrane domain 1 (Moore *et al.,* 1995; Galson *et al.,* 1996), resulting in the putative expression of C-terminally truncated CTR proteins with a single transmembrane domain (Fig. 1). In one case (hCTR-83^{+}), there is an extra 35-bp exon (exon 7a) inserted just upstream of the 48-bp exon and in the other case (hCTR-Δ127), these 2 exons plus the next downstream exon (exon 8) were omitted. The potential physiological significance of these truncated isoforms is currently not known. Of interest, three truncated forms of the luteinizing hormone receptor have been identified, and these truncated isoforms exist as soluble binding proteins that are involved in the control of free hormone levels by competing with the membrane-associated luteinizing hormone receptor for ligand binding (Koo *et al.,* 1991).

Furthermore, we have identified a hCTR mRNA that contains a variably spliced novel 85-bp (named exon 13b) inserted between exons 13 (renamed 13a) and exon 14 whose presence results in a frameshift that creates a hCTR molecule with a completely novel intracellular domain 4 (IC4) at the C terminus (Galson *et al.,* 1997). This novel IC4 is only three amino acids longer than the archetypal IC4. CTR mRNA containing exon 13b was detected by reverse transcription-polymerase chain reaction (RT-PCR) technique in kidney, placenta, BIN67 (human ovarian cell line), and some giant cell tumor of bone samples. It was not found in brain, stomach, liver, and foreskin fibroblasts, although these samples were positive for other forms of hCTR mRNA. Functional studies using transient transfection into COS cells of a cDNA coding for the hCTR-$48^{-}85^{+}$IC4

isoform (which lacks the insert of 16 amino acids in IC1) indicate that the ability of this hCTR isoform to bind salmon CT is low. It does, however, signal through cAMP induction with the same EC_{50} as the archetypal hCTR, but the magnitude of the cAMP increase is markedly lower.

Rodent CTRs do not appear to contain the variably spliced exons found in the hCTR-coding sequence except, as mentioned earlier, the exon that inserts an upstream ATG start that adds 17 amino acids to the N terminus. However, rodent CTRs contain an additional variably spliced exon (111 bp exon 8b) within the coding sequence whose presence adds 37 amino acids to extracellular domain 2 (Fig. 2) and alters ligand specificity (Albrandt *et al.,* 1993; Sexton *et al.,* 1993; Yamin *et al.,* 1994; Inoue *et al.,* 1999). The two isoforms (termed C1a:insert negative and C1b:insert positive) bind salmon CT with high affinity, but the C1b receptors have a much lower affinity (negligible) for human and rat CT than the C1a receptors (moderate). This result is particularly surprising because it would be expected that CTR would bind the endogenous CT. Both the rat and the mouse C1b forms of CTR are expressed most highly in the brain. These findings indicate a role for the second extracellular domain in ligand-binding specificity and affinity. Additionally, the characteristics of the C1b receptors suggest that they may be the receptor for some as yet unidentified neurotransmitter resembling sCT (Sexton *et al.,* 1993).

Additionally, two CTR isoforms have been cloned from rabbit tissue. One form is structurally similar to the rodent C1a isoform and the other has a deletion of 14 amino acids in the seventh TM domain encoded by a distinct exon, designated 13 (CTRΔ13) (Shyu *et al.,* 1996). The expressed receptor exhibits a reduction of binding affinity for sCT and hCT by more than 10- and 2-fold, respectively. This isoform activates adenylate cyclase but not phospholipase C. The CTRΔ13 mRNA represents less than 15% of the CTR mRNA in osteoclasts, brain, and kidney, whereas at least 50% of the CTR transcripts are represented by this isoform in skeletal muscle and lung.

CTR Gene Organization

Analysis of the structural organization of CTR genes from different species has helped define the molecular basis for generation of the distinct receptor isoforms. In addition to the variable mRNA splicing that generates different CTR protein isoforms, multiple 5′-UTR structures have been described for both mCTR (Anusaksathien *et al.,* 2001) and hCTR (Nishikawa *et al.,* 1999; Hebden *et al.,* 2000) that arise from both variable splicing and alternative promoter usage.

Characterization of pCTR genomic organization was the first among the $GPCR_{II}$ family to be reported (Zolnierowicz *et al.,* 1994). This gene was found to span approximately 70 kb, and analysis revealed that the gene contained 14 exons with 12 exons encoding the actual receptor protein (Fig. 3). While the intron lengths are varied, there is one very long intron (>20 kb) between exons 2 and 3. The first and second exons and the first 30 nucleotides of the third exon represent the 5′-untranslated sequences. The remaining 51 nucleotides of the third exon encode a hydrophobic putative signal peptide. Exons 4–6 and exons 7–13 encode the N-terminal extracellular part of the receptor and the region of transmembrane domains, respectively. Exon 14 encodes the C-terminal intracellular region and the 3′-untranslated (3′ UTR) portion of the CTR transcript, which is very long (~2 kb). The 48-bp variably spliced exon (exon 8a in pCTR), resulting in an additional 16 amino acids in IC1, was found to arise from alternative use of two potential splice acceptor sites at the 3′ end of intron 7 located 48 bp apart so as to create two differently sized exons 8. Although there is a consensus exon/intron junction located in the EC2 region (E8/E9 pCTR) among all members of the $GPCR_{II}$ family, the presence of the 111-bp insertion in this region has been reported only in rat CTR and mCTR genes (Albrandt *et al.,* 1993; Sexton *et al.,* 1993; Yamin *et al.,* 1994).

Albrandt *et al* (1995) partially characterized the hCTR gene (gene name is CALCR) using a genomic clone that extended from intron 2 through exon 14. The exon/intron boundaries were located at similar positions to the pCTR gene, except that the 48-bp insert in IC1 is contained within a distinct exon (exon 7b) located 1 kb upstream of exon 8 (Moore *et al.,* 1995; Nussenzveig *et al.,* 1995; Galson *et al.,* 1996) (Fig. 3). The N-terminally deleted isoform of hCTR (Δ125 bp:EC1) occurs as a result of the deletion of part of exons 3 and exon 4 where it is bounded by the invariant GT/AG consensus-splicing motif for exon/intron splicing (Albrandt *et al.,* 1995). Further characterization by PCR (Moore *et al.,* 1995; Galson *et al.,* 1996) and analysis of the sequence in human BAC clone GS1-117O10 (GenBank accession number AC003078) from chromosome 7 have revealed that the alternatively spliced 35-bp exon (exon 7a) whose inclusion results in a C-terminal truncation six amino acids after TM1 is located 4.2 kb downstream of exon 7 and 2.35 kb upstream of the 48-bp exon 7b. The variably spliced 71-bp exon (termed 3a) that adds 18 amino acids to the N terminus is present 8.9 kb upstream of exon 3 (renamed 3b). The variably spliced 85-bp exon (Galson *et al.,* 1997) that alters the IC4 sequence (named 13b) is located 4.4 kb downstream of exon 13 (renamed 13a) and 3.15 kb upstream of E14.

Additional 5′-UTR hCTR cDNA sequences have been described. Nishikawa *et al.* (1999) isolated a hCTR cDNA containing the equivalent of the pCTR exons 1 and 2 by 5′-RACE using a human mammary tumor cell line, MCF-7. Interestingly, they also reported a novel hCTR 5′-UTR structure with a 288-bp osteoclast-specific exon (named Oc1 here) spliced to exon 3b (Fig. 3). This new exon (located at 168711–168422 in GenBank Accession Number AC003078) lies between exons 2 and 3a in the genomic structure. Using 5′-RACE, Hebden *et al.* (2000) also detected a hCTR 5′-UTR mRNA structure in T47D breast cancer cells, kidney, and osteoclastomas (with exons 1a and 2; 1392–1360 and 1174–957, respectively, in human BAC

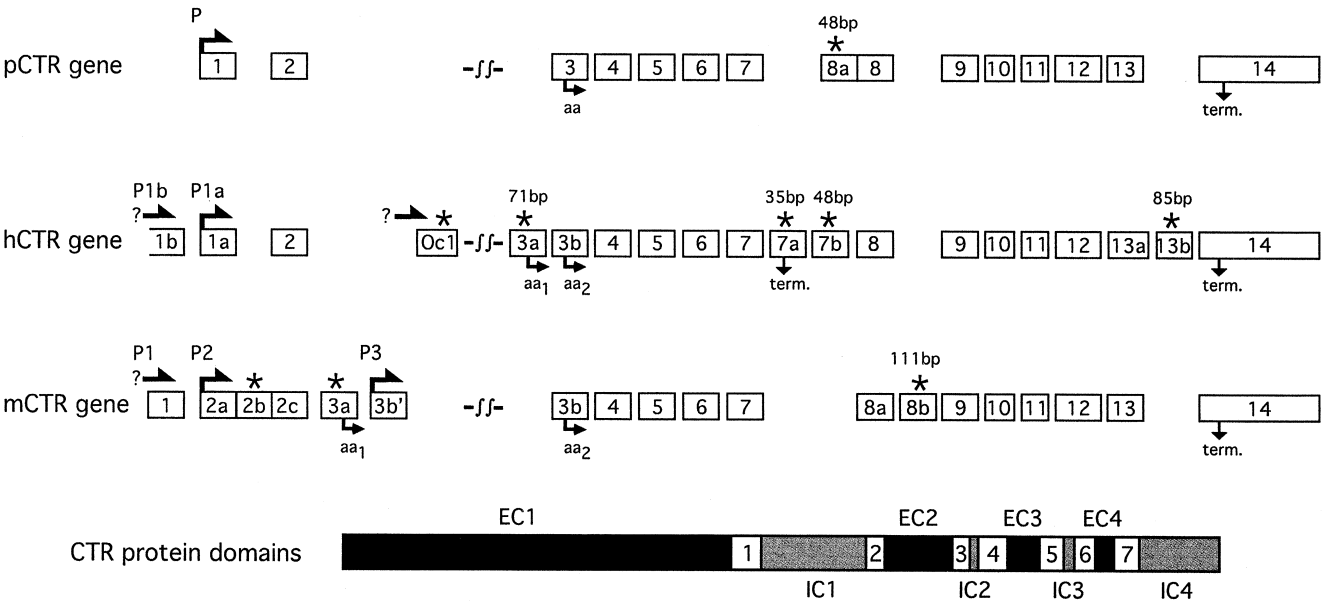

Figure 3 Genomic alignment of exons comprising porcine, human, and mouse CTR genes. Homologous exons are aligned vertically among the pCTR, hCTR, and mCTR genes (sizes are not to scale). Intron lengths are not represented, except that the position of the very long intron conserved among all three species is denoted by the double integral sign. Large gaps denote exons that have not been identified in all three species. Alternately, spliced exons are marked with an asterisk. Transcription starts of the putative promoters are indicated by arrows above the exons, and putative translation starts are indicated by arrows below the exons. Although they are not marked with an asterisk, exons used to initiate primary transcripts are always spliced out of transcripts that initiate upstream of them. Translation termini are also indicated below the exons. The hCTR exon 1b has no left side to indicate that the 5′ side has not been defined. Protein domains illustrated along the bottom show the correspondence between CTR exons and putative protein domains. The protein domains are denoted as follows: IC, intracellular domains (gray); EC, extracellular domains (black); and unprefixed numerals, transmembrane domains (white).

clone GS1-438P6, GenBank Accession Number AC005024), which resembles the pCTR 5′-UTR. While most of the 5′-RACE clones started within a few base pairs of the pCTR start, as might be expected for a TATA-less promoter, they detected multiple start positions for hCTR exon 1a, including some up to 43 bp upstream of those observed previously for hCTR and pCTR. Additionally, Hebden *et al.* (2000) reported the presence of a hCTR 5′-UTR structure that included a novel exon (named 1b) with a sequence homologous to the 5′ end of the mCTR cDNA reported by Yamin *et al.* (1994) spliced to exon 2. This novel hCTR exon lies upstream of exon 1a, but its 5′ end has not been defined. The 1059-bp intron region spliced out between exons 1b and 2 contained exon 1a. As discussed later, the presence of exon 1b or 1a in the mRNA reflects the alternative use of two different promoters in the hCTR gene. Analysis of sequence information available through the human genome sequencing project (GenBank accession numbers AC003078 and AC005024), the intron spliced out when exons 2 and 3b are joined in the mRNA is about 87 kb long and contains exons Oc1 (located ~14.8 kb 3′ of exon 2) and the 71-bp exon 3a (located 8.9 kb 5′ of exon 3b). Consequently, the length of the hCTR primary transcript initiated by the upstream hCTR promoter (from exon 1b) is at least 152 kb and contains 20 exons. The primary transcript initiated by the downstream hCTR promoter (from exon 1a) is 150 kb. It has not yet been determined whether the novel exon Oc1 represents the true 5′ end of an osteoclast-specific hCTR mRNA and thereby marks the presence of an osteoclast-specific promoter in the hCTR gene or reflects alternative splicing of a transcript generated from the upstream promoters.

Analysis of mCTR genomic clones revealed the exon structure of the original mCTR cDNA reported by Yamin *et al.* (1994) and showed that the locations of the exon/intron junctions within the coding region of the mCTR gene (exons 3–14) are identical to those of the pCTR and hCTR genes (Anusaksathien *et al.,* 2001) (Fig. 3). This includes the two putative translation start sites that are split between two exons in the murine (the 108-bp 3a and 3b) and human (the 71-bp 3a and 3b) CTR genes. Further analysis of mCTR transcripts identified novel cDNA sequences, new alternative exon splicing in the 5′-UTR, and three putative promoters (P1, P2, P3). Similarly to the hCTR 71-bp exon 3a, the mCTR exon 3a was found to be variably spliced. Some of the novel cDNA sequences add 512 bp to the 5′ side of the previously published cDNA (Yamin *et al.,* 1994), thereby extending exon 1 to 682 bp. In addition, three new exons were identified. Two of these novel exons are upstream of exon 2 and form a tripartite exon 2 (2abc) in which exon 2a is utilized by promoter P2 with variable splicing of exon 2b (which might also be considered a "retained intron"). Exon 2a is homologous to the pCTR exon 1 and the hCTR exon 1b. The third new exon (3b′) lies between 3a and 3b and is utilized by promoter P3. Exon 3b′ lies 689 bp downstream of exon 3a. The mCTR intron between exons 3b′ and 3b was found by analysis of

λ clones to be >13.9 kb and was found to be 29 kb in the mouse chromosome 6 genomic sequence (GenBank Accession number AC066688). This means that the intron spliced out between 2c and 3b is 40.2 kb. While 5′-RACE, primer extension, and RNase protection analysis combined to identify the 5′ end of mCTR exon 1, data also suggested that there might be another upstream exon in the P1 transcript. However, such an exon has not been found and the exact position of the mCTR P1 promoter remains to be determined. Therefore, the longest transcription unit, which is derived from promoter P1, is at least 77.4 kb and contains 19 exons.

Analysis of mCTR mRNAs has revealed that the three alternative promoters give rise to at least seven mCTR isoforms in the 5′ region of the gene and generate 5′-UTRs of very different lengths (from 93 bp to at least 955 bp). The structure of 5′-UTRs derived from P1 transcripts are 12c(+/−3a)3b, termed P1.1 (3a+) and P1.2 (3a−); P2 transcripts are 2a(+/−2b)2c(+/−3a)3b, termed P2.1 (2b−,3a+), P2.2 (2b−,3a−), P2.3 (2b+,3a+), and P2.4 (2b+,3a−); and the P3 transcript is P3.1 (3b′3b). Retention of the equivalent of the mCTR exon 2b (or "intron 2a") was not reported for pCTR and hCTR. This may be due to species differences or to differences in the experimental design employed. The alternatively spliced rodent-specific 111-bp coding exon (8b), which adds 37 amino acids to the extracellular domain 2 of mCTR, lies 886 bp downstream of exon 8a and 7.2 kb upstream of exon 9. The configuration of the seven mCTR 5′-UTR splice forms with the presence or absence of exon 8b has not been determined. Analysis by RT-PCR indicates that the P1 promoter (located upstream of an expanded exon 1) and the P2 promoter (located upstream of exon 2a) are utilized in osteoclasts, brain, and kidney, whereas the P3 promoter, located upstream of the novel exon 3b′, appears to be exclusively utilized in osteoclasts. Osteoclasts express all seven 5′-UTR mCTR isoforms. However, mRNA isoforms containing exon 3a are more abundant than their counterparts lacking exon 3a. It has not been possible to quantitate the relative promoter usage in osteoclasts. However, in kidney and brain, promoter P3 is not utilized and it appears that the kidney does not use exon 2b.

mCTR-P1 mRNAs have very long 5′-UTRs of >955 and >898 nucleotides that are slightly GC rich (53% GC) and contain seven AUGs before the AUG in exon 3a. Most have a pyrimidine at -3 that makes them poor translation candidates, although one such upstream ORF is 51 amino acids long (AUG at +252 in E1). One ORF (which encodes 14 amino acids) has an AUG (at +248 in E1) in a good Kozak context for translation. The occurrence of upstream AUG codons nearly always reduces the efficiency of initiation from downstream AUGs (Kozak, 1991). mCTR-P2 5′-UTRs have only 1 upstream ORF with its AUG in a poor Kozak context, range in size from 249 to 487 nucleotides, and are all GC rich (55–60% GC). The mCTR-P3 5′-UTR has no upstream AUGs, is only 93 nucleotides long, and is slightly AT rich (48% GC). One possible purpose of the generation of multiple mCTR 5′-UTRs is that mRNAs with different untranslated exons can differ in their stability, compartmentalization, and translational potential. When the translatability of mRNAs from the same gene with both a long and a short 5′-UTR has been compared, the short 5′-UTR is usually translated more efficiently (Nielsen *et al.,* 1990). Indeed, in some instances, the effect of 5′-UTR on translation can be so profound that a minor transcript from certain genes appears to be the major functional mRNA (Mitsuhashi and Nikodem, 1989; Horiuchi *et al.,* 1990). Additionally, the GC-rich, untranslated regions could have a mRNA secondary structure that may interfere with the translational process (Gehrke *et al.,* 1983; Kozak, 1986, 1989). Therefore, it is possible that translation of the seven mCTR cDNAs is regulated differentially and that the relative abundance of a particular mRNA isoform may not correlate with its contribution to the translated product.

Regulation of the CTR Gene

Although some of the human (Albrandt *et al.,* 1995), porcine CTR (Zolnierowicz *et al.,* 1994), and mouse (Anusaksathien *et al.,* 2001) genomic sequences have been cloned, little is known about the mechanism of transcriptional regulation for the CTR gene in osteoclasts or in other tissues in which it is expressed. Transfection analysis has been used to establish that the putative CTR promoters discussed earlier are functional (Zolnierowicz *et al.,* 1994; Anusaksathien *et al.,* 2001; Hebden *et al.,* 2000). A 657-bp fragment containing 357 bp of the pCTR promoter was demonstrated to drive expression of a luciferase reporter gene when transfected into the CTR-expressing porcine kidney epithelial cell line LLC-PK_1 (Zolnierowicz *et al.,* 1994). Hebden *et al.* (2001) transfected a series of hCTR-luciferase deletion constructs into human T47D breast cancer cells and demonstrated the presence of two functional promoters within the hCTR gene. As they deleted from −881 to −129 relative to exon 1a, they lost only about half the activity (the numbering has been revised to reflect +1 at the same position as pCTR). Deletions from the 3′ side were used to remove the downstream promoter (proximal to exon 1a) and define the position of the upstream promoter within a 2-kb region, which would suggest that hCTR E1b is at least 686 bp long. Using transiently transfected luciferase reporter constructs, Anusaksathien *et al.* (2001) demonstrated that the mCTR promoter P2 (proximal to exon 2a) is active in a murine kidney cell line (MDCT209), a chicken osteoclast-like cell line (HD-11EM), and a murine preosteoclast cell line (RAW264.7). Further investigation of the mCTR promoter P2 region by deletion analysis revealed that the −179 to +398 region contained maximal activity in all three cell lines. Most interestingly, the mCTR promoter P3 (proximal to exon 3b′) was only active in osteoclast-like cell lines (Anusaksathien *et al.,* 2001). Deletion analysis of the P3 promoter showed that −319 relative to exon 3b′ was sufficient for maximal activity. These transfection data con-

firmed the osteoclast specificity of mCTR promoter P3 observed by RT-PCR and provided the first evidence that the CTR gene is regulated in a tissue-specific manner by alternative promoter utilization.

The −319 mCTR promoter P3 contains five putative composite sites for the transcription factors NFAT and AP-1 (Galson *et al.,* 2000). Cotransfection of the mCTR-P3 reporter and a constitutively active NFAT (ΔNFAT; containing a deletion of the regulatory region, which allows it to localize in the nucleus independent of calcium signaling) into either uninduced RAW 264.7 or HD-11EM cells has been demonstrated to result in a large increase in activity. However, the widely expressed mCTR-P2 reporters were unresponsive to cotransfection with ΔNFAT in all three cell lines. DNA protein-binding analysis showed that the putative composite NFAT/AP-1 sites in the mCTR-P3 promoter can bind both NFAT and AP-1 proteins *in vitro*. Transient transfection of ΔNFAT into RAW 264.7 cells stimulated transcription from the endogenous CTR gene, as well as some other osteoclast-specific genes (e.g., TRAP). We conclude that calcium signaling via NFAT activation is important in regulating the expression of CTR in osteoclasts.

Comparison of the −179 mCTR-P2 region with the pCTR promoter sequence upstream from pCTR exon 1 (Zolnierowicz *et al.,* 1994) and a region of hCTR upstream of hCTR exon 1a revealed a high degree of homology and conserved sequence motifs for several transcription factors. The homology among the three species was very high in pairwise comparisons (~70%) for more than 2 kb further upstream (not shown). This would suggest that pCTR may also have the equivalent of the mCTR exon 1. Both mCTR P2 and P3 promoters lack many well-known transcription initiation site consensus sequences. These include a TATA box in the −30 region, an Inr element (YYANWYY) at +1 (Javahery *et al.,* 1994), a DPE site (RGWCGTG) downstream near +30 (Burke and Kadonaga, 1996; Orphanides *et al.,* 1996), and a BRE site (SSRCGCC) at approximately −38 relative to the start of transcription (Lagrange *et al.,* 1998). Both promoters possess a possible YY1-binding site (VKHCATNWB) at the putative transcription start, which could be involved in recruiting TFIIB (Houbaviy *et al.,* 1996; Usheva and Shenk, 1996). Typical for many TATA-less myeloid promoters, the proximal P2 promoter regions of the CTRs of all three species contain two putative Sp1 sites (Theisen and Bach, 1990), although their positions are not identical. All three mCTR-P2 homologous CTR promoters (mCTR-P2, hCTR-P1a, pCTR-P) are very GC rich and contain a high frequency of CpG dinucleotides indicative of the presence of a CpG island (Antequera and Bird, 1993). However, the P3 promoter region between exons 3a and 3b′ is only 36% GC and the −319 region is only 41% GC. The osteoclast-specific mCTR exon 3b′ is not homologous to the hCTR osteoclast-specific exon Oc1 (Fig. 3) nor do the proximal upstream regions to these exons have any significant homology.

The presence of alternative promoter usage and splicing, localized to the 5′ end of the CTR gene, may thus provide a mechanism for regulating the expression of this gene at both the translational and the transcriptional level. The existence of a mCTR promoter (P3) that is osteoclast specific is, perhaps, not surprising due to the fact that CTR is expressed in a restricted spectrum of tissues with developmental regulation. Upon proper stimulation, the osteoclast precursor, which is of monocyte/macrophage lineage, undergoes a program of cellular differentiation in which a distinct profile of genes are induced, including, for example, cathepsin K, β_3 integrin, acid phosphatase, and CTR. These genes encode protein products that confer upon the osteoclast the unique functional activities required for attachment and resorption of the mineralized bone matrix. The CTR gene appears to be induced during the terminal stages of osteoclast differentiation coincident with the acquisition of bone-resorbing capacity (Lee *et al.,* 1995). Characterization of molecular regulation of the CTR gene in osteoclasts could lead to novel approaches in treating osteoporosis, periodontal disease, inflammatory arthritis, and related bone disorders.

The function of pCTR (2.1 kb) and hCTR (4.9 kb) promoters has been assessed in transgenic mice (Jagger *et al.,* 1999, 2000). The hCTR fragment used contained both hCTR promoters defined by the transfection studies. It was found that although both of these promoters directed expression of the lacZ reporter in several embryonic and fetal tissues, which express endogenous mCTR, neither was sufficient to direct transcription in the adult kidney or bone of the transgenic mice. However, several of the tissues in which these CTR promoter-driven reporter genes were expressed represent previously unknown sites of CTR expression. Expression of the endogenous CTR gene was confirmed in each of the new sites.

The 2.1-kb pCTR*lacZ* transgene (Jagger *et al.,* 1999) was expressed in the embryonic brain and spinal cord at 11.5 days. At 15.5 days, expression was observed in the developing mammary gland, external ear, cartilage primordium of the humerus, and anterior naris (nostril). However, in the neonate and adult mouse, the transgene was only expressed in brain, spinal cord, and adult testis. No expression was observed in the kidney and osteoclasts. The 4.9-kb hCTR*lacZ* transgene (Jagger *et al.,* 2000) was expressed at additional sites not observed with the 2.1-kb pCTR*lacZ* transgene. The hCTR*LacZ* transgene was expressed at 8.5–10.5 days in the lateral side of cervical and occipital level somites and in lateral myotome and hypaxial muscle progenitors. At 11.5–16 days, expression was observed in limb buds, cornea, retina, skin, intercostal muscles, muscles of the limbs and face, and dorsal root ganglia, placenta, spinal cords, brain, anterior nares, and maxillary component of the first branchial arch. In the adult mouse, expression in the brain (olfactory bulbs, hippocampus, cerebellum, and cerebrum), ventral roots and dorsal horn of the spinal cord, nervous layer of the retina, and testis was observed. The difference in the pattern of expression of the two transgenes may be due to a species difference or to the additional amount of regulatory DNA available in the larger hCTR*LacZ* transgene. These data suggest that CTR may

play a role in morphogenesis. However, it is not clear which of the possible ligands is involved. Additionally, which CTR isoform is important is not yet known.

Homologous Downregulation of CTR

Although CT effectively inhibits osteoclast-mediated bone resorption after acute administration, continuous exposure to this ligand results in the development of a state of refractoriness, termed "escape," in which there is a loss of calcitonin-mediated inhibition of osteoclastic bone resorption. This phenomenon was first observed in bone organ cultures (Wener *et al.,* 1972; Tashjian *et al.,* 1978), but also occurs *in vivo* in patients receiving CT for the treatment of hypercalcemia and other high turnover states of bone remodeling, such as Pagets disease. Early studies suggested that the loss of responsiveness to CT was related to the downregulation of CT receptors on osteoclasts and the possible recruitment of osteoclasts that lacked CT receptors (Tashjian *et al.,* 1978; Krieger *et al.,* 1982; Nicholson *et al.,* 1987). The availability of cloned CT receptors has provided new reagents for studying regulation of the CTR and for defining the molecular mechanisms responsible for the development of refractoriness to calcitonin associated with the escape phenomenon.

The expression of the CTR appears to occur late in the sequence of osteoclast differentiation associated with the process of multinucleation and acquisition of the capacity to resorb bone (Takahashi *et al.,* 1988a,b; Hattersley and Chambers, 1989; Suda *et al.,* 1992, 1997). These findings have been confirmed in studies employing RNA samples from murine (Lee, *et al.,* 1995; Wada, *et al.,* 1995) or human (Takahashi *et al.,* 1995) bone marrow cultures that have been induced to form osteoclast-like multinucleated cells. Analysis of RNA samples prepared from cells at different stages of culture using RT-PCR with CTR-specific oligonucleotides demonstrates that the receptor is expressed in some mononuclear cells immediately prior to or in association with the process of multinucleation and competence to resorb bone.

Results from studies employing osteoclast-like cells generated in murine and human bone marrow cultures indicate that continuous treatment with CT results in the decrease in steady-state levels of CTR mRNA and downregulation of CTR binding attributed to receptor internalization (Rakopoulos *et al.,* 1995; Takahashi *et al.,* 1995; Wada *et al.,* 1995, 1996b, 1997; Inoue *et al.,* 1999; Samura *et al.,* 2000). Removal of CT from culture media results in a slow return of CTR message accompanied by the restoration of CT-binding activity (Samura *et al.,* 2000). In studies by Ikegame and co-workers (1996) using murine bone marrow cultures, they observed that the effect of CT treatment on CT binding was dependent on the stage of the cultures in which the CT was administered. If the calcitonin treatment was initiated at the beginning of the culture, osteoclasts developed normally, but these cells expressed minimally detectable levels of CTR as assessed by CT binding and CTR mRNA expression. If cultures were treated after day 6 of culture with CT, CTR mRNA levels declined rapidly and remained suppressed as long as CT was present. They speculated that the escape phenomenon was related to two independent mechanisms: one attributable to the development of osteoclasts with minimal or reduced CTR expression and the other related to the downregulation of CTR expression associated with receptor internalization and reduced receptor synthesis. It is of interest that the pattern of CTR mRNA expression after CT treatment is different in nonosteoclast lineage cells. In these cells, CT treatment only partially decreased steady-state CTR mRNA levels, although CT-binding activity was lost (Wada *et al.,* 1995; Findlay *et al.,* 1996). Removal of CT resulted in the gradual return of CTR mRNA to pretreatment levels with restoration of CT binding. In contrast, in murine bone marrow-derived osteoclasts treated in a similar fashion, CTR mRNA levels were not restored even after removal of CT. These results indicate that the regulation of CTR transcription and mRNA processing differs in osteoclasts and cells of nonosteoclast lineage.

Analysis of the intracellular signaling pathways responsible for the process of CT-induced desensitization has yielded conflicting results. This appears to be related to the source of the osteoclast-like cells employed in the experimental models. In osteoclast-like cells generated from murine bone marrow cultures, CTR downregulation appears to be principally dependent on activation of the protein kinase A pathway (Suzuki *et al.,* 1996; Wada *et al.,* 1996a). In contrast, Samura and co-workers (2000) demonstrated that in osteoclast-like cells prepared from human peripheral blood, CT-mediated desensitization was dependent on activation of the protein kinase C pathway. These findings indicate that the signaling pathways responsible for CTR regulation in human and murine osteoclasts may differ.

Studies have provided additional insights into the molecular mechanisms underlying the loss of CT responsiveness associated with CT treatment of osteoclasts. In studies by Wada *et al.* (1997), they observed that CT treatment of murine osteoclast-like cells derived from bone marrow cultures resulted in decreased steady-state levels of CTR mRNA that appeared to be principally related to increased rates of CTR mRNA decay. Nuclear run-on assays to assess the effects of CT treatment on CTR gene transcription indicated that CT treatment had no effect. They noted that the 3′-untranslated end of the murine CTR gene contained multiple copies of the AUUUA motif, as well as other A/U-rich sequences, which have been shown to regulate the stability of RNA transcripts. In contrast, studies by our own group in collaboration with Inoue and co-workers (1999) indicated that the downregulation of CTR mRNA levels by CT treatment was in part related to direct effects on gene transcription. Additional studies will be necessary to define the molecular mechanisms underlying CT-induced escape in osteoclasts.

In conclusion, cloning of the CTR has helped provide insights into the extreme diversity and pleiotropy of the *in vivo* activities of CT. These effects can be accounted for

based on the widespread distribution of CT receptors, including tissues not directly involved in the regulation of mineral ion homeostasis. The presence of multiple structurally and functionally distinct receptor isoforms that are expressed in a tissue- and cell-specific fashion provides a unique system for producing organ-specific responses to this ligand. The physiological relevance of many of these effects has not been established, but these activities potentially can be exploited for the development of novel applications for the use of CT in the treatment of disorders of skeletal and nonosseous tissues. The availability of new reagents derived from the cloning of the CTR gene will also permit further elucidation of the molecular mechanisms responsible for the regulation of the CTR gene. This should provide important insights into the mechanisms underlying the "escape"phenomenon and help define the possible role of CT in development.

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CHAPTER 35

Calcitonin Gene Family of Peptides

Structure, Molecular Biology, and Effects

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Introduction

The physiopathology of calcitonin (CT) is of considerable pertinence to basic scientists and clinicians who are interested in bone and calcium metabolism. However, both the importance and the complexity of this topic have increased markedly due to the awareness that there are multiple peptides that originate from the "calcitonin gene family of peptides" and due to the finding that some of the precursors involved in the biosynthesis of these peptides possess great importance in normal physiological mechanisms as well as in human illness.

Mature human CT originates from the calcitonin-I (CALC-I) gene. CALC-I is a member of a family of genes: CALC-I, II, III, IV, and V. With the exception of CALC IV, which is on chromosome 12, all these genes are on chromosome 11. In addition to mature CT, the mRNAs, which originate from this gene family, produce other bioactive hormones: CT gene-related peptides I and II (CGRP-I and -II), adrenomedullin (ADM), amylin (see Fig. 1), and several other circulating precursor or derivative peptides, some of which also have biological functions.

Several common features characterize the classic hormones of the calcitonin gene family (Fig. 2). CT, the CGRPs, ADM, and amylin all contain two N-terminal cysteines that form a disulfide bridge, resulting in a ring structure at the amino terminus. Furthermore, the carboxyl-terminal amino acids of these peptides are amidated. The midregions of CGRP, CT, amylin, and ADM form an α-helical structure. Interestingly, when the sequences of CT, CGRP, and amylin are aligned with a gap introduced in the CT sequence to maximize homology, the 12 identical matches and five conservative amino acid substitutions suggest gene duplication of a common ancestral gene. Importantly, as will be discussed later, CT gene family peptides exert their bioeffects by binding to the same family of receptors.

Human Calcitonin and Its Preprohormonal Components

Biochemistry

Mature human CT is a single chain peptide of 32 amino acid residues (Fig. 2). The molecular mass of the hormone is 3418 Da. A disulfide bridge connects the cysteines at positions 1 and 7 to form a 7 amino acid ring structure at the amino terminus. At the carboxyl terminus, there is an amidated proline.

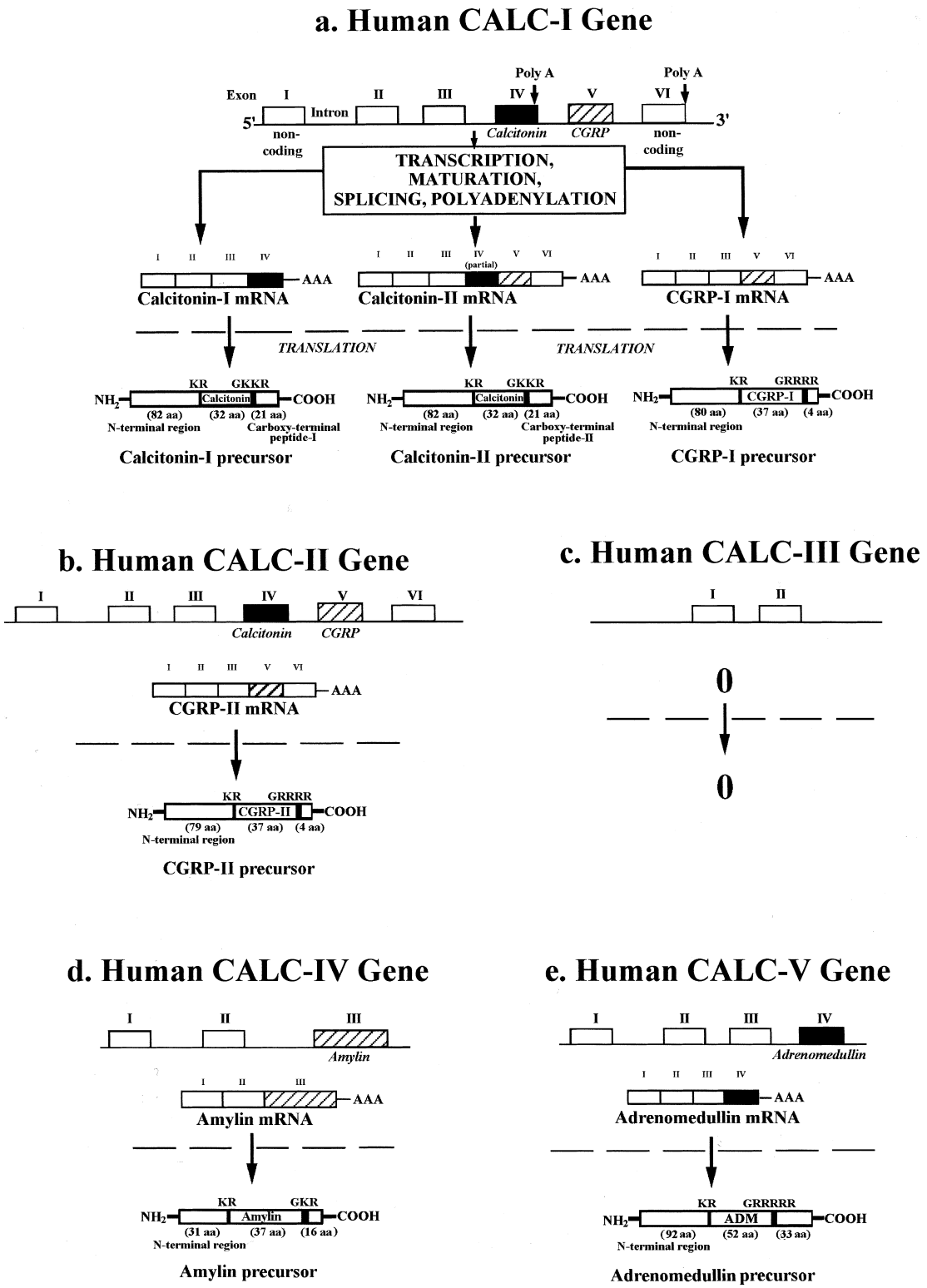

Figure 1 The human calcitonin (CT) gene family: organization of genes, mRNAs, and their hormone precursors. Based on their nucleotide sequence homologies, five genes belong to this family: CALC-I (CT/CGRP-I), CALC-II (CGRP-II), CALC-III, CALC-IV (amylin), and CALC-V (adrenomedullin) genes. (a) The CALC-I primary transcript is processed into three different mRNAs: CT, CT-II, and CGRP-I. The different products are generated by the inclusion or exclusion of exons by a mechanism termed splicing. Exons I – III are common for all mRNAs. Exon IV codes for CT, and exon V codes for CGRP-I. CT mRNA includes exons I + II + III + IV. CT-II mRNA includes exons I + II + III + IV (partial) + V + VI. CGRP-I mRNA is composed of exons I + II + III + V + VI. Each mRNA codes for a specific precursor. CT mRNA codes mainly for an N-terminal region, mature CT, and a specific C-terminal peptide (i.e., katacalcin, PDN-21, or CCP-I) that consists of 21 amino acids. The N-terminal region includes a signal peptide of 25 amino acids and an N-terminal peptide of 57 amino acids (i.e., NProCT or PAS-57). The CT-II precursor differs from the CT-I precursor by its specific C-terminal peptide, CCP-II. CCP-I differs from CCP-II by its last 8 amino acids. CGRP-I mRNA codes for an N-terminal region, mature CGRP-I, and a cryptic peptide. The commitment of the primary transcript in the different splicing pathways is determined, in part, by tissue specificity. Although there is some overlap, CGRP-I mRNA is expressed mainly in nervous tissue, and CT mRNA is the major mRNA product in thyroid tissue and other tissues, whereas CT-II was found to be expressed in liver. (b) The CALC-II gene codes only for a CGRP-II precursor. Its organization is similar to the CALC-I gene, containing 6 exons. Sequence

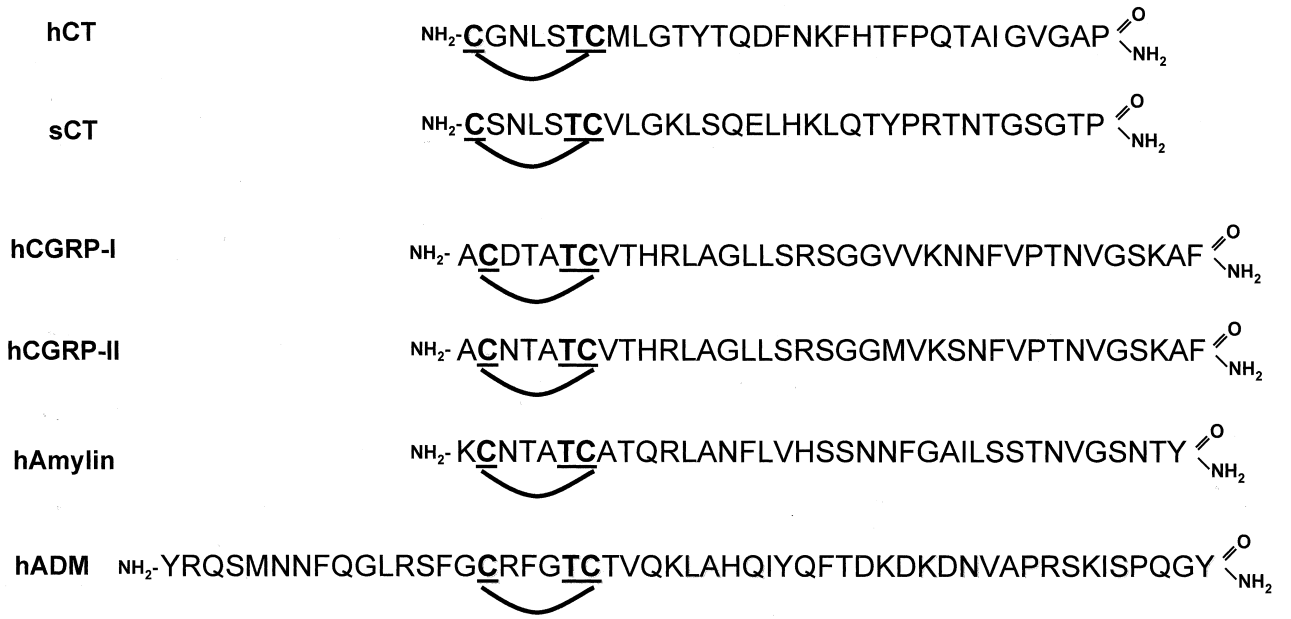

Figure 2 Amino acid sequences of human calcitonin (hCT), salmon CT (sCT), human CGRP-I (hCGRP-I), human CGRP-II (hCGRP-II), human amylin, and human adrenomedullin (hADM). Two structural features that are essential for full functional activity are conserved between the peptides: they contain two N-terminal cysteines that form a disulfide bridge resulting in an N-terminal loop and a C-terminal amide. Modified from Becker (2001).

The polypeptide precursor of CT, preprocalcitonin (Pre-ProCT) (molecular mass 15,466 Da), contains 141 amino acid residues (Le Moullec *et al.,* 1984) (Fig. 3). The CALC-I gene encodes information for its primary structure. The leader sequence (signal peptide) is composed of 25 amino acid residues.

As is common for leader sequences of all proteins, methionine (number 84 in Fig. 3) is the initial residue. Within the leader sequence, there is a customary stretch of mostly hydrophobic residues (amino acid residues 77–67 in Fig. 3). The function of the leader sequence is to assist in transport of the ribosomal precursor molecule into the cysternae of the rough endoplasmic reticulum.

Early in posttranslational processing, the leader sequence is cleaved from the Pre-ProCT precursor molecule by a signal peptidase. The resultant prohormone, procalcitonin (ProCT, also termed PAN-116), may be glycosylated. It consists of 116 amino acid residues (molecular mass 12,795 Da), which are folded into their appropriate three-dimensional conformation. At the amino terminus portion of ProCT, there is a 57 amino acid peptide called nProCT (also called PAS-57); its molecular mass is 6221 Da (Fig. 4). The immature CT, which is placed centrally within ProCT, consists of 33 amino acid residues, including a carboxyl-terminal glycine. There is a dibasic amino acid cleavage site (Lys-Arg), which is adjacent to the amino terminus side of immature CT. The final 21 amino acid residues comprise the CT carboxyl-terminal peptide-I (CCP); it is also termed carboxyl-terminal flanking peptide-I or PDN-21 (formerly called katacalcin). Human CCP occurs in two possible forms that differ by their terminal eight amino acid residues (CCP-I and CCP-II) (Fig. 4). These alternative structures arise from different forms of CT mRNA (Fig. 1). Situated between the amino terminus end of CCP-I or CCP-II and the carboxyl terminus of immature CT, there is a tribasic amino acid cleavage site (Lys-Lys-Arg).

Posttranslational Processing of Procalcitonin

The biosynthetic secretory pathway for CT involves a complex series of progressive modifications, which eventuate in the final exocytosis of the mature secretory product. Topographically, highly organized traffic from the endoplasmic reticulum must pass through the Golgi apparatus,

Figure 1 *(continued)*
homologies are important. Examination of the exon 4-like region of CALC-II indicates that CT mRNA is unlikely. Splicing at the site equivalent to the exon 3–exon 4 junction in hCT mRNA results in a stop codon within the reading frame of the precursor polypeptide. Although CALC-II appears to be a pseudogene for CT, it is a structural gene for CGRP-II. The CGRP-II hormone differs from CGRP-I by 3 amino acids. (c) The CALC-III gene contains only 2 exons. Their sequences have homologies with exons 2 and 3 of CALC-I and -II genes. The CALC-III gene does not seem to encode a CT- or CGRP-related peptide hormone and is probably a pseudogene, which is not translated into a protein. (d) The CALC-IV gene codes for a precursor containing the amylin peptide. This gene contains only three exons. The third exon codes for amylin. This 37 amino acid peptide has marked homology with CGRP peptides. It has been suggested that CT and CGRP exons are derived from a primordial gene and that the different CT/CGRP/adrenomedullin/amylin genes have arisen by duplication and sequence-divergent events. (e) The CALC-V gene is translated into adrenomedullin. This gene contains 4 exons. Adrenomedullin (ADM) is coded by the fourth exon. The amino-terminal peptides, encoded by exons 2 and 3, also have some bioactivity. Modified from Becker (2001).

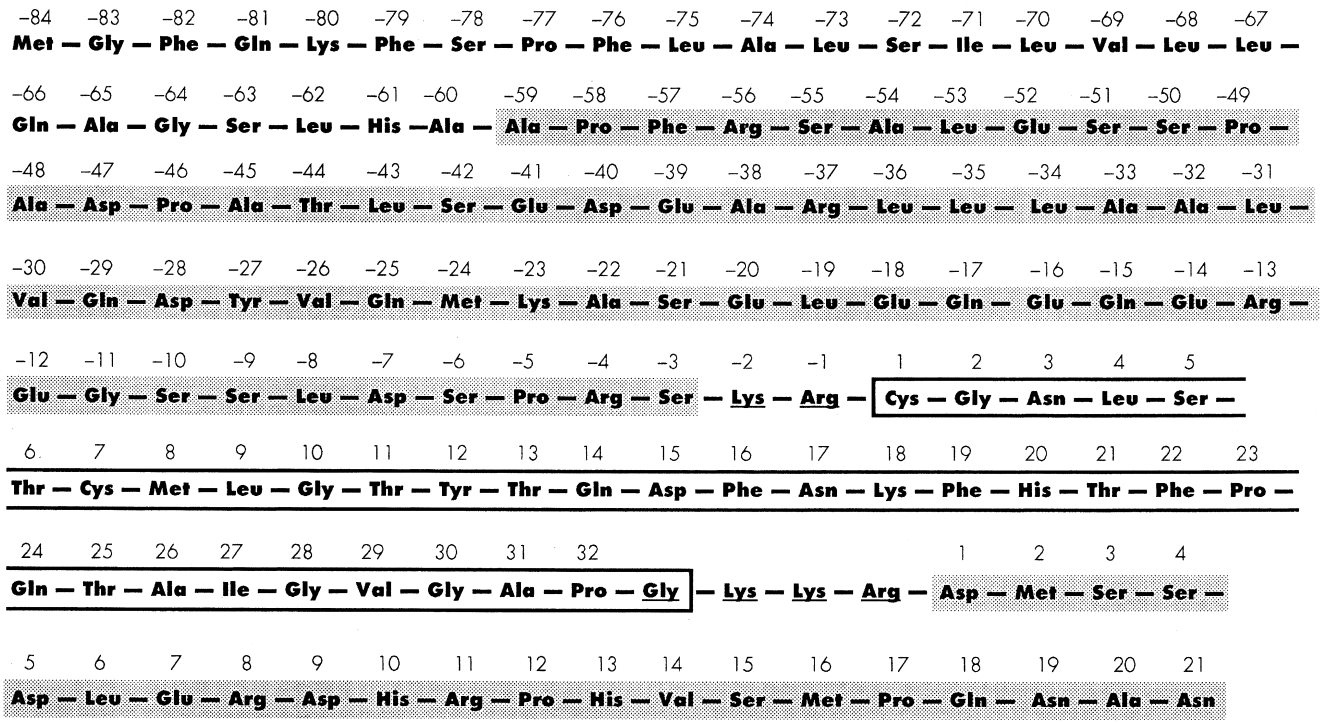

Figure 3 Sequence of human Pre-ProCT. This protein represents one of the two CT-containing molecules arising from the CALC-I gene and is the product of calcitonin-I mRNA (see Fig. 1). At the amino terminus, the first 25 amino acid residues comprise the signal peptide, the next 57 amino acid residues in the initial shaded area comprise N-procalcitonin (nProCT or PAS-57); the 33 amino acid residues in the bold enclosed area comprise immature CT; the underlined Gly residue at the carboxyl terminus of CT is removed during amidation of CT; the 21 amino acid residues in the final shaded area comprise calcitonin carboxyl peptide-I (CCP-I, PDN-21, or katacalcin). The underlined Lys and ARg residues between nProCT and immature CT and the underlined Lys, Lys, and Arg residues between immature CT and CCP-I are basic amino acid cleavage sites. The alternative Pre-ProCT that arises from calcitonin-II mRNA of the CALC-I gene differs only in the CCP portion (see Fig. 4). The amino acid sequence and numbering system were derived from Le Moullec *et al.* (1984).

the densecore secretory vesicles, and, eventually, the cell surface. This regulated secretory pathway for a CT molecule that is composed mostly of the mature, bioactive form differs from the constitutional, unregulated, secretory pathway by which, in all likelihood, mostly ProCT would be secreted. Although this constitutional pathway may be present to a small extent in normal persons, it seems to be paramount in certain disease states.

Regulated Secretion of Calcitonin and Its Precursor Peptides

The mechanisms by which the large precursor, ProCT, is serially processed and by which its component peptides are sorted into nascent secretory vesicles have not been fully clarified. However, much that has been learned from the study of other propeptides is also applicable to ProCT.

After the biosynthesis and folding of ProCT, subsequent proteolytic processing occurs, both within the Golgi apparatus and, later, within the secretory granules (Chanat and Huttner, 1991). Cisternae of the Golgi apparatus are arranged into a series of compartments, the final one being the *trans*-Golgi; this is the exit compartment of the apparatus. Immature secretion vesicles bud off from this compartment and, both here and within the vesicles, endoproteolytic cleavage occurs (Fig. 5). This cleavage of ProCT, and the consequent release of the immature CT, is accomplished by a prohormone convertase (PC) enzyme, which has not yet been identified. PC enzymes, which cleave propeptides preferentially at the carboxyl terminus of basic residues, are Ca^{2+} dependent; they often carry out their endoproteolysis in a strict temporal sequence. The appropriate order of proteolysis is, in part, modulated by autocatalytic self-activation, which, in turn, may be influenced by neuroendocrine "chaperone" peptides that aid in protein folding. For reasons detailed later, it is likely that the initial or preferential cleavage site of immature CT is at its amino terminus region, yielding a conjoined polypeptide of CT plus the CT carboxyl-terminal peptide (CT:CCP).

During early posttranslational processing, the nProCT segment may act as a signal for sorting its parent ProCT molecule to nascent secretion vesicles of the regulated secretory pathway; such a role has been demonstrated for the N-terminal 26 amino acid peptide of the prohormone of adrenocorticotropin (ACTH), pro-opiomelanocortin (POMC) (Cool and Loh, 1994). Furthermore, chromogranin B may function as a

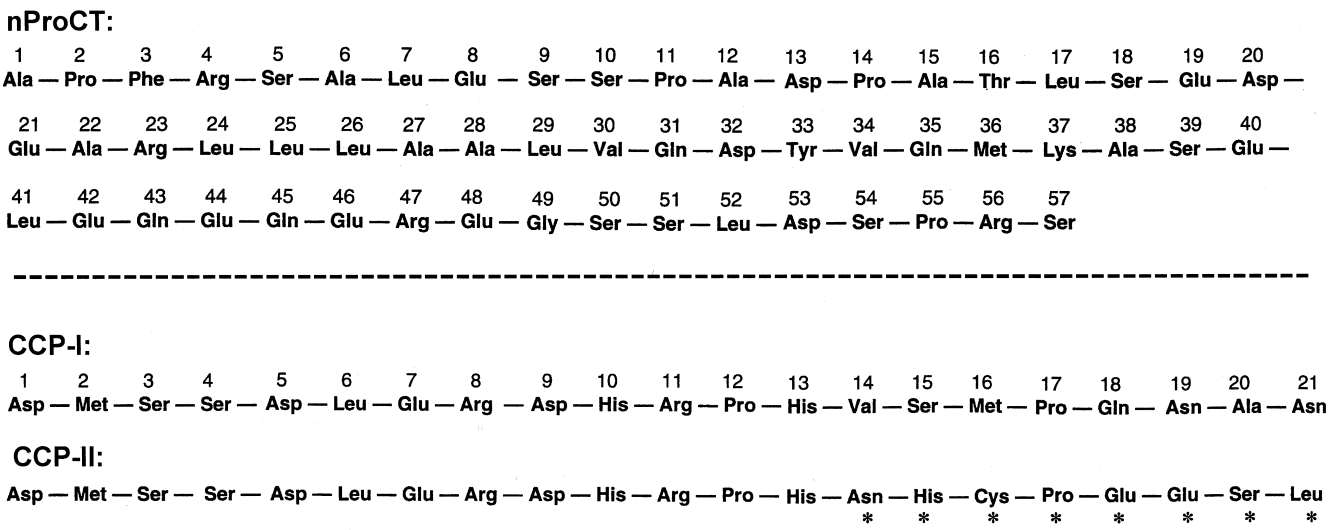

Figure 4 Sequence of human nProCT (top) and the two CCP peptides (bottom).

helper protein to favor *trans*-Golgi sorting to the regulated secretory pathway as it does for ACTH.

Within the newly formed secretion vesicles, proteolytic cleavage releases immature CT. Then, as amidation proceeds, mature CT is produced and is concentrated progressively within these vesicles (Treilhou-Lahille *et al.,* 1986). The ensuing tight aggregation of hormones within the vesicle causes its subsequent electron-dense appearance. These secretory vesicles are destined to serve as storage repositories for later secretion; without the appropriate external

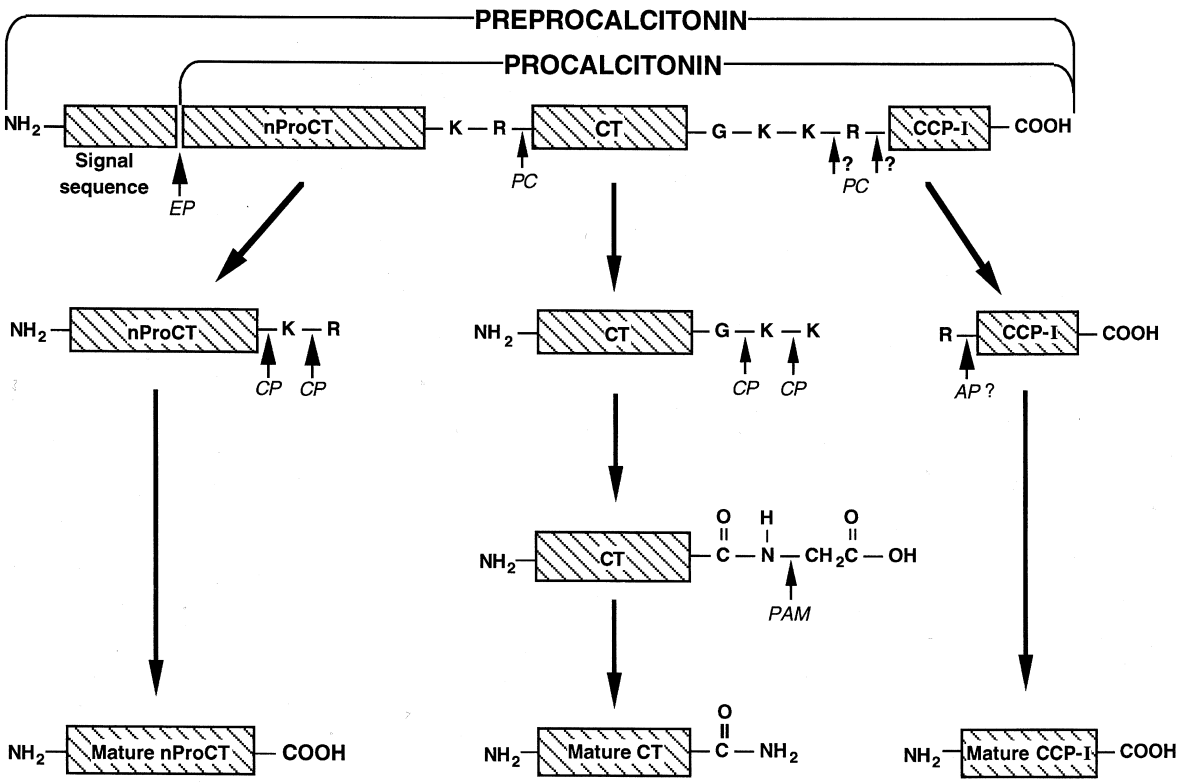

Figure 5 Enzymatic processing of Pre-ProCT and its constituents. EP, an endopeptidase; PC, a prohormone convertase; CP, a carboxypeptidase; AP, an aminopeptidase; PAM, peptidylglycine α-amidating monoxygenase and its constituent enzyme peptidyl-α-hydroxyglycine α-amidating lyase (PAL); K, lysine; G, glycine; R, arginine. The PC cleaves the propeptide at the carboxyl terminus of the dibasic paired Lys-Arg residues between nProCT and immature CT, and either between Lys and Arg or at the end of the Lys, Lys, Arg basic triplet, which is located at the junction between immature CT and CCP-I. If the cleavage is between Lys and Arg, an aminopeptidase enzyme would remove residual Arg. A carboxypeptidase removes residual Lys-Lys (or the Lys-Lys-Arg) from the immature CT prior to the action of PAM and removes Lys-Arg from the carboxyl terminus of nProCT.

stimulus, they have a relatively long half-life. Ultimately, in response to the appropriate signal at the plasma membrane, there is a brief increased concentration of cytosol-free Ca^{2+}; this induces secretion. In this process, the secretory vesicles further migrate via an intracellular microtubular system toward the periphery of the cell, fuse with the apical portion of the plasma membrane, and, by exocytosis, discharge their hormonal contents in a quantal release. Studies in normal humans suggest that these secretory vesicles contain, in addition to mature CT, nProCT, CT:CCP, and probably some free immature CT (Treilhou-Lahille *et al.,* 1986; Snider *et al.,* 1996).

Location of Calcitonin in the Body

Systematic studies have been made of immunoreactive CT (iCT) in tissues of humans and monkeys (Becker *et al.,* 1979, 1980a). In humans, the highest concentration of iCT is in the thyroid gland, where it is located within the C cells, mostly found in the central portion of each lobe. Scattered C cells may also be found in adjacent tissues (i.e., parathyroid glands and thymus). However, a survey of approximately 20 tissues of other regions of the human body has yielded iCT values for many tissues that are appreciably higher than in the blood. The highest levels have been found in small intestine, thymus, urinary bladder, lung, and liver. In both intact and thyroidectomized monkeys, these tissues also have considerably elevated levels. In the monkey, extrathyroidal tissue levels of iCT do not diminish following thyroidectomy (except for the high levels in the liver of the intact animal, which decrease markedly). When one considers the weights of most of these extrathyroidal tissues, their total iCT content is considerable. Gel filtration of several tissue extracts (e.g., lung, thymus, liver, stomach) demonstrates that the iCT consists primarily of mature CT, with very little of the peptide precursors. These patterns do not differ from that of the thyroid. In nearly all of the tissues where appreciable amounts of iCT are found, neuroendocrine cells have been identified, and in some of these tissues, immunohistochemical staining reveals the presence of iCT. These extrathyroidal neuroendocrine cell contents contribute to the serum content of iCT, and these cells can be induced to secrete both locally and distally under the influence of various stimuli.

In normal conditions, it is likely that the extrathyroidal iCT originates from iCT-containing neuroendocrine cells, as well as hormonal binding to receptor tissues. However, in several pathological conditions, iCT production and its corresponding mRNA are augmented greatly, emanating ubiquitously from nonneuroendocrine parenchymal cells (Müller et al., 2001).

Mature Calcitonin

A mature peptide may be defined as a final product of a precursor propeptide. If the peptide is a bioactive hormone, it must possess the required structure to exert an effect upon its receptor. An immature peptide hormone may be defined as one that either has not yet undergone proteolytic separation from its precursor propeptide or, if separated, has not yet undergone the final biochemical process(es) that is requisite for its full bioactivity.

As is the case for a very large number of bioactive peptides, mature CT possesses an α-amide moiety at its carboxyl terminus. In part, amidation may confer upon the hormone a structure or configuration that is important for its bioactivity and may also increase the resistance of the molecule to enzymatic degradation (Rittel *et al.,* 1976). Initially, prior to amidation, there usually is proteolysis at an endoproteolytic cleavage site (e.g., the Lys-Lys-Arg locus within the ProCT), and there is a prerequisite glycine residue at the carboxyl-terminal side of the amino acid residue that is to undergo the subsequent amidation.

The sequential steps leading to amidation are shown in Fig. 6. The parent enzyme that plays the key role in the amidation is peptidylglycine α-amidating monooxygenase (PAM) (Eipper *et al.,* 1992). The preliminary step performed by PAM is oxidation of the α-hydrogen of glycine to form an α-OH. Within the PAM protein, a second enzyme resides, peptidyl-α-hydroxyglycine α-amidating lyase (PAL). This enzyme acts on the intermediate molecule; it catalyzes amidation of the adjacent proline of CT, thus removing glycine in the form of a glyoxylate. The product of this two-step enzymatic action is mature 32 amino acid CT with an amidated proline at its carboxyl terminus. In most peptides that have been studied, amidation of the carboxyl terminus glycine residue occurs only after the prior complete proteolytic cleavage of the precursor molecule.

Bioactions of Calcitonin

The direct bioactivity of CT is linked inextricably to the location and nature of its receptors. In general, the relevant actions of CT in the human have been difficult to determine. Most of the *in vivo* and *in vitro* studies have been performed only in the laboratory animal; some of the study animals had been parathyroidectomized and some were intact; most investigators have utilized species of CT other than human (salmon, porcine, eel); and most investigators have utilized pharmacologic and not physiologic doses. Despite hundreds of studies, the precise physiologic role of CT remains uncertain. Nevertheless, it appears that biologically relevant effects occur in blood, bone, the central nervous system (CNS), the respiratory system, the gastrointestinal system, the reproductive system, and the kidney.

Calcitonin Effects on Serum Calcium and Phosphate

Acutely, CT decreases the serum calcium of laboratory animals. When human CT is administered to rats, this hypocalcemic activity is blunted by the deletion of the carboxyl-terminal amide group, by the shortening of the peptide chain, or by opening the disulfide ring (Rittel *et al.,* 1976). In human studies, the effects of CT on serum cal-

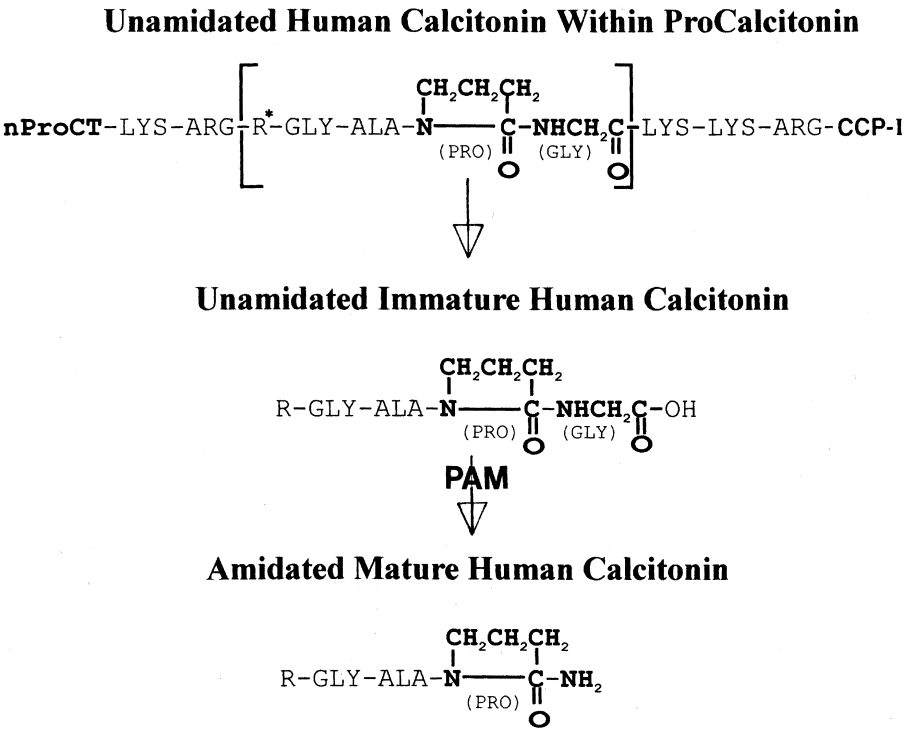

Figure 6 Sequential steps involved in the amidation of CT (see text). R^*, 29 amino acids of human CT not shown.

cium vary with the species of hormone used, its dosage, its method of administration, and the concurrent bone turnover rate of the human subject. In some studies of normal persons, CT is hypocalcemic (Gennari *et al.,* 1981), but, usually, it does not influence serum calcium levels (Gnaedinger *et al.,* 1989). However, in patients with high bone turnover, such as in Paget disease or in immobilized children, CT administration usually is hypocalcemic. In normal humans and dogs, pharmacologic doses of salmon CT result in hypocalcemia that is characterized by marked fluctuations, an occasional biphasic hypocalcemic response, and paradoxical above-baseline increases of serum calcium; these patterns may be partly due to parathyroid hormone overcompensation. In thyroparathyroidectomized humans and dogs, induced hypocalcemia may be more pronounced, but there still may be marked fluctuations to and above the baseline (Mohamadi *et al.,* 1975). Serum calcium is normal in hypercalcitonemic patients with medullary thyroid cancer (MTC).

The hypophosphatemic effect of CT is dose dependent and, in some studies, parallels the hypocalcemic effect.

CALCITONIN ACTIONS IN THE OSSEOUS SYSTEM

Mature CT plays an important role in skeletal homeostasis, being a key modulator of bone resorption (Zaidi *et al.,* 1994). The hormone inhibits bone resorption by inducing an acute quiescence of cell motility (Q effect); this occurs within 1 min and is followed by a more gradual retraction of the osteoclasts (R effect) (Moonga *et al.,* 1992; Alam *et al.,* 1993a). Retraction of the pseudopods, which occurs in most of the osteoclasts, is associated with the formation of intracellular retraction fibers and a cessation of membrane ruffling; the final result is a small, rounded, nonmotile cell (Gravel *et al.,* 1994). Both cyclic adenosine monophosphate (cAMP) and intracellular Ca^{2+} are second messengers for the Q and R effects, and both are G protein mediated. However, there are distinct differences: the Q effect is coupled to adenylate cyclase, acting via a cholera toxin-sensitive Gs; and the R effect is thought to be coupled to a pertussis toxin-sensitive G protein, resulting in an increased cytosolic-free calcium concentration (Alam *et al.,* 1993a). These studies have not determined whether there are one or two receptor complexes involved. Other investigators have demonstrated that protein kinase C participates in the effect (Su *et al.,* 1992). Chronic administration of CT reduces the number of osteoclasts.

CT inhibits other components of the osteoclast, such as the release of acid phosphatase. Carbonic anhydrase II is expressed at a high level in the osteoclast and plays an important role in the bone resorptive activity of osteoclasts. CT diminishes expression of this enzyme in a dose-dependent manner (Zheng *et al.,* 1994). Also, osteoclasts were reported to contain large amounts of focal adhesion kinase in their cytosol; this tyrosine kinase may assist in the maintenance of contact between the osteoclasts and the mineralized matrix upon which they act. CT inhibits this kinase (Berry *et al.,* 1994). Similarly, in the rabbit, expression of the bone matrix protein osteopontin, which participates in the attachment of osteoclasts to bone matrix, may be inhibited, acting via cAMP-dependent protein kinase and cal-

cium–protein kinase pathways (Kaji *et al.,* 1994). Thus, by various mechanisms, mature CT diminishes osteoclastic activity, a phenomenon that is so marked that its *in vitro* effect on bone resorption can be used as a bioassay to measure picomolar concentrations of the hormone (Zaidi *et al.,* 1994). The escape from CT inhibition of osteoclastic bone resorption appears to be due in large part to a rapid desensitization of the osteoclast (Samura *et al.,* 2000). As a reflection of the decreased bone turnover, CT reduces the excretion of urinary hydroxyproline, both acutely and chronically (Tolino *et al.,* 1993). The hormone appears to interact with the osteoprotegerin/osteoprotegerin ligand, thus influencing RANK (Mancini *et al.,* 2000). Some data suggest that CT may also exert a stimulatory effect on the osteoblast. The hormone increases the concentrations of IGF-I and IGF-II in cultures of human osteoblast cell lines in a dose- and time-dependent manner (Farley *et al.,* 2000). Also, it inhibits TNF-α production (Ballica *et al.,* 1999).

Vitamin D influences the osseous system, and the interrelationships between CT and this vitamin require further study. Several investigators have demonstrated that CT stimulates 1,25-dihydroxyvitamin D production *in vivo* in humans and in rats (Darger *et al.,* 1986) and, specifically, 1α-hydroxylase activity in the proximal straight tubule (Wongsuranat and Armbrecht, 1991). Furthermore, the 24-hydroxylase enzyme system, which participates in the deactivation of vitamin D metabolites in rat intestine, is diminished by CT administration (Beckman *et al.,* 1994). Conversely, in rats, the vitamin D status modulates the responsivity of the renal tubules to CT. For example, in this animal, an increased tubular reabsorptive capacity for calcium is induced by CT; this effect is blunted in vitamin D deficient animals (Su *et al.,* 1988).

Indirect evidence often cited for a relationship between CT and the osseous system relates to serum levels of the hormone. In general, normal men have higher levels than women. Some authors have reported lower levels in postmenopausal than in premenopausal women, and others have reported higher levels. In elderly women, there may be a low level of mature CT, with a consequent increased proportion of high molecular weight forms of CT (Bucht *et al.,* 1995). Notwithstanding, because of its inhibitory effects on the osteoclast, CT is used successfully in the therapy of disorders of bone loss or of rapid bone turnover and also to diminish the egress of osseous calcium into the blood in hypercalcemic conditions (see Chapter 82).

Further advances will undoubtedly emerge from the evaluation of the osteopenic CT gene knockout mouse model (Hoff *et al.,* 1998).

Calcitonin Actions in the Central Nervous System

CT has specific binding sites within the CNS. The intracerebral injection of CT suppresses food and water intake in rats (Chait *et al.,* 1995). Experimentally, the hormone also increases body temperature, acting on specific regions of the thalamus and hypothalamus (Sellami and de Beaurepaire, 1993). In the rat, intracerebral administration of CT decreases the frequency and amplitude of spontaneous growth hormone secretory pulses (Tannenbaum and Goltzman, 1985). In the human, large doses of salmon CT reduce the serum concentrations of testosterone, LH, and FSH, probably acting at the hypothalamic level (Mulder, 1993). CT activates the brain serotonergic system in rats, acting via a direct central effect and also via a hypocalcemic mechanism. In normal humans, salmon CT may decrease serum prolactin.

The antinociceptive activity of CT may be mediated in part via central cholinergic influences (Chen and Lee, 1995); also, the integrity of the brain serotonergic system is required for this analgesic effect (Coldado *et al.,* 1994). Furthermore, CT interacts with the opioid system (Martin *et al.,* 1993). Chronic administration of salmon CT to humans with migraine headaches increases the levels of β-endorphin, as well as ACTH and cortisol. In this respect, there have been many studies using pharmacologic dosages of CT for the control of pain secondary to osteoporosis (Pontiroli *et al.,* 1994) or painful osteolytic metastases or for other forms of pain not associated with bone involvement. Some of these studies have had convincing results, whereas some, particularly those involving nonosseous pain, have been less impressive.

Calcitonin and the Respiratory System

The total amount of iCT in normal lungs exceeds that of any tissue of the human body, including the thyroid gland (Becker *et al.,* 1979, 1984). The hormone is found within pulmonary neuroendocrine (PNE) cells, which are situated near the basement membrane and often extend to the lumen of the airway (Becker *et al.,* 1980b). In the newborn, they are grouped into strategically located organoid clusters and are termed neuroepithelial bodies (Becker, 1993). Similar cells are found in the trachea and larynx. The great number of PNE cells in the fetus and newborn and the response of these cells to stimuli such as cigarette smoke or hypoxia strongly suggest that CT plays a role in pulmonary maturation and pathophysiology (Tabassian *et al.,* 1989). CT affects transcellular and intracellular movements of calcium, and hence may exert an intrapulmonary paracrine action. The inhibition by CT of prostaglandin and thromboxane synthesis and its augmentation of prostacyclin production by endothelium may modulate local pulmonary blood flow. CT may increase cartilagenous growth (Burch, 1984) and hence may influence chondrogenesis of the bronchial tree. The hormone also interacts with other peptides; for example, CT blocks the bronchoconstrictor effects of bombesin-like peptides and substance P.

The molecular configuration of the iCT contained within normal PNE cells has been studied in the hamster; in this species, long-term cultures of the PNE cell have been established (Nylén *et al.,* 1987; Linnoila *et al.,* 1993). Hamster PNE cells appear to contain mature CT, perhaps in a dimeric form, and the iCT that is secreted by PNE cells following acute stimulation is mostly the mature hormone. In this regard, putative malignancies of the human PNE cells differ; both the bronchial carcinoid and the small cell lung

cancer store predominantly mature CT, but secrete predominantly precursor forms (Becker *et al.,* 1983; Nylén *et al.,* 1987).

GASTROINTESTINAL EFFECTS OF CALCITONIN

In humans, CT pharmacologically increases gastric acid and pepsin secretion, decreases pancreatic amylase and pancreatic polypeptide, and modulates small intestinal motility (Demol *et al.,* 1986). The effects of the hormone on gastrin release are variable. Human CT administration decreases serum levels of gastrin, insulin, and pancreatic glucagon. Serum motilin is decreased, as is gastric inhibitory peptide. Somatostatin levels in the serum are increased. The small intestinal secretion of potassium, sodium chloride, and water is augmented. Thus, at high concentrations, CT increases the net secretion of water and electrolytes from the human jejunum and ileum, and it has been postulated that these effects may be a cause of the diarrhea seen in some patients with medullary thyroid cancer (Gray *et al.,* 1976). Physiologic doses of CT do not appear to influence the gastrointestinal absorption of calcium or phosphate in humans.

CALCITONIN AND THE REPRODUCTIVE SYSTEM

In the human male, CT is over 10-fold higher in the seminal plasma than in venous plasma, strongly suggesting that it is produced within the genital tract (Davidson *et al.,* 1989). In this respect, neuroendocrine cells are found in the urethral epithelium and in the prostate gland. The CT of the seminal fluid consists predominantly of high molecular weight forms.

CT occurs in both the uterus and the placenta and has been reported to be secreted by human placental tissue (Balbanova *et al.,* 1987). In the rat, CT messenger RNA is found in the glandular epithelial cells of the uterus at the time of implantation. This expression is abolished by the antiprogestin drug mifepristone, a drug that blocks implantation. Furthermore, progesterone administration stimulates CT mRNA in the uteri of ovariectomized animals. Estrogen, which is inactive alone, is synergistic to this progesterone action (Ding *et al.,* 1994).

There are receptors to CT in the human placenta, both in the syncytiotrophoblast brush border that faces the mother and in the basal plasma membranes that face the fetus (Lafond *et al.,* 1994). In this respect, CT is known to induce an increase in human chorionic gonadotropin (hCG) secretion by human placental cells at term (Rebut-Bonneton *et al.,* 1992). The intraplacental presence of CT receptors, as well as cells containing iCT, suggests a role of CT in implantation (Kumar *et al.,* 1998) and in the regulation of placental function; nevertheless, little attention has been devoted to this important possibility. In one study, thyroidectomized pregnant ewes demonstrated an increased placental transfer of calcium from the dam to the fetus, a phenomenon that was abolished by the daily injection of the dams with salmon CT (Barlet, 1985).

The measurement of serum CT during pregnancy in the human has yielded inconsistent results, ranging from no appreciable change to an increase. These differences probably reflect the differing specificity of the antisera used.

Human breast milk contains large amounts of immunoreactive CT, much of it being composed of high molecular weight moieties (Bucht and Sjoberg, 1987). It is likely that these peptides emanate from the neuroendocrine cells of the breast ducts. The possible role of CT as a modulator of mineral and electrolyte concentrations of milk merits study.

CALCITONIN ACTIONS IN THE KIDNEY

The kidney is a principal site of CT degradation (Hysing *et al.,* 1991); much of this may be accomplished by the cell surface enzyme, neutral endopeptidase (NEP). In addition, the abundance of CT receptors in the kidney bears witness to the multiple actions of CT within this organ (Kurokawa, 1987). However, interpretations of some elicited actions are clouded by the fact that CT appears to activate both cAMP and protein kinase pathways, leading to opposite biological responses that depend on the phase in the cell cycle of the target renal cell (Chakraborty *et al.,* 1991). In humans, the intravenous administration of CT stimulates diuresis and increases the fractional excretion rates of sodium, chloride, magnesium, and potassium. Urinary calcium and phosphate excretion increases, as do the urinary levels of adenosine 3′,5′-cyclic monophosphate (Gnaedinger *et al.,* 1989) and *N*-acetyl-*β*-*D*-glucosaminidase. However, in some species (rat, mouse), CT stimulates the renal tubular reabsorption of calcium and magnesium, probably within the thick ascending limb of the Henle loop (Carney *et al.,* 1992). The CT stimulation of urinary excretion of phosphate occurs within the proximal tubule and may be due to the inhibition of Na/PO_4 cotransport (Muff *et al.,* 1994). Interestingly, high levels (up to 20-fold serum levels) of iCT (but not mature CT) are excreted in the urine. Urine iCT levels have been determined and characterized in normal adults (Snider *et al.,* 1978) and children (Silva *et al.,* 1981) and have been demonstrated to be useful for the detection and follow-up of medullary thyroid cancer and also to detect C-cell hyperplasia (Silva *et al.,* 1979a,b). Moreover, urine iCT levels are increased in some patients with lung cancer (Becker *et al.,* 1980c).

METABOLIC EFFECTS

CT increases plasma glucose and lactate in the rat and causes peripheral insulin resistance by inhibiting the insulin-stimulated incorporation of glucose into glycogen.

SUMMARY OF CALCITONIN ACTIONS

It is difficult to clearly delineate the biologically relevant roles that CT may play. However, although it is impossible to extirpate all iCT-producing cells in the body, the development of a CT gene knockout model (Zhang *et al.,* 2001) should make this task more feasible. The highest concentration of iCT is found within the thyroid gland, but not the highest total content. Also, the experimental effects of thyroidectomy vary considerably among different species. Although lower than

normal, serum and urine iCT levels usually remain measurable following thyroidectomy in the human (Silva *et al.,* 1978). However, in thyroidectomized humans, there is no response of iCT to a calcium infusion (Silva *et al.,* 1978) or to pentagastrin (Weissel *et al.,* 1991). Provided thyroid hormone is replaced, thyroidectomy in humans has little or no important biochemical or pathologic consequences, and calcium homeostasis remains largely intact. In addition, bone density is not affected (Hurley *et al.,* 1987). Nevertheless, when intravenous calcium is administered to patients with prior hypothyroidism or with thyroid ablation who are maintained on thyroid hormone, the subsequent return to normocalcemia is delayed as compared to persons with intact thyroid glands (Williams *et al.,* 1966). A cautious but still valid hypothesis, in part made nearly two decades ago, is that CT maintains bone mineral in emergency situations (i.e., to combat hypercalcemia) and may play a role in the conservation of body calcium stores in certain physiologic states (i.e., growth, pregnancy, lactation). Furthermore, immunoreactive CT is present in many normal tissues, and hence hemocrine, paracrine, neurocrine, and/or solinocrine (i.e., intraluminal) secretions of this hormone are undoubtedly important; its functions remain to be fully elucidated.

Physiopharmacologic Stimuli of Calcitonin Secretion

Many agents have been reported to stimulate the gene expression and secretion of CT. Often, the findings are difficult to extrapolate to the human because of the very diverse experimental conditions: e.g., *in vivo* vs *in vitro,* species of animal, different tissues being investigated, nature of the agent, pharmacologic vs physiologic dose of the stimulating agent, route of administration, acuity or chronicity of the stimulus, and assay used to quantitate the CT response. Furthermore, the effective pharmacologic secretagogues vary with the location of the hormone production.

In normal humans, an intravenous calcium infusion usually raises serum iCT (Silva *et al.,* 1974; Hurley *et al.,* 1988), as does pentagastrin (Guilloteau *et al.,* 1990). In normal persons, iCT persists in the serum, despite induced hypocalcemia (Body and Heath, 1983). Hypermagnesemia but not hyperphosphatemia induces iCT release from the thyroid gland.

The malignant C cell of the rat [i.e., medullary thyroid carcinoma (MTC)] exhibits an elevation of cytosolic-free Ca^{2+} in response to very small changes in extracellular Ca^{2+} (Fried and Tashjian, 1986). Such cytosolic changes induce secretion of CT. However, the response of the *nonmalignant* C cell to similar calcium perturbations may be considerably less (Selawry *et al.,* 1975). Endogenous hypercalcemia, such as that due to neoplasia, multiple myeloma, and sarcoidosis, often is associated with increases of serum iCT in humans; however, this increase may sometimes be related to the condition per se and not hypercalcemia. In hyperparathyroidism, serum iCT has been variably reported to be normal or increased.

The increase of serum iCT in response to gastrin, and perhaps to pancreozymin and glucagon, raises the question as to whether these or other gastrointestinal hormones regulate its secretion (Cooper *et al.,* 1971; Selawry *et al.,* 1975). In this respect, endogenous hypergastrinemia, as occurs in pernicious anemia, is associated with an increased serum iCT (Becker *et al.,* 1980d). Also, there is specific binding of 1,25-dihydroxyvitamin D to malignant human C cells (MTC), raising the possibility of the modulation of CT by this steroid hormone, but it is unknown whether this occurs in normal C cells.

In hamsters and in intact and thyroidectomized men, cigarette smoke increases serum iCT acutely and chronically probably due to the nicotine content of the smoke (Tabassian *et al.,* 1988, 1989, 1990, 1993). Nicotine per se increases serum CT in hamsters. Chronic exposure to cigarettes induces PNE cell hyperplasia. There are cholinergic–nicotinic receptors on the PNE cell, and cultured PNE cells of newborn hamsters secrete CT in response to nicotine (Nylén *et al.,* 1993). This alkaloid stimulates the growth of PNE cells and, in the hamster, also exerts this effect transplacentally on the fetus (Nylén *et al.,* 1988). Catecholamines do not appear to influence CT secretion (Epstein *et al.,* 1983).

Dietylnitrosamine, a carcinogenic agent with nicotinic characteristics, induces PNE cell hyperplasia in hamsters; pulmonary and serum levels of CT are increased; and hypercalcitonemia occurs (Linnoila *et al.,* 1984). The chronic exposure of hamsters to 60% oxygen for 3 months increases CT levels in the lungs and serum, probably due to PNE cell hyperplasia (Nylén and Becker, 1993). Also, in these animals, the combination of diethylnitrosamines with hyperoxia induces tumor-like hyperplasia of the PNE cells and increased serum iCT (Nylén *et al.,* 1990). In addition, the acute induction of hypoxemia in hamsters raises serum iCT (E. S. Nylén *et al.,* unpublished observations).

Measuring Mature Serum Calcitonin

Serum iCT has been measured by bioassay, radioreceptor assay, and immunoassay. Bioassay techniques (e.g., induced hypocalcemia in the laboratory animal, *in vitro* generation of adenylate cyclase from renal cell membranes, inhibition of ^{45}Ca release from prelabeled mouse calvaria) demonstrated that not all of the iCT that was immunologically detectable was bioactive. This is because the great majority of assays used prior to the 1990s cross-reacted with immature CT found within ProCT, within the free CT:CCP-I and CT:CCP-II conjoined peptides, and, alone, with the free immature form (Snider *et al.,* 1997) (see Fig. 5).

The principal clinical use of mature CT measurement is the detection and follow-up of patients with medullary thyroid cancer (MTC). It had been known since the 1970s that there was heterogeneity of calcitonin-containing peptides in the serum of MTC patients; therefore, mature CT was selected as the definitive marker to facilitate obtaining uni-

form normative and diagnostic criteria for C-cell hyperplasia as well as for medullary thyroid cancer.

The development of polyclonal antisera raised to specific regions of the mature CT molecule had provided useful normative data (Snider *et al.,* 1977); however, these values included the immature CT within the larger molecular weight precursors. In addition, early standard preparations for mature CT were insufficiently pure. Then, prior extraction and concentration of the serum with silica cartridges resulted in lower values that were more specific for mature CT (Body and Heath, 1983). The advent of monoclonal antisera further improved accuracy and specificity.

However, quantification of mature CT requires the specific detection of the amidated carboxyl terminal portion of the molecule. Usually, this utilizes a double-antibody method: one antibody reacts with the amidated carboxy terminus and the second one reacts with another portion of the molecule (usually the midportion). Such an assay will not cross-react with the immature CT within CT precursor molecules (Seth *et al.,* 1989; Guilloteau *et al.,* 1990; Motté *et al.,* 1988; Perdrisot *et al.,* 1990). This assay, which is available commercially, supersedes all others for the measurement of mature CT (Engelbach *et al.,* 2000). When using such an assay, the basal levels of mature CT are <10 pg/ml (<3 fmol/ml). Importantly, nearly all of the studies in the literature that have studied serum iCT levels relating to age, influence of the menstrual cycle, pregnancy, lactation, diurnal rhythm, influence of hormones, effect of secretagogues, physiopharmacologic manipulations, determinations of endogenous secretion rate (thought to be ~100 to 200 μg/day), half-life (thought to be ~10 min), degradation studies, and so on have not been verified using a specific and sensitive assay for mature CT.

Calcitonin Carboxyl-terminal Peptides I and II

CCP (Fig. 4) is the 21 amino acid peptide that, within the ProCT molecule, is connected to the carboxyl terminus of immature CT by the Lys-Lys-Arg cleavage site. CCP-I (molecular mass 2437 Da) is derived from the calcitonin-I mRNA. CCP-II (molecular mass 2505 Da) is derived from the calcitonin-II mRNA and is produced by splicing part of exon 4 to exon 5 of the CALC-I gene (Fig. 1). These two peptides differ in their last eight amino acid residues (Minvielle *et al.,* 1991; Cohen *et al.,* 1992). In their free forms, neither of these peptides are amidated. CCP-I is found in thyroid and MTC tissue and in the sera of normal persons and those with MTC (Born *et al.,* 1991). Although some investigators have reported that CCP-I is secreted in an equimolar amount with CT, other studies are not in accord. In several studies, serum CCP-I increased with calcium infusion. Some workers have found that serum levels increase in normal persons following a pentagastrin injection (Blind *et al.,* 1992). Serum levels of CCP-I have been used for detection and follow-up of patients with MTC. CCP-II has been found in thyroid, in MTC cells, in pituitary tumor, and in normal neurologic tissue (Cohen *et al.,* 1994). The serum level and bioactivity of CCP-II have not been studied sufficiently CCP-I does not influence osteoclast or serum calcium levels; its bioactivity is unknown (Zaidi *et al.,* 1991).

Clinical Conditions Characterized by Increased Serum Procalcitonin and Its Component Peptides

Normal persons secrete measurable serum levels of intact ProCT, nProCT, CT:CCP-I peptide, mature CT, and CCP-I (Snider *et al.,* 1997) (Fig. 7). Collectively, we refer to these precursor and component peptides as calcitonin precursors (CTpr).

Moreover, in several pathological states, there is an augmented biosynthesis and secretion of CTpr. These conditions include extrathyroidal neuroendocrine cell hyperplasia, medullary thyroid cancer, nonneuroendocrine tumors, and, last but not least, severe inflammation–infection–sepsis.

Extrathyroidal Neuroendocrine Cell Hyperplasia

In addition to C-cell hyperplasia of the thyroid (a sporadic or familial condition, which often eventuates in MTC), extrathyroidal neuroendocrine cell hyperplasia is also associated with increased serum iCT. Thus, the study of some chronic nonneoplastic pulmonary conditions with region-specific antisera has demonstrated a slight to moderate increase of serum iCT that is consistent with an apparent increase of CT-precursor forms. Usually, levels of free mature CT remain within the normal range. These conditions include patients with chronic bronchitis, chronic obstructive pulmonary disease, and chronic pulmonary tuberculosis (Becker *et al.,* 1981; Kelley *et al.,* 1994a). In this respect, iCT-containing PNE cells are hyperplastic in some chronic lung diseases (Becker *et al.,* 1980b, 1981; Johnson *et al.,* 1988). Similar phenomena have been induced in experimental animals (Linnoila *et al.,* 1984; Tabassian *et al.,* 1993). The authors have also encountered mildly increased levels of serum iCT in some patients with chronic intestinal disorders (e.g., regional ileitis). Insufficient studies have been performed to fully characterize the nature and distribution of the precursor forms secreted in extrathyroidal neuroendocrine cell hyperplasia.

Medullary Thyroid Cancer

The measurement of serum mature CT to detect and to follow the course of the neoplasm of the thyroid C cells, MTC, has proven to be a very important clinical procedure. In addition, it has had considerable usage to detect persons with the familial forms of MTC [although its use for this latter indication has been much diminished by the advent of its identification by RET protooncogene mutations (Mulligan *et al.,* 1995)]. The use of region-specific antisera and separatory techniques has demonstrated that MTC secretes

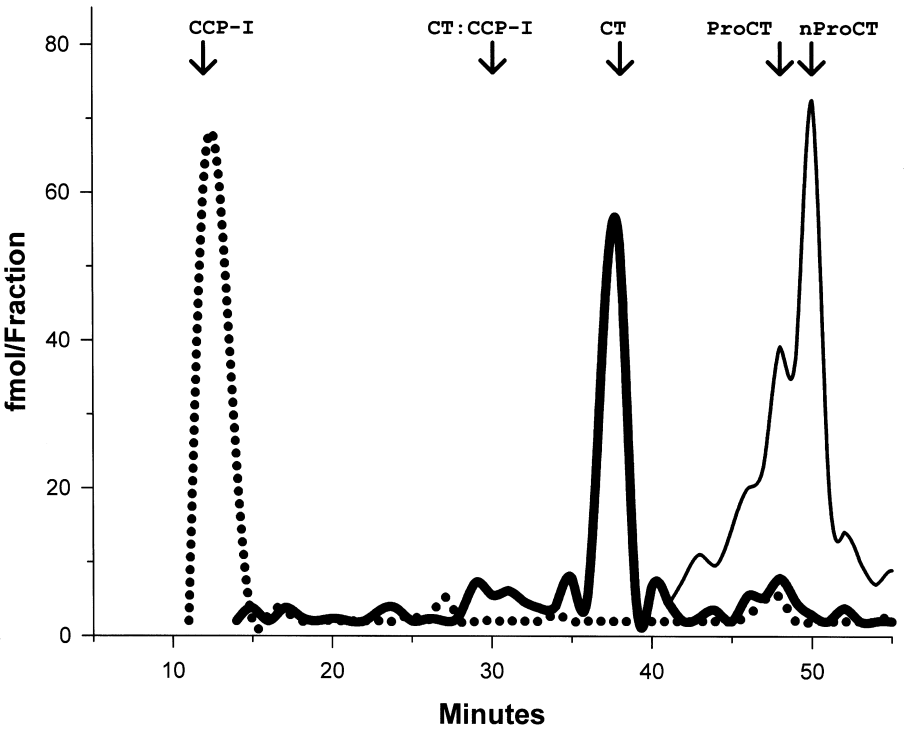

Figure 7 Procalcitonin and its component peptides in normal human serum. CT radioimmunoassay (thick line), CCP-I radioimmunoassay (dashed black line), and nProCT radioimmunoassay (thin line). Modified from Snider *et al.* (1997).

large amounts of high molecular weight forms of the hormone (i.e., CTpr) as well as considerable quantities of mature CT (Becker *et al.,* 1978; Ham *et al.,* 1980; Snider *et al.,* 1977) (Fig. 8).

Extrathyroidal Neuroendocrine Tumors

Two neoplasms of the lung, the highly malignant small cell lung cancer and the more benign bronchial carcinoid, are putative tumors of the PNE cells. Usually they secrete iCT (Becker and Gazdar, 1983, 1985). In these latter tumors, although serum levels of mature CT are increased, the proportion of high molecular weight forms usually is greater than in MTC (Becker *et al.,* 1978, 1983; Bertagna *et al.,* 1978; Nylen *et al.,* 1987). Other neuroendocrine tumors also may secrete iCT (e.g., pheochromocytoma, and pancreatic islet cell tumor).

Nonneuroendocrine Tumors

Increased levels of serum CTpr may occur in patients with nonneuroendocrine tumors. In such patients [e.g., epidermoid carcinoma of lung, breast cancer (Silva *et al.,* 1979)], it seems likely that hyperplasia of adjacent or intermixed neuroendocrine cells may play a role in this phenomenon (Kelley *et al.,* 1994).

Profile of Calcitonin Precursors Secreted by Tumors

Studies of patients with increased serum iCT due to various forms of neoplasia (Becker *et al.,* 1978; Snider *et al.,* 1977) demonstrated substantial amounts of high molecular weight iCT, which has been positively identified as CTpr (i.e., procalcitonin, nProCT, CT:CCP-I, CCP-I), as well as the mature CT hormone (Conton *et al.,* 1988; Ghillani *et al.,* 1989). Nearly always, the ratio of three of these large molecular weight CTpr (i.e., procalcitonin, nProCT, and CT:CCP-I), as compared with mature CT, is high (Becker *et al.,* 1978). Thus, in all of these tumors, synthesis and secretion of CTpr are increased, and posttranslational processing is incomplete. On a clinical level, when diagnosing or following MTC, a mature CT assay should be employed. However, when following the course of other neuroendocrine malignancies (e.g., small cell cancer of lung, carcinoid tumor) or of a nonthyroidal nonneuroendocrine malignancy (e.g. epidermoid carcinoma of the lung), an assay for CTpr should be used.

Increased Serum Calcitonin Precursors in Severe Inflammation–Infection–Sepsis

Marked insults, such as burns, trauma, surgical procedures, pancreatitis, or bacterial infections, commonly induce a hypersecretion of various proinflammatory cytokines,

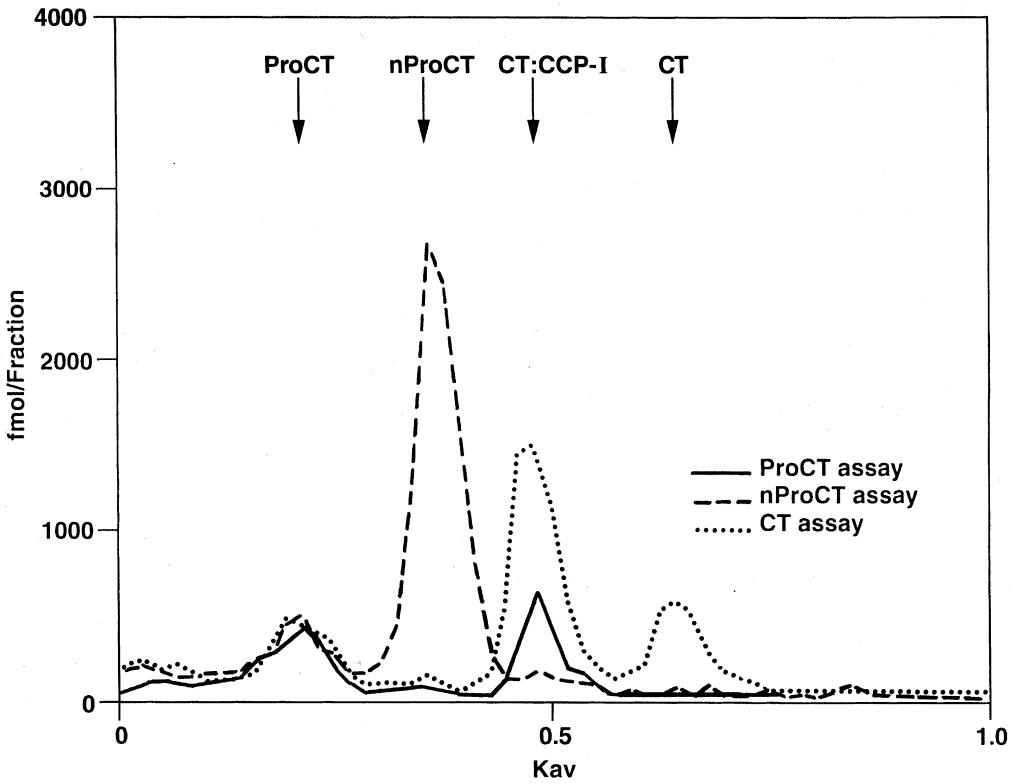

Figure 8 Gel filtration of serum of MTC patient. Note that the peak corresponding to the elution position of the CT:CCP-I peptide does not cross-react equally in both CT and ProCT assays. This may be due to the presence of some CT dimer (Tobler *et al.,* 1983), as it has a similar molecular mass.

arachidonic acid metabolites, and other humoral substances that cause a syndrome of systemic inflammation: vasodilation, chemoattraction of hematopoietic cells, activation of macrophages, and capillary endothelial leakage. The appellation of "sepsis" is given to this clinical condition (Bone, 1995). In severe cases, this may lead to "multiple organ dysfunction" characterized by myocardial insufficiency, circulatory hypoperfusion, hypoxemia, cerebral obtundation, renal failure, coagulopathy, and shock. Essentially, the patient becomes severely ill due primarily to a hyperresponsivity of the humoral reaction to the initial injury.

Calcitonin Precursors as a Marker for Severe Systemic Inflammation–Infection–Sepsis

In the United States, sepsis afflicts one-half million persons yearly and the mortality rate is 30 to 40%. Sepsis is defined as the systemic response to an injury that often is infectious (Bone, 1995). The systemic inflammatory response to an infection or marked injury originates from the elaboration by the host of a multitude of pro- and anti-inflammatory cytokines. Several studies over the past two decades have indicated that CTpr, including the ProCT prohormone, are increased markedly in severe inflammation and are often further augmented during microbial infection (Nylén *et al.,* 1992; Assicot *et al.,* 1993). In sepsis, circulating levels may be increased hundreds to thousands fold. In fact, CTpr are the most reliable markers of sepsis on an intensive care unit; they tend to correlate both with severity of illness and mortality and can be used to study the daily clinical course of the patient. Importantly, because of incomplete processing, there is very little or no elevation of serum mature CT. Assays for CTpr have become the best clinical marker for the presence, severity, and course of these conditions (Whang *et al.,* 1998; Müller *et al.,* 2000). The principal peptide components, which are increased, include the intact ProCT, the free nProCT, the free conjoined CT:CCP-I, and free CCP-I (Figs. 9 and 10). It is best to use an assay that measures more than one CTpr (e.g., Nylen *et al.,* 1992; Assicot *et al.,* 1993) because of the varying profiles of the increases occurring in the serum among different patients (Snider *et al.,* 1997).

The most proximal stimulus to increased CTpr in these conditions may be endotoxin, which, in turn, stimulates an increase of tumor necrosis factor α (TNF-α) and other cytokines. Indeed, in humans and experimental animals, injection of endotoxin or TNF-α results in a prolonged increase of serum CTpr (Preas *et al.,* 2001; Whang *et al.,* 2000). The initial hyperendotoxinemia may result from gram-negative bacterial infection or from translocation across the bowel wall of the *Escherichia coli* (or its endo-

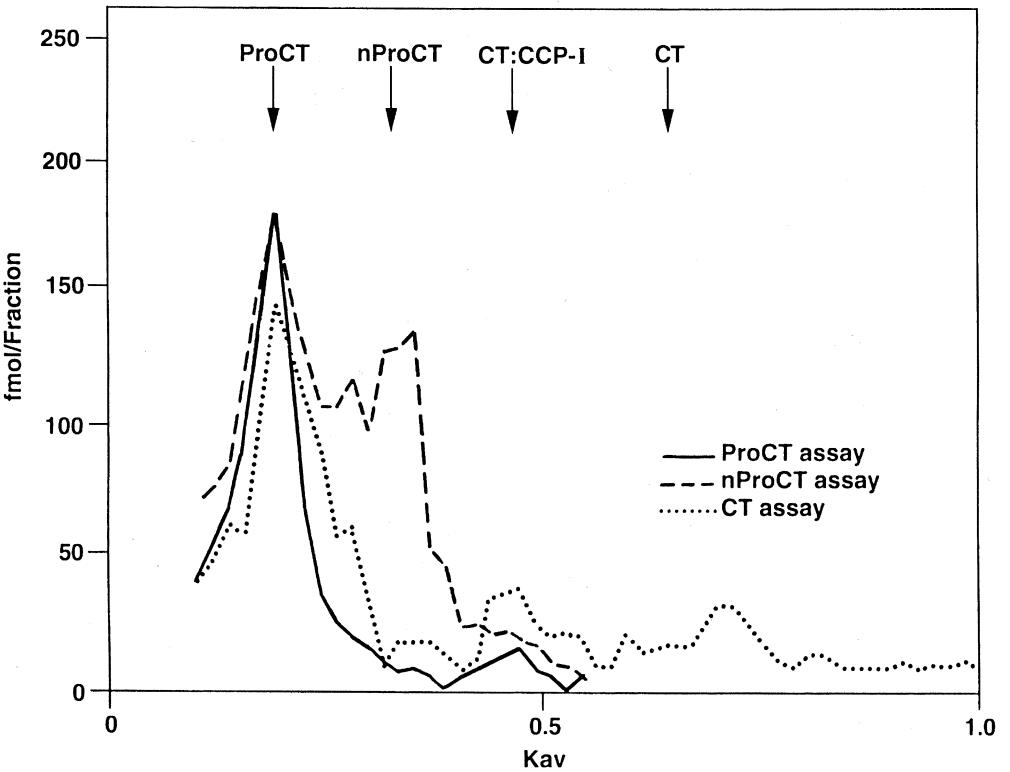

Figure 9 Gel filtration of serum of a septic burn patient with pneumonitis. No peak indicative of the presence of an uncleaved peptide-containing nProCT plus immature CT has been found in any of our studies to date. This suggests that the initial PC enzymatic cleavage may be at the basic amino acid cleavage site (Lys-Arg) situated between nProCT and the amino terminus of immature CT.

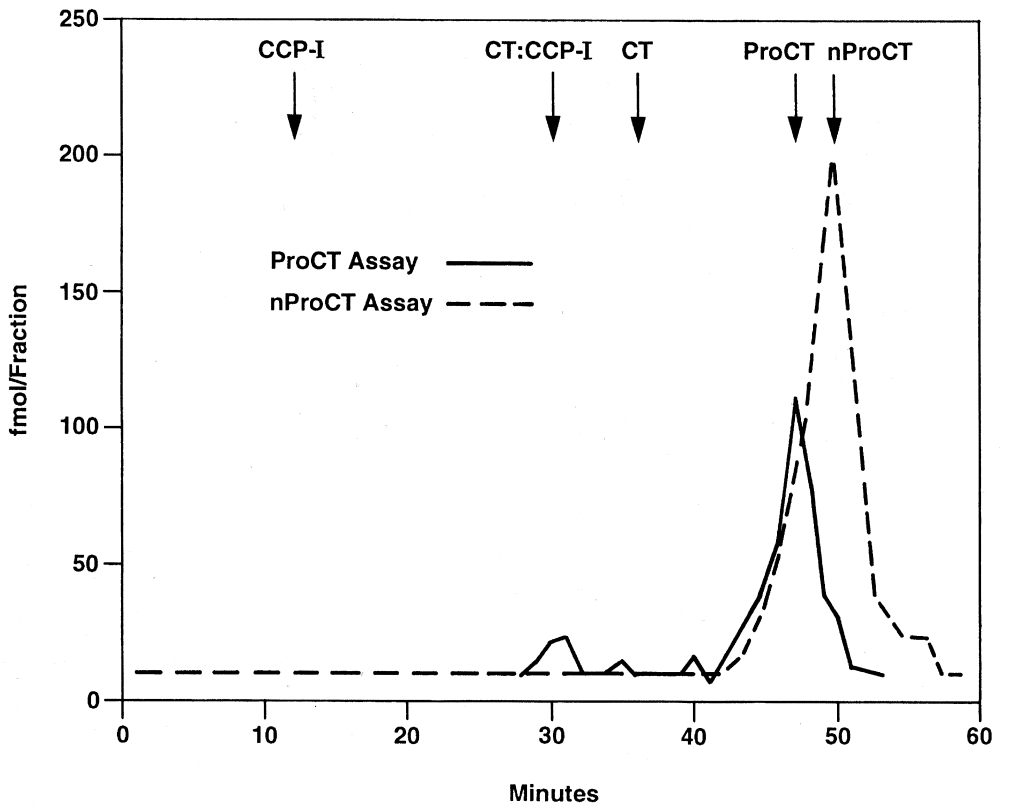

Figure 10 HPLC of serum from a burn patient with sepsis. Note the marked increase of nProCT and ProCT, as well as a moderate increase of the CT:CCP-I peptide.

toxin), which normally inhabit the gut (Ryan *et al.,* 1992). In this respect, burns and other severe injuries are known to induce such a translocation. However, sepsis induced by gram-positive bacteria also leads to an increase in circulating CTpr levels. Thus, other microbial products induce CTpr production.

Origin of Increased Calcitonin Precursors in Severe Systemic Inflammation

Studies in septic hamsters, pigs, and humans have revealed detectable CT mRNA not only in the thyroid gland, but also in all extrathyroidal tissues studied (brain, gut, kidney, liver, lung, testes, muscle, etc.) In contrast, in control animals, CT mRNA is detectable only in thyroid (i.e., C cells), and lung (i.e., PNE cells). In septic humans and animals, *in situ* hybridization studies have demonstrated that multiple cell types within these organs and tissues are involved. Thus, in sepsis, the entire organism is transformed into an endocrine gland (Müller *et al.,* 2001a). A similar phenomenon has been found for the expression of CGRP and ADM, two other peptides of the CALC gene family (Müller *et al.,* 2001b). It appears that increased gene transcription is induced by microbial infection-specific sepsis response elements in the gene promotor. Thus, these findings indicate that calcitonin gene products can follow either a classical hormonal expression or, alternatively, a cytokine-like expression pathway. In this regard, these gene products are a prototype of "hormokine" mediators (Müller *et al.,* 2001a,b).

Procalcitonin as a Toxic Factor in Systemic Inflammation

The correlation between serum CTpr levels and the severity of systemic inflammation/infection/sepsis suggested that one of these peptides, in particular ProCT, might be contributing to the morbidity and mortality of the host. Indeed, injection of ProCT into septic hamsters was found to increase mortality greatly. In follow-up studies, immunoneutralization of ProCT with antisera raised to different regions of the molecule has improved the clinical course of experimentally induced sepsis markedly (i.e., severe peritonitis) in hamsters and pigs (Nylén *et al.,* 1998; Wagner *et al.,* 2000). Studies in the septic pig have demonstrated marked amelioration of the disturbed physiologic and metabolic parameters among treated animals. The mechanism of the toxicity of ProCT in sepsis is under study.

Calcitonin Precursors and the Constitutive Pathway

The extraordinarily augmented and ongoing hypersecretion of CTpr in the face of a normal or only slightly increased serum level of mature CT in severe systemic inflammation indicates not only hypersynthesis, but also suggests a marked shift to the constitutive pathway of secretion, resulting in an incomplete processing of precursors.

As mentioned previously, the *trans*-Golgi normally identifies and selects certain proteins to be routed to two different secretory pathways: regulated and constitutive. The constitutive secretory pathway is present in all cells. Here, the vesicles that bud off from the *trans*-Golgi are less dense than those of the regulated pathway, and they migrate rapidly to the plasma membrane (Burgess and Kelly, 1987). Hence, in contrast to the slow transit time of the dense vesicles of the regulated system, which await periodic stimuli by their appropriate secretagogues for their exocytosis, the constitutive pathway vesicles are involved in a nonstoring, continuous secretion of newly synthesized peptide(s); it is a bulk-flow system. The vesicles fuse continuously with the plasma membrane, and the extrusion of their hormonal material may be calcium independent (Ma *et al.,* 1995). In severe systemic inflammation, a choice by the *trans*-Golgi to route ProCT to the constitutive pathway may be the major factor determining the exceptional spectrum of secreted components. Experimentally, such a shift to constitutional secretion has been shown to occur by the induction of dysfunctional prohormone convertase enzymes (Jung *et al.,* 1993; Naggert *et al.,* 1995), by the loss of Golgi recognition molecules (Bershadsky and Futerman, 1994; Chung *et al.,* 1989), by diminished intracellular calcium (Ma *et al.,* 1995), by alkalinization of cytoplasmic organelles (Scheele *et al.,* 1994; Xu and Shields, 1994), or by depolarization of the plasma membrane (Mostov, 1995; Schneeberger and Lynch, 1992; Simons and Van Meer, 1988; Simons and Wandinger-Ness, 1990). In this regard, septic serum, as well as some cytokines, may damage plasma membranes (Madara and Stafford, 1989; Todd and Mollitt; 1994).

Calcitonin Gene-Related Peptides I and II

The CALC-I gene produces either CT or CGRP-I. This is accomplished by alternative processing of the primary RNA transcript (Fig. 1) (Amara *et al.,* 1982) (see Chapter 36). CGRP-I (also termed CGRP-α) (molecular mass 3789 Da) is a 37 amino acid hormone, which, in humans, differs from CGRP-II (or CGRP-β) (molecular mass 3794) by three amino acids (Steenbergh *et al.,* 1985) (Fig. 2). CGRP-II originates from the CALC-II gene, for which there is no alternative processing of the RNA transcript. As is the case for CT, there is a carboxyl-terminal amide and a disulfide bridge at the amino terminus. CGRPs are biosynthesized from a larger Pre-ProCGRP, which has an nProCGRP that is very similar to nProCT. In addition, within the ProCGRP, there is a small carboxyl-terminal peptide that flanks the CGRP molecule.

CGRPs are predominantly neuropeptides; they are found mostly in the brain, ganglia, spinal cord, and peripheral nerves. They also are found in C cells and in PNE cells of the lung, where often they coexist with CT. Serum

levels are detectable in normal persons, and although values may be increased by induced hypercalcemia, this does not seem to be a physiologically relevant response. Values are commonly increased in the serum of patients with MTC, often in those with small cell cancer of the lung (Kelley *et al.,* 1994b).

CGRP may cause a slight diminution of bone resorption, the physiologic relevance of which is unknown (Owan and Ibraki, 1994) (see Chapter 36). The hormone inhibits osteoclast bone resorption by inducing quiescence (Zaidi *et al.,* 1987). This inhibition is mediated in part via cAMP (Akopian *et al.,* 2000). CGRP increases the number of bone colonies in cultured rat bone marrow (Bernard and Shih, 1990). In this respect, CGRP has been shown to have direct effects on osteoblast-like cells (Drissi *et al.,* 1997). Interestingly, CGRP increases intracellular-free Ca^{2+} concentrations in CGRP receptor-positive osteosarcoma cells (Drissi *et al.,* 1999). This increase is not coupled to adenylate cyclase, suggesting a downstream involvement of phospholipase C. CGRP-containing nerves appear to play a role in bone remodeling (Konttinen *et al.,* 1996). Although CGRP administration induces hypocalcemia in some laboratory animals, it does not do so in the human.

CGRP is a potent vasodilator and may diminish blood pressure. In this regard, it is noteworthy that the CT/CGRP gene knockout mouse model has elevated baseline blood pressure (Gangula *et al.,* 2000). The hormone may play a role in pulmonary pathophysiology. For example, there is an increase of CGRP-containing PNE cells in infants with fetal and neonatal lung disease. Serum CGRP is increased slightly in sepsis. Undoubtedly, the major physiologic role of CGRP is that of a neuromodulator. There are many receptor sites for CGRP in the nervous system and in peripheral tissues. CGRPs are discussed in greater detail in Chapter 36.

Amylin

The third hormonal member of the CALC gene family of peptides is amylin (Fig. 2), a 37 amino acid peptide (molecular mass 3903 Da) that was isolated from amyloid deposits in an endocrine pancreatic tumor and also in patients with type 2 diabetes mellitus (Cooper, 1994) (see Chapter 36). Amylin is produced from the CALC-IV gene located on chromosome 12. The hormone, which is cosecreted with insulin from pancreatic β cells, has an anti-insulin action; it diminishes insulin-stimulated glucose utilization (Castillo *et al.,* 1995). It inhibits the synthesis of glycogen in muscle and increases glycogen synthesis in the liver. Within the brain, amylin has an anorectic effect and also inhibits gastric acid secretion and gastric emptying (Samson *et al.,* 2000). In the kidney, the hormone stimulates plasma renin secretion. In common with CGRP, amylin exerts a vasodilator action. Amylin has an anti-inflammatory action, as do CT and CGRP (Clementi *et al.,* 1995).

Amylin affects osseous metabolism (Chapter 36). Intravenous administration of this hormone in dogs and in humans with Paget disease causes hypocalcemia (Wimalawansa *et al.,* 1992). In the dog, this hypocalcemia is associated with hypercalciuria; however, the increased urine calcium is insufficient to cause the decrease of serum calcium (Miles *et al.,* 1994). Amylin inhibits bone resorption by inducing quiescence of osteoclasts. In neonatal mouse calvariae, it reduces both basal and parathyroid hormone-induced bone resorption (Alam *et al.,* 1993b). Interestingly, amylin also influences bone formation; *in vitro,* it stimulates osteoblast proliferation in mice and increases mineralized bone volume (Cornish *et al.,* 1995). In young rats, the daily subcutaneous administration of amylin increases bone volume; there is no effect on ionized calcium (Romero *et al.,* 1995).

Serum levels of amylin are elevated in some patients with insulin resistance, in obesity, and in hypertension. It is unknown whether amylin contributes to an insulin-resistant state or whether the formation of islet amyloid noted in type 2 diabetes participates in the pathogenesis of this disease (Tenidis *et al.,* 2000).

Adrenomedullin

Adrenomedullin is a bioactive peptide that was originally found in pheochromocytoma tissue (Fig. 2) This 52 amino acid peptide (molecular mass 6029) is also amidated at the carboxyl-terminal and possesses an intramolecular disulfide bond between cysteine residues at positions 16 and 21, thus forming a 6-residue ring structure (Ishimitsu *et al.,* 1994a). These characteristics, plus its moderate amino acid identity with CGRP and a slight amino acid identity with amylin, suggest that it may be considered part of the CALC gene family (Wimalawansa, 1997). As is the case for CALC-I, -II, and -III genes, its gene is found on human chromosome 11. In contrast to CT and CGRP, which possess a disulfide ring structure at the amino terminus, and in contrast to amylin, which has a disulfide ring nearly at the amino terminus, adrenomedullin contains an additional 15 amino acid residues that are situated on the amino terminus side of its disulfide ring. However, much of this latter peptide extension is not needed for hormonal bioactivity. Within the proadrenomedullin molecule, there is an N-terminal 20-residue peptide that may be biologically active (Shimosawa *et al.,* 1995).

Adrenomedullin is found in adrenal medulla, heart, lung, kidney, vascular smooth muscle, endothelium, and plasma (Hinson *et al.,* 2000). The hormone activates adenylate cyclase through a G protein-coupled mechanism and mobilizes intracellular Ca^{2+}. Various pharmacologic stimulants of its secretion include cortisol, aldosterone, II-1α, TNF-α and-β, and lipopolysaccharide (Sugo *et al.,* 1995).

Adrenomedullin is a potent vasorelaxant; it dilates the pulmonary vascular bed, increases local blood flow, and produces a dose-related hypotension. Other actions include bronchodilation, inhibition of ACTH release from the pituitary gland, decreased angiotensin-induced aldosterone release, and natriuresis (Kanazawa *et al.,* 1994; Petrie *et al.,*

Table I Affinity of the CT Gene Family of Peptides According to the Type of Receptor and to RAMP Expression

Calcitonin receptor	
RAMP-1	CGRP and amylin; CT slight
RAMP-2	Calcitonin; CGRP and amylin slight
RAMP-3	Amylin; CGRP slight
Calcitonin receptor-like receptor	
RAMP-1	CGRP
RAMP-2	Adrenomedullin
RAMP-3	Adrenomedullin

2000). In the brain, adrenomedullin exerts vasorelaxant and antidipsogenic effects and may function as a neurotransmitter–neuromodulator.

In both *in vivo* and *in vitro* studies, adrenomedullin has been found to be a potent stimulator of osteoblasts (Cornish *et al.,* 1997). This has been postulated to play an important paracrine role in bone growth and maintenance (Hinson *et al.,* 2000). The effect of the hormone on the osteoclast is unknown.

Moderate increases of serum ADM occur in renal failure and in sepsis. The increased serum levels noted in patients with hypertension perhaps exert a protective role in this condition (Ishumitsu *et al.,* 1994).

Receptors of the Calcitonin Gene Family

Bioactivity of the peptides of the CT gene family is exerted by binding to their receptors. These receptors are G protein coupled; guanidine nucleotide guanosine triphosphate mediates their functions by binding to specific mediator proteins. There are two subgroups of receptors for the CT gene family: CT receptors (CRs) and CT receptor-like receptors (CRLRs) (see Chapter 34). Each of the CT gene family of peptides binds with differing affinities to these receptors. Consequently, some of their bioeffects overlap.

The complicated and unique overlapping of receptivity of CRs and CRLRs is due to their multipotentiality. Accessory proteins act upon these receptors, thus altering their specific responsivity and hence the physiologic profile of action of the CT gene peptides. These accessory proteins, which are called receptor activity-modifying proteins (RAMPs), alter the phenotype of the receptors, act on CRs by modification of its gene expression, and act on the CRLR by influencing transport to the plasma membrane. The presence, concentration, and/or timing of one or more of the three RAMPs (RAMP-1, -2, and -3) determines the specific cellular phenotype of the receptor that is ultimately expressed on the cell surface (Table I) (McLatchie *et al.,* 1998; Sumpe *et al.,* 2000; Martinez *et al.,* 2000). The profile of RAMP expression and activity is altered by the local milieu and is subject to humoral influences (Frayon *et al.,* 2000). This extraordinarily elegant system allows for a diversification of receptor function, thus modulating the action of the CT gene products according to ambient needs. Perhaps other receptors for individual members of the calcitonin gene family remain to be cloned.

Conclusion

CT was discovered in the early 1960s (Copp *et al.,* 1961; Hirsch *et al.,* 1963), at which time it was assumed to be a single hormone with a yet to be determined role in human physiology. Since then, it has been found to be only one peptide among a vast array of related circulating peptides comprising precursors, known hormones, and potential hormones. The discovery of this broad calcitonin gene family of peptides should provide great insight into the physiology of the human in health and disease.

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CHAPTER 36

Amylin and Calcitonin Gene-Related Peptide

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Introduction

Calcitonin gene-related peptide (CGRP) and amylin are homologous 37 amino acid peptides, the genes for which have a common ancestral origin (see Fig. 1). Both have been reviewed recently (Wimalawansa, 1997). They have in common a 6 amino acid ring structure at the N terminus created by a disulfide bond between positions 2 and 7. In addition, C termini are amidated. CGRP-1 is generated by alternative processing of mRNA from the calcitonin gene, located on the short arm of chromosome 11. This gene has six exons, the first four of which produce the mRNA for the precursor of calcitonin, preprocalcitonin. This is subsequently converted to calcitonin itself and N- and C-terminal flanking peptides. An alternative mRNA for the CGRP precursor, preproCGRP, is formed from exons 1,2,3,5, and 6. This peptide is cleaved to produce an N-terminal flanking peptide similar to that from procalcitonin and CGRP-1 itself, which is coded by exon 5. The alternative splicing of calcitonin mRNA is tissue specific—in parafollicular cells of the thyroid, calcitonin mRNA is produced predominantly, whereas in the nervous system, CGRP-1 mRNA is produced predominantly. A second form of CGRP, CGRP-2, differs from CGRP-1 by only three amino acids in the human and one amino acid in the rat (see Fig. 1). It is produced by a separate gene also on the short arm of chromosome 11, thought to have arisen as a result of exon duplication (Zaidi *et al.,* 1987a). Both CGRPs have almost 20% homology with calcitonin, which is also amidated and contains a disulfide bridge.

Amylin has 43% sequence identity with CGRP-1, 49% with CGRP-2, and 13% with calcitonin in the human. It was originally isolated from amyloid deposits in the pancreases from patients with insulinoma or diabetes mellitus (Cooper *et al.,* 1987; Westermark *et al.,* 1987). Human amylin appears to be produced from a single gene on the short arm of chromosome 12, consisting of three exons. Like calcitonin and CGRP, amylin is synthesized as a prepropeptide, an 89 amino acid precursor that is subsequently processed to proamylin (67 amino acids) and hence to amylin itself. Amylin is the only known hormonal product of this gene.

Amylin is produced principally in β cells of the islets where its tissue content is less than 1% that of insulin. It is cosecreted with insulin, and evidence shows that insulin and amylin genes share transcriptional regulators (German *et al.,* 1992). Thus, hyperglycemia stimulates amylin secretion (O'Brien *et al.,* 1991), whereas hypoglycemia reduces it (Alam T *et al.,* 1992). Destruction of the β cell with streptozotocin reduces amylin secretion (Ogawa *et al.,* 1990). The excursion in circulating insulin levels following a glucose challenge appears to be greater than that seen with amylin (Mitsukawa *et al.,* 1990). Amylin secretion, however, may be more sustained following glucose administration (Mitsukawa *et al.,* 1990). In some experimental models, dissociation of the secretion of the two peptides has been achieved but this does not commonly occur *in vivo.*

Amylin has also been detected in tissues of the gastrointestinal tract, with tissue concentrations about 1% of those in the pancreas being found in the pyloric antrum (Nakazato *et al.,* 1989). Amylin or its mRNA has also been found in lung, dorsal root ganglion, hypothalamus, neuroendocrine tumors, and in an osteoblast cell line (Gilbey *et al.,* 1991).

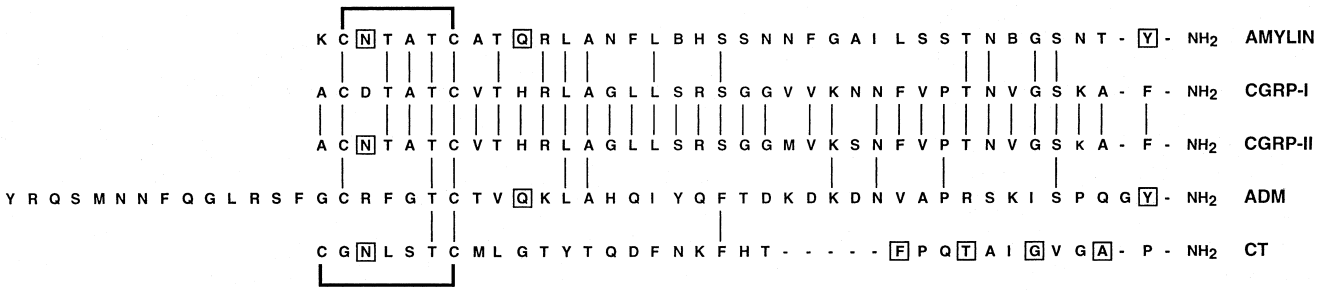

Figure 1 Amino acid sequences of amylin, CGRP, adrenomedullin (ADM), and calcitonin (CT) in humans. There is a disulfide bond in each peptide, as indicated by the dark line. Each peptide is amidated at its C terminus. Amino acids are identified as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine. Identical amino acids are indicated by vertical lines and squares.

Extraskeletal Actions of Amylin and CGRP

Fuel Metabolism

The effects of amylin and CGRP on fuel metabolism appear to be comparable. In skeletal muscle the primary effects are probably on glycogen synthesis (which is inhibited) and glycogenolysis (which is stimulated). Thus, muscle glycogen stores are decreased by amylin or CGRP, and this is accompanied by diminished glucose uptake and increased glycolysis, leading to increased lactate production. In the liver, glucose output is increased, probably as a result of increased gluconeogenesis. This may be contributed to the increased flow of lactate from skeletal muscle, but has also been observed in some studies of isolated hepatocytes, implying an additional direct effect of amylin on liver cell function. Thus, in both skeletal muscle and liver, amylin and CGRP act as noncompetitive antagonists of insulin and produce insulin resistance (Young *et al.,* 1992). Amylin does not influence adipocyte metabolism, however, so the insulin resistance it produces is selective and could lead to a redirection of fuel from carbohydrate storage in liver and muscle to triglyceride storage in adipose tissue.

As well as antagonizing the actions of insulin (Hettiarachchi *et al.,* 1997; Gebremedhin *et al.,* 1998), amylin also reduces its secretion (Furnsinn *et al.,* 1994), possibly by altering glucose sensing at the β cell (Wagoner *et al.,* 1993). Overexpression of the amylin gene results in the development of diabetes in transgenic mice (Verchere *et al.,* 1996), but paradoxically, an amylin analog (pramlintide) reduces postprandial blood glucose excursion, probably by retarding gastric emptying (Thompson *et al.,* 1997). In some species, amylin can, in certain conditions, polymerize to form the β-pleated sheets characteristic of amyloid. Amyloid is toxic to pancreatic β cells (Lorenzo *et al.,* 1994), and its presence in type 2 diabetes may contribute further to reduced insulin secretion in that condition. Amino acids 25–29 in human amylin appear to be critical for amyloid formation. There is no homology between amylin and CGRP in this region, which may account for the failure of CGRP to form amyloid deposits.

Cardiovascular System

CGRP is one of the most potent vasodilators known and its receptors are distributed widely throughout the vascular system. In some vessels, it appears to act directly on smooth muscle, whereas in others, CGRP receptors are on endothelial cells, which release a diffusible mediator, probably nitric oxide. CGRP-containing nerves are also present in the heart, and CGRP exerts both positive inotropic and positive chronotropic effects. The wide distribution of CGRP-containing nerves throughout the vascular system makes it likely that CGRP has an important role in the regulation of regional blood flow, including renal and cerebral blood flow. CGRP administration is associated with a fall in systemic blood pressure. Amylin shares the vasodilatory of CGRP but it is significantly less potent (Young *et al.,* 1993). Both peptides cause hypertrophy of cardiomyocytes (Bell *et al.,* 1995).

Nervous System

CGRP is distributed throughout the nervous system, being found in sensory, motor, and autonomic nerves, as well as in the hypothalamus, thalamus, and hippocampus. It is one of the most abundant peptides in the nervous system and it is frequently colocalized with other neuropeptides, such as substance P. This suggests that it may have a neuromodulatory role, acting in concert with other neurotransmitters. CGRP modulates pain perception and also appears to act centrally as an anorexiant (Lutz *et al.,* 1994).

Amylin also appears to have a role in the nervous system. It is found in some sensory neurons (Mulder *et al.,* 1995) and modulates appetite, memory, and motor function (Morley *et al.,* 1995; Clementi *et al.,* 1996). Through the formation of oligomers, it may produce neuronal apoptosis (Tucker *et al.,* 1998).

Other Tissues

These peptides increase renin secretion (Wookey *et al.,* 1996) and may also interact with other hormonal axes, as circulating amylin concentrations are higher in patients with

primary hyperparathyroidism (Valdemarsson *et al.,* 1996), and CGRP is a growth hormone secretagogue (Nakamura *et al.,* 1998). They reduce gastric acid secretion (Rossowski *et al.,* 1997) and inhibit gastric emptying (Kolterman *et al.,* 1995; Young *et al.,* 1995). There is also evidence that both peptides can modulate inflammatory responses (Clementi *et al.,* 1995).

Peptide Access to the Bone Microenvironment

The principal route by which amylin reaches bone is the circulation, which, in turn, derives its amylin from pancreatic secretion. Circulating amylin levels are of the order of 5 pmol/liter, rising to 10–20 pmol/liter following a meal. Amylin secretion is pulsatile, with peaks occurring at about 5-min intervals (Juhl *et al.,* 2000). Levels are probably higher in obese subjects and those with type 2 diabetes (Butler *et al.,* 1990; Mitsukawa *et al.,* 1990; Hartter *et al.,* 1991; Sanke *et al.,* 1991; Reid *et al.,* 1993), but appear to be decreased by leptin (Karlsson *et al.,* 1998). There is one report of amylin production from a human osteoblast-like cell line (Gilbey *et al.,* 1991), raising the possibility of amylin production locally within the bone microenvironment, but we have been unable to confirm the presence of amylin mRNA in primary rat osteoblasts (D. Naot *et al.,* unpublished data).

Early reports suggested that CGRP circulated in concentrations of 30–40 pmol/liter (Zaidi *et al.,* 1986; Schifter, 1991), but it has been suggested more recently that the concentration is closer to 1 pmol/liter (Born *et al.,* 1991). Circulating concentrations are increased by sex hormone replacement therapy in postmenopausal women (Spinetti *et al.,* 1997). Some circulating CGRP may be secreted by the parafollicular cells of the thyroid and the balance is released by nerve endings. It is likely that bone may be exposed to significantly higher concentrations of CGRP as a result of local release of CGRP from nerve terminals. Sensory nerve fibers containing CGRP are widely distributed in bone, including bone marrow (Bjurholm, 1991; Hukkanen *et al.,* 1992; Ahmed *et al.,* 1994). Innervation is richest at the epiphysis and periosteum (Hill and Elde, 1991). When defects are created surgically, the development of CGRP-containing nerves is noted several days later, often in association with new blood vessels (Aoki *et al.,* 1994), suggesting a role in callus formation and bone healing. Similar responses are seen following fractures (Hukkanen *et al.,* 1993). It is interesting to note that CGRP-containing nerves are also seen in the growing deer antler (Gray *et al.,* 1992) and surrounding developing teeth (Fristad *et al.,* 1994). It is possible that CGRP aids bone growth in all these circumstances through its direct effects on osteoblast function. The intimate association of these nerves with blood vessels suggests that they may also have a role in regulating blood flow to sites of bone healing or growth.

There has been a report of CGRP mRNA in osteosarcoma cell lines and in human osteoblasts, raising the possibility that this peptide is produced locally in bone (Drissi *et al.,* 1997).

Effects on Osteoclasts

CGRP

Following the discovery of CGRP, its common origin and sequence homology with calcitonin led to an investigation of its effects on bone resorption. This was first approached by injection of the peptide into intact animals (Tippins *et al.,* 1984). Tippins and colleagues (1984) found that CGRP had a calcitonin-like effect in the rat and rabbit, lowering circulating calcium concentrations. In the rabbit, it was approximately equipotent with calcitonin, although in the rat, concentrations 100- to 1000-fold higher than those of calcitonin were required to produce hypocalcemia. However, in the rabbit, higher concentrations of CGRP produce hypercalcemia. In the chicken, the peptide causes only hypercalcemia.

These results led to more detailed assessments of CGRP effects on bone resorption. Yamamoto *et al.* (1986) studied the effect of human CGRP on ^{45}Ca release from prelabeled neonatal mouse calvariae (Fig. 2). CGRP produced a comparable degree of inhibition of both basal and parathyroid hormone-stimulated resorption, but the half-maximal concentration of CGRP was 500-fold higher than that for human calcitonin. Others have produced similar results in fetal rat bone organ cultures (D'Souza *et al.,* 1986; Roos *et al.,* 1986; Tamura *et al.,* 1992; Zhang *et al.,* 1994). The antiresorptive effect of CGRP blocks the stimulation of bone resorption produced by a variety of osteolytic factors.

Studies of disaggregated neonatal rat osteoclasts have confirmed that both CGRP-1 and CGRP-2 directly inhibit bone resorption in these cells (Fig. 3). These two peptides are equipotent, but the effect requires nanomolar concentrations, in contrast to calcitonins, which require only picomolar

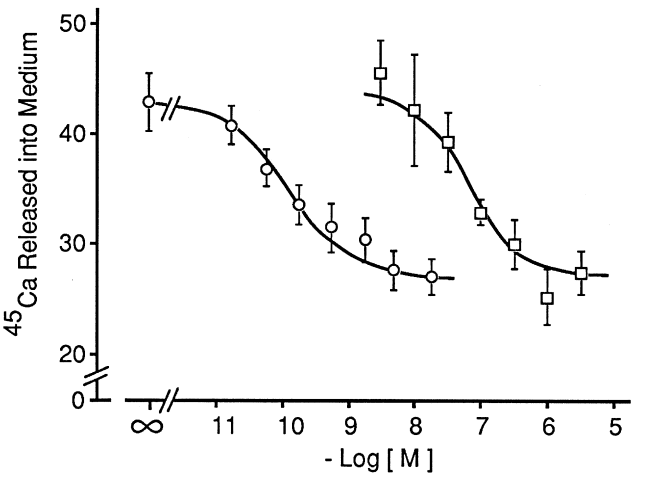

Figure 2 Effect of human calcitonin and human CGRP on PTH-stimulated bone resorption in neonatal mouse calvariae. Data are mean ±SE, $n = 6$. From Yamamoto *et al.* (1986), with permission.

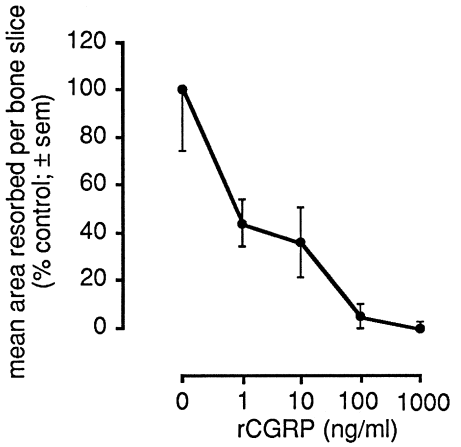

Figure 3 Effect of rat CGRP on bone resorption by isolated rat osteoclasts; 1 ng/ml CGRP = 0.26 nmol/liter. From Zaidi *et al.* (1987), with permission.

concentrations to produce comparable inhibition (Zaidi *et al.,* 1987b,c). More recent studies in isolated osteoclasts have further characterized the effects of CGRP on these cells (Alam *et al.,* 1991, 1993a,b). It inhibits osteoclastic bone resorption by inhibiting cell motility, probably via cylic AMP production, as this effect is reproduced by forskolin. Inhibition of cell motility is blocked by the CGRP fragment, CGRP-(8-37). Osteoclast retraction, which appears to be mediated by changes in intracellular calcium, is not seen with CGRP, although it is produced by calcitonin. These data imply that CGRP activates only the cyclic AMP intracellular messenger system, whereas calcitonin also acts on osteoclasts via changes in intracellular calcium. This may contribute to the greater potency of calcitonin in inhibiting bone resorption.

CGRP has also been shown to be active in other osteoclast models. Tamura *et al.* (1992) demonstrated cyclic AMP production in osteoclast-like multinucleated cells formed in cocultures of mouse osteoblasts and bone marrow cells in the presence of calcitriol. Concentrations 60-fold higher than those of human calcitonin were required, and CGRP in high concentrations displaced calcitonin from its specific binding site.

Evidence also suggests that CGRP may act on osteoclast precursors. Specific binding of CGRP to mouse bone marrow cells (Mullins *et al.,* 1993) and macrophages has been demonstrated, and CGRP inhibits the development of osteoclasts in macrophage–osteoblast cocultures (Owan and Ibaraki, 1994). We have addressed this question in cultures of mouse bone marrow, where CGRP inhibits the formation of mononuclear cells staining with tartrate-resistant acid phosphatase, as well as inhibiting the subsequent fusion of these cells to form multinucleated osteoclasts. Similar effects were seen with calcitonin at 1000-fold lower concentrations (Cornish *et al.,* 2001). Akopian *et al.* (2000) have reported reduced formation of human osteoclasts following treatment with CGRP. These data suggest that CGRP binds

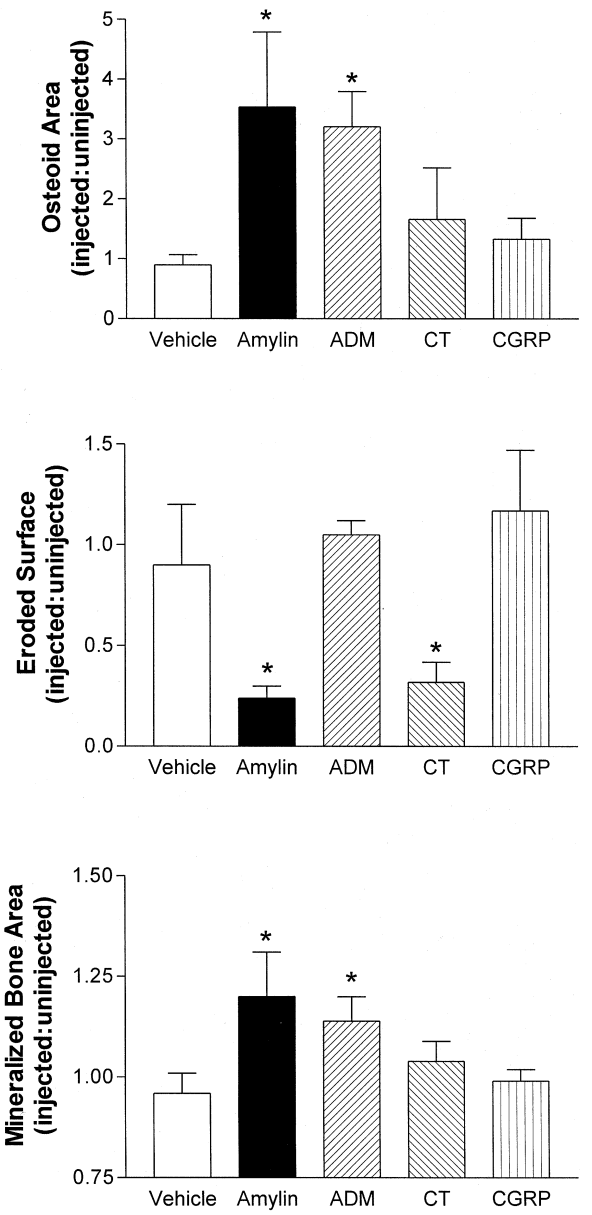

Figure 4 Comparison of the *in vivo* effects of rat amylin, human adrenomedullin, rat calcitonin (CT), and rat CGRP on bone histology in adult mice. Animals were injected daily with 4.1×10^{-9} mol of each peptide over the periosteum of one calvaria for 5 days and then sacrificed 1 week later. Data are expressed as the ratio of each index measured in the injected hemicalvaria to that measured in the contralateral, uninjected hemicalvaria. $n = 5$ in each group. Data are mean ±SE. Significant differences ($p < 0.05$) between injected and uninjected hemicalvariae are indicated by asterisks. From Cornish *et al.* (1999d), with permission.

specifically to osteoclast precursors and regulates osteoclast development.

Despite this consistent evidence of an effect of CGRP on osteoclastic resorption *in vitro*, a study of *in vivo* injection of CGRP over the calvariae of adult mice detected no significant inhibition of resorption (Cornish *et al.,* 1995) (Fig. 4). However, Valentijn *et al.* (1997) have demonstrated incomplete suppression of post-ovariectomy increases in bone

resorption in rats with CGRP treatment. This required the use of CGRP in a dose 500 times higher than that of calcitonin, which produced a greater therapeutic effect. Thus CGRP is antiresorptive *in vivo*, but its low potency suggests that this is unlikely to contribute to normal skeletal physiology except, possibly, in the region immediately adjacent to CGRP-producing cells.

Amylin

Within a short time of the description of amylin, its effect on indices of bone resorption had been assessed in studies paralleling those just described for CGRP. Thus its hypocalcemic actions in rats (Datta *et al.,* 1989; MacIntyre, 1989; Zaidi *et al.,* 1990), rabbits (Datta *et al.,* 1989), and humans (Gilbey *et al.,* 1991b; Wimalawansa *et al.,* 1992) were reported (Fig. 5), and similar effects have been described in the goat (Min *et al.,* 1999). In the rat, 30-fold higher doses of amylin were required to produce comparable hypocalcemia to that from calcitonin, and human studies showed a similar trend. The effects of amylin on serum calcium in the rat, however, were significantly greater than those of CGRP (Zaidi *et al.,* 1990).

More detailed studies of the action of amylin on isolated osteoclasts have shown that its actions are qualitatively similar to those of CGRP. Thus amylin inhibits the motility of mature osteoclasts by way of increasing intracellular cyclic AMP concentration. It is 10-fold more potent than CGRP but an order of magnitude less potent than human calcitonin (Alam *et al.,* 1993b). Amylin does not produce the osteoclast retraction seen with calcitonin. In contrast, CGRP and amylin were equipotent in stimulating cyclic AMP production in osteoclasts produced by coculture of osteoblasts and bone marrow cells (Tamura *et al.,* 1992), although 60-fold less potent than human calcitonin. In the putative osteoclast precursor, the macrophage, amylin stimulated cyclic AMP production but was 100-fold less potent than CGRP (Owan and Ibaraki, 1994). This effect was blocked by the CGRP antagonist, CGRP(8-37). We have also studied the effect of amylin on osteoclast development in mouse bone marrow cultures (Cornish *et al.,* 2001). As with CGRP, there is an inhibition of both the formation of mononuclear osteoclast-like cells and the fusion of these cells.

Amylin has also been shown to reduce bone resorption in organ culture. It is approximately equipotent with CGRP in inhibiting calcitriol-stimulated resorption in fetal rat long bones (Tamura *et al.,* 1992). Amylin reduces both basal and parathyroid hormone-stimulated bone resorption in neonatal mouse calvariae, and cyclic AMP production is also stimulated in this model (Pietschmann *et al.,* 1993; Cornish *et al.,* 1994). In the studies of Cornish and co-workers (Fig. 6), inhibition of resorption was seen at concentrations as low as $10^{-9}M$.

These results should be interpreted in the light of the marked propensity for amylin to adhere to the surfaces of laboratory plasticware (Young *et al.,* 1992), suggesting that the actual concentrations of amylin in all *in vitro* experiments may be one to two orders of magnitude less than the amount added to the media. Thus, both osteoclast and calvarial studies imply that amylin may regulate bone resorption at physiological concentrations. This activity is dependent on the presence of the carboxyl-terminal amide group. In its absence, the potency of amylin in reducing osteoclastic resorption is comparable to that of CGRP (Datta *et al.,* 1989; Alam *et al.,* 1993b). Amlyin's inhibition of bone resorption in neonatal mouse calvariae only occurs with the intact molecule, in contrast to the situation with amylin action on osteoblasts. Amylin fragments, which act as antagonists in the osteoblast, do not modify the effect of amylin on osteoclasts (Cornish *et al.,* 1998b).

The effect of amylin on resorption *in vivo* has been studied histomorphometrically in several different models. Cornish

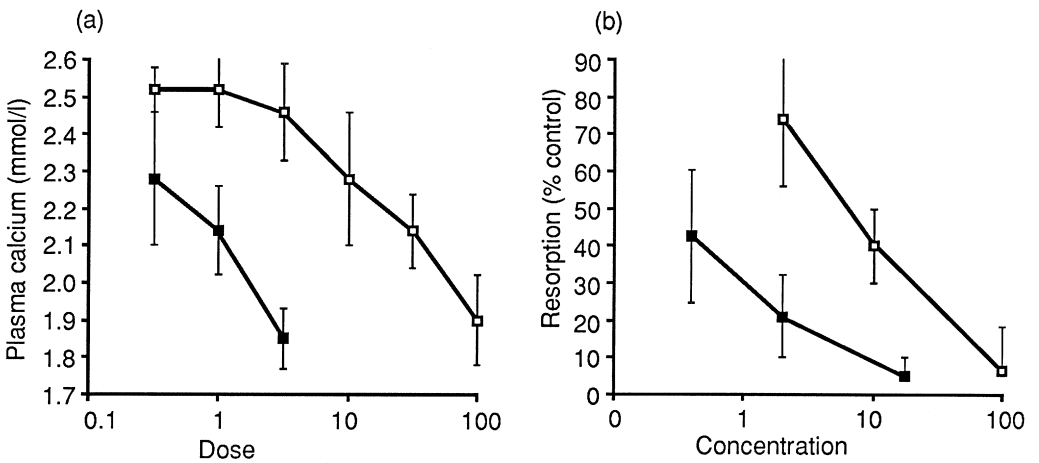

Figure 5 Effect of human amylin and human calcitonin on (a) plasma calcium in the rat and (b) bone resorption by isolated osteoclasts *in vitro.* Doses are given in pmol/rat (a) and in pmol/liter (b). Data are mean ±SE. From MacIntyre (1989), with permission.

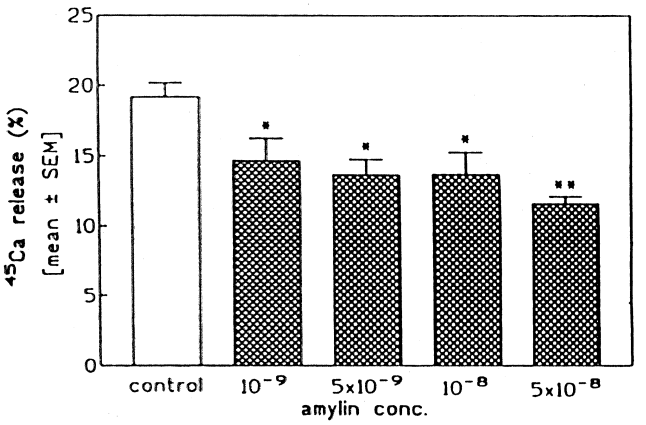

Figure 6 Effect of amylin on basal bone resorption in neonatal mouse calvariae. Based on data in Cornish *et al.* (1994).

et al. (1995) demonstrated 60–70% reductions in indices of bone resorption following daily local administration of amylin over the calvariae of adult mice. Very similar changes in resorption indices were seen following systemic administration of amylin to adult male mice for 1 month (Cornish *et al.,* 1998a), although the aminoterminal octapeptide of amylin is without effect on resorption in the same model (Cornish *et al.,* 2000). In ovariectomized rats, intact amylin reduces urinary excretion of deoxypyridinoline and reduces bone loss (Horcajada-Molteni *et al.,* 2000) (Fig. 8). In contrast, the earlier experiment of Romero *et al.* (1995), in which amylin was administered systemically to rats, showed only a nonsignificant trend toward reduced resorption, and Borm *et al.* (1999) found no change in resorption markers in 23 diabetic patients receiving the amylin analogue pramlintide for 1 year. The latter study needs to be interpreted with caution, as it used an amylin analog of unknown activity on bone and it was uncontrolled.

Effects on Osteoblasts

CGRP

At the time that the effects of CGRP as a calcitonin analog in bone were being investigated, data began to emerge suggesting that it may also have an effect on osteoblasts. Michelangeli *et al.* (1986) demonstrated that CGRP increased cyclic AMP formation in an osteogenic sarcoma cell line (UMR 106-01) that had an osteoblastic phenotype and was not calcitonin responsive. The same group subsequently studied mixed bone cell cultures obtained by sequential digestion of neonatal chicken, rat, or mouse calvariae and again demonstrated the presence of a cyclic AMP response to CGRP when none to calcitonin was detectable (Michelangeli *et al.,* 1989). Other studies have demonstrated CGRP binding (Seitz *et al.,* 1986; Datta *et al.,* 1990) and cyclic AMP production (Thiebaud *et al.,* 1991; Bjurholm *et al.,* 1992; Tamura *et al.,* 1992) in normal or transformed osteoblast-like cells. Aminoterminal peptides and the disulfide bridge were both necessary to maintain activity of CGRP on osteoblasts (Thiebaud *et al.,* 1991).

A variety of other effects of CGRP on osteoblasts have been demonstrated. Cornish *et al.* (1995) showed small increases in osteoblast proliferation in response to CGRP but only at concentrations 1000-fold higher than those of amylin required to produce an effect (Fig. 7). Gupta *et al.* (1994) demonstrated a stimulation of Na^+/H^+ exchange in UMR 106 cells. From these studies, they concluded that activation of more than one intracellular second messenger pathway is necessary to account for their findings and, in particular, that cyclic AMP generation alone is not an adequate explanation. Subsequently, Kawase *et al.* (1995) have demonstrated an effect of CGRP on intracellular calcium concentrations and on potassium fluxes and membrane polarization (Kawase and Burns, 1998) in the same cell line. In another osteoblast cell line, CGRP increases intracellular calcium concentrations but has no detectable effect on cyclic AMP (Drissi *et al.,* 1999).

There is also evidence that CGRP may act on preosteoblasts (Thiebaud *et al.,* 1991; Tamura *et al.,* 1992), influencing their development from precursor cells (Mullins *et al.,* 1993). Bernard and Shih (1990) have demonstrated that the number and size of bone colonies developing in bone marrow cultures are increased by CGRP and that systemic treatment with CGRP increases the number of bone colonies developing in marrow cultures.

The effects of CGRP on cytokine/growth factor production have been assessed. Sakagami *et al.* (1993) found that CGRP increased cyclic AMP and interleukin-6 production in a preadipocyte-like stromal cell line. CGRP also inhibited the proliferation of these cells. In primary osteoblasts, however, CGRP increased IGF-1, IGF-2, and osteocalcin mRNA, and this effect on IGF-1 has been confirmed by others (Vignery and McCarthy, 1996). In primary rat osteoblast cultures, CGRP has also been shown to substantially reduce tumor necrosis factor-α production and to weakly stimulate interleukin-6 production by these cells (Millet and Vignery, 1997). Thus CGRP may regulate the function of both precursor cells and mature osteoblasts by modulating the production of cytokines and growth factors.

The small amount of data regarding the effects of CGRP on osteoblast function *in vivo* is conflicting. Cornish *et al.* (1995) found no significant effect of local injection of CGRP on osteoblast indices in adult mice (Fig. 4). Valentijn *et al.* (1997) found no change in bone formation rates in ovariectomized rats treated with CGRP, but observed increases in bone formation indices, bone volume, and bone density of mice with osteoblasts overexpressing CGRP (Ballica *et al.,* 1999).

We have raised the possibility that these relatively weak effects of CGRP on osteoblast function may be mediated by receptors that have a higher affinity for amylin than for CGRP (Cornish *et al.,* 1999b). In cultures of fetal rat osteoblasts, amylin increased cell number, thymidine, and phenylalanine incorporation at 100-fold lower concentrations

than those of CGRP and its maximal effects were about twice as great as those of CGRP. There was no additivity between maximal doses of the peptides on these indices. The CGRP receptor blocker, CGRP-(8-37), completely blocked the effect of CGRP at blocker concentrations $\leq 10^{-9}\,M$. In contrast, the amylin receptor blocker, amylin-(8-37), completely blocked the effects of CGRP when the blocker was present in concentrations as low as $10^{-11}M$. In converse experiments studying the blockade of maximal doses of amylin, amylin-(8-37) $10^{-10}M$ was effective, whereas a 100-fold greater concentration of CGRP-(8-37) was necessary to achieve the same effect. It was concluded that amylin and CGRP probably act through a common receptor to stimulate osteoblast growth and that this receptor has a higher affinity for amylin than for CGRP.

Amylin

The binding of amylin to osteoblast-like cells was demonstrated soon after the discovery of the peptide (Datta *et al.,* 1990), and binding of amylin and stimulation of cyclic AMP production were also demonstrated in a pre-osteoblastic cell line (Tamura *et al.,* 1992). However, these studies did not demonstrate cyclic AMP production in response to amylin in primary osteoblast cultures, although CGRP produced cyclic AMP in these cells (Tamura *et al.,* 1992). Cornish *et al.* (1995) demonstrated stimulation of proliferation of fetal rat osteoblasts by amylin in concentrations as low as $10^{-11}M$, as shown in Fig. 7. Similar effects have been shown in human osteoblasts by Villa *et al.* (1997) and by ourselves (unpublished data). Amylin-(1-8) also stimulates osteoblast proliferation, although its half-maximally effective concentration is 10-fold higher than that of the intact peptide (Cornish *et al.,* 1998b). This peptide also stimulates thymidine incorporation in neonatal mouse calvariae (Cornish *et al.,* 1999c).

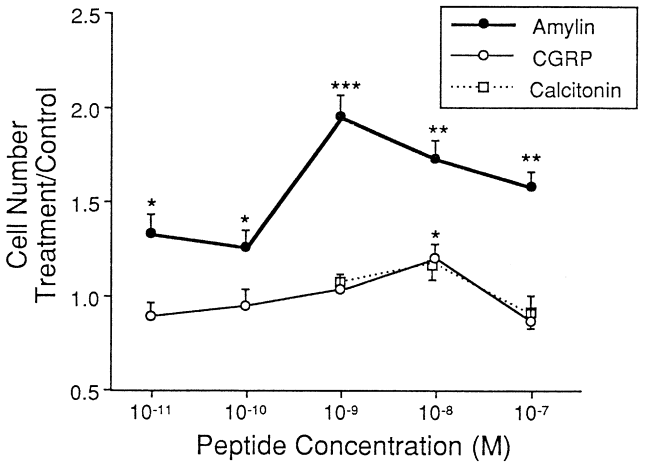

Figure 7 Dose dependence of the effects of rat amylin (●) rat CGRP-1(○), and rat calcitonin (□) on numbers of primary rat osteoblast-like cells in culture over 24 hr. $n = 6$ in each group. Data are mean ±SEM. Statistical significance of differences from control: $^{*}p \leq 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$. Reprinted with permission from Cornish *et al.* (1995).

In vivo studies of the effect of amylin on osteoblastic function are now available. Jacobs *et al.* (1992) commented on an increase in cortical endosteal osteoblast numbers in normal rats treated with amylin, but not in diabetic rats similarly treated. Subsequently, the same group showed a transient elevation of serum osteocalcin in rats given daily injections of amylin (Romero *et al.,* 1995). Cornish *et al.* (1995) (Fig. 4) have shown two- to four-fold increases in histomorphometric indices of osteoblast activity in adult mice to whom amylin was administered locally over the calvariae daily for 5 days, and we have now shown 30–100% increases in these indices following daily systemic administration of amylin

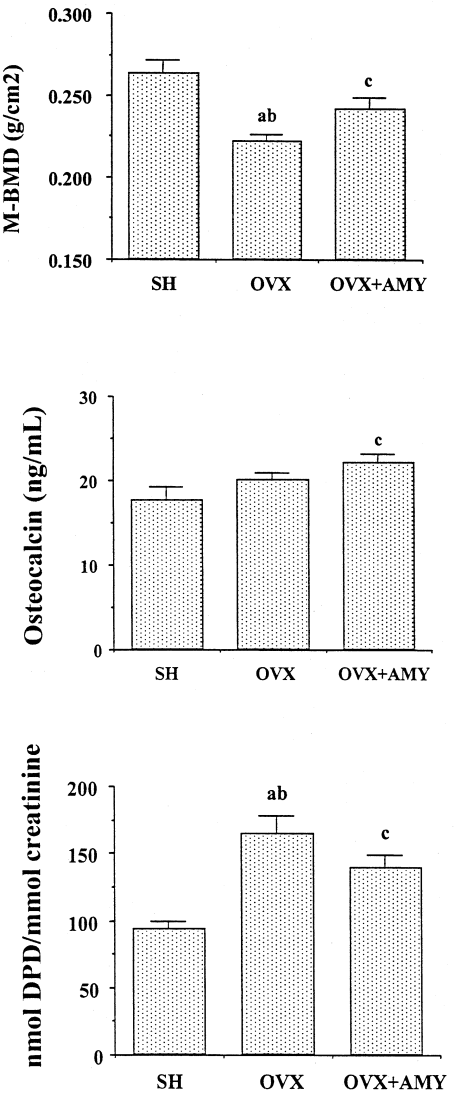

Figure 8 Effects of daily systemic administration of amylin (3 μg/100 g) to ovariectomized rats for 90 days. Indices assessed were distal metaphyseal bone mineral density of the femur (M-BMD), serum osteocalcin concentration, and urinary excretion of deoxypyridinoline (DPD). SH, sham operated; OVX, ovariectomized; AMY, amylin. a, significantly different from sham animals, $p < 0.01$; b, significantly different from amylin-treated group, $p < 0.05$; c, significantly different from sham, $p < 0.05$. Data from Horcajada-Molteni *et al.* (2000), with permission.

over 1 month (Cornish *et al.,* 1998a). Horcajada-Molteni *et al.* (2000) demonstrated increases in serum osteocalcin concentrations in ovariectomized rats treated systemically with amylin (Fig. 8), although the small human study with pramlintide did not detect any changes in osteoblast function (Borm *et al.,* 1999).

These *in vivo* studies have now been extended to amylin-(1-8). When administered by local injection over the calvariae of female mice, this peptide increased the double-labeled surface threefold. The effect was dose dependent from 0.4 to 40 n*M* and greater than that of an equimolar dose of hPTH-(1-34). The mineral apposition rate was increased by 40 n*M* amylin-(1-8) but not by hPTH-(1-34). Daily systemic administration of this peptide to sexually mature male mice for 4 weeks almost doubled histomorphometric indices of osteoblast activity (Cornish *et al.,* 2000). Thus, a number of studies have found evidence of an anabolic action of amylin and its amino terminus in osteoblasts.

Effects on Bone Mass

Studies in which the effects of these peptides on the total amount of mineralized tissue in bone has been assessed have mostly been referred to already. In the study of Cornish *et al.* (1995) involving local injection of intact amylin over the calvariae of adult mice, there was a substantial decrease in bone resorption, an increase in bone formation, and a 20% increase in mineralized bone area after only five daily injections (Fig. 4). An equimolar dose of CGRP in the same model produced no significant effects on any of these parameters. By comparison, calcitonin inhibited resorption but did not significantly affect formation and caused a small, nonsignificant increase in mineralized bone area.

Romero and colleagues (1995), administered amylin systemically to normal male rats for 18 days, producing no changes in histomorphometric indices of formation or resorption but increasing cancellous bone volume of the proximal tibia by 25%. In diabetic animals, a similar upward trend in bone volume was evident but was not statistically significant. In a similar study in normal male mice, Cornish *et al.* (1998a) showed increases in total bone volume in the proximal tibia of 70% (Fig. 9), and there were also increases in cortical width, tibial growth plate width, tibial length, body weight, and fat mass. A similar experiment using amylin-(1-8) increased bone volume by 36%, tibial cortical thickness by 8%, and resulted in increased bone strength as assessed by three-point bending (Cornish *et al.,* 2000). There were no between-group differences in soft tissue mass in the study with the amylin octapeptide, but growth plate width was again increased, suggesting an effect on chondrocytes also. This effect has now been confirmed *in vitro* in studies demonstrating the increased proliferation of canine and human chondrocytes in response to amylin, amylin-(1-8), and adrenomedullin (Cornish *et al.,* manuscript submitted). In the study of systemic administration of intact amylin to ovariectomized rats, reported by

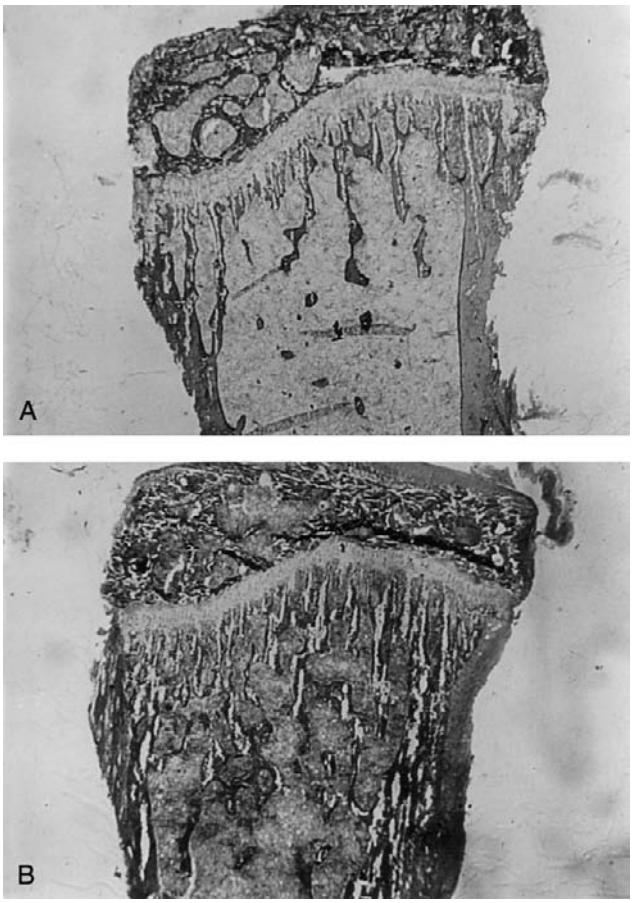

Figure 9 Photomicrographs of proximal tibiae of mice treated systemically with (A) vehicle or (B) amylin 10 μg/day for 4 weeks. Trabecular bone volume is increased 70% in amylin-treated animals. Reprinted from Cornish *et al.* (1998), with permission.

Horcajada-Molteni *et al.* (2000), both distal metaphyseal and total femoral bone densities were higher in animals receiving peptide (Fig. 8). The negative result of the uncontrolled study with pramlintide in patients with type I diabetes has already been referred to (Borm *et al.,* 1999).

In the study of Valentijn *et al.* (1997), ovariectomized rats were given daily subcutaneous injections of CGRP-α for 28 days. This treatment decreased bone resorption and caused a modest reduction in postovariectomy bone loss from 60 to 46%. In a study of transgenic mice overexpressing the CGRP gene in osteoblasts, Ballica *et al.* (1999) demonstrated a 5% increase in distal femoral bone density at the age of 12 weeks, confirming the potential impact of these peptides on bone mass, but also suggesting that the impact of amylin is greater. A quite different approach to determining the effect of CGRP on bone mass was used by Hill *et al.* (1991). Reasoning that most of the CGRP that gains access to bone does so via sensory nerves, they studied the effect of sensory denervation using capsaicin treatment in rats. This intervention produced no change in tibial histomorphometry although the osteoclast surface in the mandible was decreased. Whether this is attributable to loss of CGRP or to other effects of capsaicin treatment is not known.

Effects on Calcium Metabolism *in Vivo*

The most apparent effect of amylin and CGRP on systemic calcium metabolism is the induction of hypocalcemia, which has been demonstrated in a number of species, as discussed earlier. This is probably substantially attributable to inhibition of osteoclastic bone resorption. However, amylin, like calcitonin, may have a direct calciuretic effect on the kidney. Rat amylin binds to the renal porcine calcitonin receptor with an affinity comparable to that of porcine calcitonin itself. Both peptides comparably stimulate cyclic AMP production via this receptor (Sexton *et al.,* 1994). CGRP does not compete for binding to this receptor. However, in rat renal tubular membranes, CGRP and amylin stimulated cyclic AMP production with comparable half-maximal concentrations, and the effects of both peptides were blocked by CGRP(8-37) (Osajima *et al.,* 1995). Consistent with these results is the finding of Miles *et al.* (1994) that amylin infusion doubles urinary calcium excretion in dogs. However, the increased urinary loss of calcium accounted for less than 10% of the fall in serum calcium, suggesting that reduced osteoclastic resorption was the principal contributor to the hypocalcemic effect. These changes were accompanied by a doubling of PTH but no change in circulating calcitonin concentrations.

In the rat, CGRP, like amylin, is hypocalcemic with a potency slightly greater than that of amylin (Young *et al.,* 1993). However, in the chicken it has the opposite effect, elevating serum calcium within 20 min of administration (Ancill *et al.,* 1990; Bevis *et al.,* 1990). The mechanism of this effect has not been elucidated.

Adrenomedullin

It is not appropriate to discuss the actions of amylin and CGRP on bone without mentioning those of the closely related peptide, adrenomedullin, a 52 amino acid peptide (Kitamura *et al.,* 1993). It was originally identified in 1993 in a human pheochromocytoma and has since been found to be present in normal adrenal medulla and in many other tissues, including the atria, ventricles, endothelial cells, lungs, brain, kidneys, and bone (Ichiki *et al.,* 1994; Montuenga *et al.,* 1997; Hinson *et al.,* 2000). It circulates in picomolar concentrations in both rats and humans (Kitamura *et al.,* 1994; Hinson *et al.,* 2000). It is a potent vasodilator, acting directly on the renal, cerebral, mesenteric, pulmonary, and systemic circulations, including the vascular supply of the skeleton (Kato *et al.,* 1996). Its hemodynamic effects are probably mediated via receptors on vascular smooth muscle cells and possibly endothelial cells. Binding to a number of other tissues, including lung, heart, kidney, and hypothalamus, has also been demonstrated (Hinson *et al.,* 2000). Adrenomedullin shows approximately 20% sequence identity with amylin and CGRP and slightly less with calcitonin (Muff *et al.,* 1995). Like amylin and CGRP, it has an N-terminal ring created by a disulfide bond and is amidated at its C terminus. Adrenomedullin differs from the others in that it has a linear N-terminal extension, consisting of 15 amino acids in the human and 13 in the rat.

Because of its structural similarity to amylin and CGRP, the effects of adrenomedullin on the skeleton were assessed (Cornish *et al.,* 1997). At concentrations of 10^{-12} *M* and greater, adrenomedullin dose dependently increases cell number and [^{3}H]thymidine incorporation in cultures of fetal rat osteoblasts. This effect was also seen with adrenomedullin(15-52), adrenomedullin(22-52), and adrenomedullin(27-52) but not with adrenomedullin(40-52). Adrenomedullin also increases [^{3}H]thymidine incorporation into cultured neonatal mouse calvariae and phenylalanine incorporation into both isolated osteoblasts and calvariae (Cornish *et al.,* 1997), and we have also shown that it stimulates proliferation of primary cultures of human osteoblasts (J. Cornish *et al,* unpublished observations). Adrenomedullin injected daily for 5 days over the calvariae of adult mice increased indices of bone formation two- to threefold and increased mineralized bone area (Cornish *et al.,* 1997). When administered systemically to mice for 4 weeks, it produces very similar effects to those of amylin-(1-8). Like that peptide, adrenomedullin is without effect on osteoclast activity, and its effects on osteoblasts are blocked by amylin receptors blockers, such as amylin-(8-37). These similarities suggest that the actions of amylin and adrenomedullin on the osteoblast might share a common mechanism.

Receptors

In nonosseous tissues, there are believed to be separate specific receptors for amylin and CGRP, with some evidence pointing to more than one class of receptor for the latter. Amylin, CGRP, and calcitonin are able to displace each other from specific binding sites, implying significant cross-reactivity of each with the receptors of other peptides. Whether this leads to significant biological effects at the peptide concentrations found *in vivo* is unknown.

The fact that the actions of amylin on osteoblasts and osteoclasts can be dissociated, suggests that they may be mediated by different receptors. Data available at the present time are consistent with the actions of amylin and CGRP on bone resorption being mediated by the calcitonin receptor. As noted earlier, current data suggest that the actions of amylin, CGRP, and adrenomedullin on osteoblasts may all be mediated by a common receptor, which has a lower affinity for CGRP than for the other two peptides. We have shown that the proliferative effects of amylin and adrenomedullin are dependent on the presence of the IGF-1 receptor, although neither of these peptides appear to compete for binding to this receptor, implying a less direct mechanism for its involvement (Cornish *et al.,* 1999a).

Uncertainty still surrounds the identities of the receptors for this family of peptides in nonbone cells. A CGRP receptor was identified in 1995 by Kapas and Clark, but work by

McLatchie *et al.* (1998) casts doubt on the significance of this receptor. They suggest that the principal CGRP receptor is in fact the so-called calcitonin receptor-like receptor (CRLR). Cells expressing this receptor alone are relatively unresponsive to CGRP, but in the presence of a peptide they have termed receptor activity modifying protein-1 (RAMP1), the CRLR is both glycosylated and translocated to the cell surface, conferring CGRP sensitivity. Separate proteins, RAMP2 and RAMP3, interact with this receptor to produce an adrenomedullin receptor. Other groups have confirmed that these CRLR-based receptors account for most of the specific binding of adrenomedullin and CGRP in a variety of rat tissues (Chakravarty *et al.,* 2000). When RAMPs 1 or 3 interact with the calcitonin receptor, an amylin receptor is created (Christopoulos *et al.,* 1999; Muff *et al.,* 1999). Primary rat osteoblasts express mRNAs for all three RAMPs, the calcitonin receptor-like receptor, and the putative adrenomedullin receptor identified by Kapas *et al.,* (1995), but not that for the calcitonin receptor (Naot *et al.* 2000). Thus, the nature of the receptor mediating the osteoblast effects described earlier is uncertain, but it could be an adrenomedullin receptor.

The receptors referred to earlier are all seven transmembrane G protein-coupled receptors, thought to act via adenylyl cyclase and/or calcium-inositol signaling pathways. It should be noted that the effects of these peptides on cyclic AMP concentrations in osteoblasts are modest (in comparison with parathyroid hormone, for instance) and that amylin and adrenomedullin activate mitogen-activated protein kinase in osteoblasts (Abrahamsen *et al.,* 2000). Further work is needed to delineate fully the second messenger pathways mediating their effects in bone.

Si1gnificance in Calcium Metabolism

What role amylin and CGRP play in normal bone metabolism and bone pathology remains to be determined. It has been hypothesized that amylin secretion following a meal directs the absorbed calcium and protein from the meal into new bone synthesis by increasing bone growth at a time when the substrates are available (MacIntyre, 1989; Zaidi *et al.,* 1993). Amylin may also contribute to the relationship between body mass and bone density. Body mass, or more particularly fat mass (Reid *et al.,* 1992a,b), is the principal determinant of bone density in women. While this might be mediated to some extent by the effect of weight on skeletal load bearing and by adipocyte production of estrogen, neither of these explanations is adequate to explain the published results (Reid *et al.,* 1992a,b, 1993, 1994, 1995). Because both insulin and amylin are hypersecreted in obesity and because both may potentially act directly or indirectly to increase bone mass, they may contribute significantly to this relationship. Indeed, circulating insulin levels are directly related to bone density in normal postmenopausal women (Reid *et al.,* 1993), and because amylin is cosecreted with insulin, it would seem likely that a similar relationship for this peptide exists.

Because most of the CGRP gaining access to bone does so via sensory nerves, it is likely that this peptide is involved in the response of bone to injury and to other stimuli, such as exercise. The association of CGRP-containing nerves with healing and growing bone supports this contention.

The effects of both amylin and CGRP are, broadly, to increase bone formation and reduce bone resorption. As such, they are attractive candidates for the treatment of the generalized bone loss of osteoporosis and to promote healing of local bone defects. Their efficacy has now been demonstrated in several different animal models, although much remains to be done in determining which fragments or analogs are the most promising candidates and what their other effects might be. Further studies are under way that should determine whether compounds mimicking the effects of amylin and CGRP will find a place as therapeutic entities.

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CHAPTER 37

Estrogens and Progestins

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Introduction

Since the publication of the first edition of this book in 1996, the field of estrogen and progestin action has witnessed several major advances that impact bone, which are the emphasis of this chapter. Estrogen (E) is the major sex steroid affecting the growth, remodeling, and homeostasis of the skeleton, whereas the importance of progesterone (P) is less well defined. It is becoming increasingly clear that E regulates the processes of osteoblast (OB)-mediated bone formation and osteoclast (OC)-mediated bone resorption at multiple levels; including progenitor cell recruitment, proliferation, differentiation, and programmed cell death. Additionally, a second estrogen receptor distinct from the classical receptor has been identified, and loss-of-function mutations for these two receptor isoforms produce different skeletal phenotypes in mice. Taken together, these findings demonstrate that the effects of E on bone and cartilage are highly complex. Similar receptor isoforms for progesterone receptor have been identified in OBs, which also contribute to the varying actions of P on the skeleton. Added to these variables are the existence of steroid receptor coregulators; nuclear transcription factors that modulate steroid receptor interactions with the transcriptional machinery. Nevertheless, the actions of E and P at the molecular, cellular, and bone tissue level are currently being elucidated.

The topics covered in this chapter include estrogen and progesterone receptor structure and function, roles of the receptor isoforms and receptor coregulators, receptor isoform expressions in bone tissues, effects of E and P treatment on bone cells *in vitro* and on the skeleton *in vivo,* and consequences of estrogen and progesterone receptor gene deletions on skeletal structure and bone metabolism. Additionally, due to the increasing clinical and scientific interest in these hormones, the actions of E-related compounds [selective estrogen receptor modulators (SERMs), E metabolites, and phytoestrogens] on the skeleton and bone cells are discussed.

General Estrogen Actions on the Skeleton

Mechanism of Action of Steroid Hormones

Estradiol and progesterone are the principle circulating sex steroids in females, which also function in males. All steroids are thought to diffuse passively into cells and are retained intracellularly by complexing specific receptor proteins, which are steroid and target tissue specific. The steroid receptor (ER and PR) concentration in bone is low relative to reproductive tissues and suggests a more limited range of actions. Steroid hormone receptors are a subclass of a larger (100 member) family of zinc finger-containing, nuclear hormone receptors that function as transcriptional regulators (Tsai and O'Malley, 1994). The ligands are bound with high affinity (K_d 10^{-10} to 10^{-8} M), but reversibly, to their respective receptors. Binding to the receptors triggers a receptor "activation," which induces a conformational change, dissociation of accessory proteins, receptor dimerization and oligomerization, and, in some cases, posttranslational modifications such as phosphorylation. The activated receptors either bind directly to regulatory DNA elements (hormone response elements) in the promoters of target genes or bind indirectly with other DNA-binding transcription factors (e.g., AP-1 factors) via protein–protein interactions, which then bind to the promoter. Once bound to the DNA, directly or indirectly, the activated receptor complex recruits transcription factors and/or coregulator proteins, which in turn bind to the core proteins of the transcriptional

activation complex to regulate gene transcription (Bevan and Parker, 1999; McKenna *et al.,* 1999; Robyr *et al.,* 2000).

The diffusion of steroids into the cell and the formation of the ligand–receptor complex are rapid, occurring within minutes of the injection of the steroid into an animal. Following steroid–receptor complex formation (1–4 min posttreatment), this complex binds to nuclear acceptor sites (2–5 min). This nuclear binding apparently, directly or indirectly, regulates the transcription of "early" (5–30 min) or "late" (2–4 hr) genes. Changes in protein profiles due to changes in "late" gene expression begin to occur 6–8 hr posttreatment. The major physiological effects of steroids in reproductive and skeletal tissues begin to occur by 12 to 24 hr after steroid treatment. Cell and tissue responses that occur at 36 to 72 hr after treatment probably represent secondary effects of steroid regulated paracrine/autocrine factors. The 2 to 3-hr period elapsing between the steroid receptor binding to the nuclear acceptor sites and the delayed, but major, transcriptional response of the late structural genes has been termed the "lag" phase. This is a period during which early response genes, coding for transcription factors and other proteins, are activated to subsequently regulate late gene expression. The latter process has been termed "the cascade model of steroid hormone action" (Landers and Spelsberg, 1992).

Estrogen Receptor Isoforms

Receptor Isoforms

The discovery of a second isoform of the ER, termed ERβ, as the product of a different gene in addition to that of the first described and extensively studied ERα, has added a new level of complexity to our understanding of E action (Kuiper *et al.,* 1996; Mosselman *et al.,* 1996). As depicted in Fig. 1, ERα and ERβ possess a high degree of homology in their DNA-binding domains and a moderate degree of conservation in their C-terminal ligand-binding domains (Kuiper and Gustafsson, 1997). The former explains why the two receptor isoforms interact with the same DNA response elements and the latter explains why both isoforms bind many natural estrogens, SERMs, and antiestrogens often with the same, but sometimes different, affinities (Kuiper *et al.,* 1997). In contrast, ERα and ERβ are most divergent in their N-terminal A/B domains, which harbor the ligand-independent AF-1 transactivation function and which interact with proteins of the core transcriptional machinery to regulate gene expression. The molecular size and tissue/cell type distribution of ERα and ERβ also differ significantly. Concentrations of the β isoform are high in prostate, ovary, vasculature, brain, and bladder, but are low or absent in uterus, breast, kidney, pituitary, and epididymis (Kuiper *et al.,* 1996, 1997). Interestingly, the latter tissues contain high levels of the ERα species. In certain cell types, transfection-reporter gene analysis using an E-response element reporter gene construct indicates that ERβ is a weaker regulator of gene transcription than ERα (Kuiper *et al.,* 1996, 1997; Paech *et al.,* 1997; Hall and McDonnell, 1999). It has also been shown that in response to E treatment, ERα interacts with AP-1 factors and binds to AP-1 regulatory elements, a site where ERβ is inactive when complexed with E (Paech *et al.,* 1997). In contrast, ERβ activates AP-1 regulatory elements in response to treatment with SERMs, e.g., tamoxifen and raloxifene (Paech *et al.,* 1997). Similar results for ERα and ERβ with these ligands have also been noted for transactivation through Sp1 elements (Saville *et al.,* 2000). Studies indicate that ERβ may function as a transdominant inhibitor of ERα at subsaturating E levels, and that in some systems the partial agonist activity of tamoxifen manifested through ERα is completely abolished upon coexpression of ERβ (Hall and McDonnell, 1999).

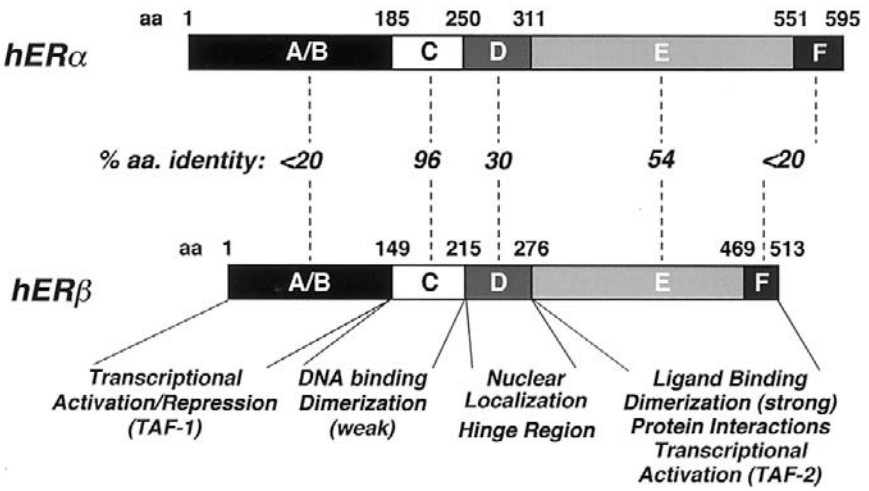

Figure 1 Model of human estrogen receptor isoforms, their domains, functions, and homologies to each other.

STEROID RECEPTOR COREGULATORS

The activity of steroid nuclear receptors is modulated by the family of steroid receptor coregulators, which is composed of coactivators and corepressors (Bevan and Parker, 1999; Robyr *et al.,* 2000; McKenna *et al.,* 1999). Each receptor isoform (ERα and ERβ, or A and B isoforms of the progesterone receptor, PR) adopts a unique conformation that is dependent on the particular isoform and the agonist/antagonist/mixed agonist nature of the ligand (Brzozowski *et al.,* 1997; Paige *et al.,* 1999). It is the unique conformation adopted by each receptor isoform on ligand binding that determines the relative interaction of the receptor with either coactivators or corepressors and thereby the differential transactivation of hormone-responsive promoters. Coactivators, when bound to "active" receptor conformations (usually formed with agonists), mediate favorable interactions with the basal transcriptional machinery, stabilize the preinitiation complex and, overall, stimulate gene transcription. Conversely, corepressors bind preferentially to "inactive" receptor conformations (usually formed with antagonists or unliganded receptor) and prevent the interaction of the receptor with coactivators, thus resulting in nonproductive transcription factor complexes, which suppress gene transcription.

As an example, the opposing transcriptional activities of the two isoforms of PR have been demonstrated to be due, on the one hand, to preferential binding of the coactivators GRIP1 and SRC-1 by agonist-bound PR_B and, on the other hand, by the ability of PR_A to bind the corepressor SMRT but not coactivators (Giangrande *et al.,* 2000). Given the weaker transactivating activity of ERβ (Hall and McDonnell, 1999), an analogous pattern of coregulator interaction may occur with ERα and ERβ. Interestingly, in the presence of mixed agonist/antagonists, when the receptor adopts conformations that are intermediate between active and inactive states, the relative level of coactivators to corepressors can determine transcriptional responses. In this regard, changing the coactivator/corepressor ratio has been shown to modulate ERE promoter activity in the presence of 4-hydroxytamoxifen and ERα (Smith *et al.,* 1997). Consequently, it is plausible that alterations in the expressions of the various coregulators in bone cells, e.g., during differentiation, would affect the responsiveness to E and P.

Expression and Distribution of ERα and ERβ in Bone Cells and Skeletal Tissue

OSTEOBLASTS AND OSTEOBLAST PROGENITOR CELLS

While the effects of E on bone growth and turnover are well documented, the inability to detect estrogen receptors in bone cells fostered the belief that E acts indirectly on bone via other calciotropic hormones, such as parathyroid hormone, 1,25-dihydroxyvitamin D_3, or calcitonin. However, in 1988, two groups reported the detection of high-affinity ERs (now known to be ERα) in cultured normal human osteoblastic (hOB) cells (Eriksen *et al.,* 1988), as well as human (TE85) and rat (ROS 17/2.8) osteosarcoma cell lines (Komm *et al.,* 1988). In additon, both groups were able to detect ER mRNA and E effects on endogenous gene expression in osteoblastic cells. Subsequently, others have reported the presence of ERα in human SaOS (Etienne *et al.,* 1990), mouse MC3T3-E1 (Masuyama *et al.,* 1992), and rat UMR-106-01 (Davis *et al.,* 1994) osteoblastic cells. Available evidence indicates that the expression of both ERα and ERβ increases during OB differentiation *in vitro*. Thus, in fetal rat calvarial-derived OB cultures, the level of ERα mRNA was initially low in proliferating cells but increased at the onset of alkaline phosphatase expression and progressively increased thereafter to reach a maximum level in fully differentiated OBs (Bodine *et al.,* 1998). In a human fetal OB line, expression of ERβ mRNA was also found to increase steadily as cells differentiated and then deposited a mineralized matrix (Arts *et al.,* 1997). However, the level of ERβ transcripts in primary rat calvarial cultures was maintained at a high level throughout differentiation (Onoe *et al.,* 1997). Overall, various sensitive techniques have demonstrated the presence of ERs in normal and transformed osteoblastic cells in culture from several species.

It remains uncertain whether osteoblast progenitor cells express ERα and/or ERβ and thus represent additional targets for E action. However, because the expression of both ER isoforms appears to increase during OB differentiation, the receptor levels in progenitor cells would be expected to be low. ERα mRNA has been detected in a murine bone marrow stromal cell line, and the levels were upregulated by 1,25-dihydroxyvitamin D3 (Bellido *et al.,* 1993). More recently, the expression of ERα mRNA was found to be increased upon glucocorticoid-induced differentiation of human bone marrow stromal cells (Oreffo *et al.,* 1999), consistent with findings from rat calvarial OBs. There have been few reports on the effects of E on OB progenitor cells. Although E modulates the expression of certain OB marker genes in an osteogenic murine stromal cell line (Mathieu and Merregaert, 1994), proliferation and osteogenic differentiation of primary rat bone marrow stromal cells were unaffected by E (Rickard *et al.,* 1995).

OSTEOCLASTS

E may also exert direct effects on cells of the osteoclast (OC) lineage. The expression of ERα mRNA and protein has been reported in isolated avian and human OCs and giant cell tumors of bone (Oursler *et al.,* 1991, 1994). The human leukemic cell line FLG 29.1, which can be induced by phorbol esters to cells exhibiting many features typical of OCs, was also shown to possess ERα and estrogen/raloxifene-binding sites (i.e., E receptors) (Fiorelli *et al.,* 1995, 1997). In addition, OC precursors present in murine hemopoietic blast cell cultures have been shown to express ERα mRNA (Kanatani *et al.,* 1998). The presence of ERβ in mature OCs or their precursors has not been reported.

Skeletal Distribution

Available evidence suggests that only a subset of osteogenic cells in bone tissue express ER. Although the distribution of ERα or ERβ in bone is incompletely known at present, the pattern of expression of the two isoforms appears to overlap considerably. Both ERα and ERβ have been localized to hypertrophic chondrocytes in growth plate cartilage (Ben-Hur *et al.,* 1993; Nilsson *et al.,* 1999), although ERα is additionally present in chondrocytes of the proliferating zone (Ben-Hur *et al.,* 1993; Kusec *et al.,* 1998). In bone, the general consensus for ER distribution is that both receptor isoforms are localized predominantly to osteoblasts and lining cells of trabecular bone, with lower levels in osteocytes and low to undetectable levels in osteoclasts. However, other patterns of distribution of ERα have been obtained. For example, immunofluorescence detection of ERα in human female and pig bone revealed strongest reactivity in about 50% of the osteocytes with indistinct staining of OBs and lining cells (Braidman *et al.,* 1995). Using a more sensitive *in situ* reverse transcriptase (RT) polymerase chain reaction (PCR) technique to identify ERα mRNA in actively remodeling human fracture callus, Hoyland *et al.* (1997) reported that OBs opposing bone surfaces expressed the highest levels of ERα, whereas osteocytes and OCs expressed lower levels. These results were confirmed in another study in which the ERα mRNA and protein were similarly predominantly localized to osteoblasts and lining cells of trabecular bone from human and rabbit, with no clear expression in osteocytes or OCs (Kusec *et al.,* 1998).

The *in situ* RT-PCR technique has been used to demonstrate a loss of ERα mRNA expression from OBs and osteocytes in males with idiopathic osteoporosis, suggesting a case of estrogen resistance (Braidman *et al.,* 2000). In an immunolocalization study of mouse and human trabecular bone and fracture callus, ERβ was identified in OBs, osteocytes, and OCs, with the latter cells exhibiting cytoplasmic rather than nuclear immunoreactivity (Vidal *et al.,* 1999). Similar results were obtained for ERβ mRNA in neonatal rat bone in which the transcripts were expressed predominantly by OBs covering metaphyseal trabecular bone surfaces with lower signals in osteocytes and bone marrow (Windahl *et al.,* 2000b). In rats, the relative levels of ERα and ERβ mRNA have been reported to be lower in cortical compared to trabecular bone, with ERα being the dominant species in both locations (Lim *et al.,* 1999; Onoe *et al.,* 1997). The weak expression of ERβ in cortical bone is an intriguing observation given that the most striking skeletal phenotype in ERβ knockout mice is an increase in cortical bone mineral content in postpubertal females (Windahl *et al.,* 1999). In any case, these *in vitro* and expression studies indicate that the concentrations of ERα are higher than ERβ in bone tissue, as well as in the OBs at different stages of differentiation. It is unclear, however, what concentration of each receptor isoform is required for responsiveness in OB cells and whether one isoform modulates the activity of the other when both are expressed, as has been reported in other cell types.

In Vivo Effects of Estrogens and Progestins on Bone

Introduction

Sex steroids influence most if not all aspects of bone development, growth, and remodeling. The physiological actions of sex steroids contribute to (a) sexual dimorphism of the skeleton, (b) timing of epiphyseal closure, (c) determination of peak bone mass, (d) maintenance of mineral homeostasis during reproduction, and (e) maintenance of bone mass, architecture, and mineral homeostasis in adults. The important role of gonadal hormones in bone metabolism is well established. Despite the recognition that gonadal insufficiency is the most important risk factor for osteoporosis, the mechanisms of action of sex steroids on bone *in vivo* are poorly understood and have received surprisingly moderate attention by investigators. Because human studies have provided a very limited understanding regarding the underlying cellular and molecular mechanisms that mediate hormonal action, studies in laboratory animals have proven extremely useful in identifying an impressive range of physiological actions of gonadal hormones on bone and mineral homeostasis. Additionally, a common contemporary use of animals involves preclinical studies evaluating the effectiveness of pharmaceuticals as interventions to prevent or treat bone loss. The strengths and weaknesses of the most well-established animal models for osteoporosis have been reviewed (Turner *et al.,* 2000).

This section emphasizes the effects of sex steroids on the skeleton of placental mammals. The reader is referred to a prior review for a discussion of the important and unique effects of sex steroids on the skeleton during reproduction in oviparous vertebrates (Turner *et al.,* 1994a).

Effects of Sex Steroids on Bone Mass

Ovariectomy leads to a bone deficit in adult rodents and primates (Kalu *et al.,* 1991; Lindsay *et al.,* 1978; Jayo *et al.,* 1990). These changes are due to endocortical bone modeling, destructive cancellous bone modeling, and remodeling (Wronski *et al.,* 1988a; Turner *et al.,* 1987b). Similar changes occur in rats (Goulding and Fisher, 1991) and monkeys (Mann *et al.,* 1990) following administration of GnRH agonists, a chemical intervention that decreases serum estradiol levels comparable to ovariectomy. This important role of E in mediating skeletal changes is further implicated by the observation that the skeletal changes following treatment of rats with ICI 182,780, a potent estrogen receptor antagonist, are similar to ovariectomy (Sibonga *et al.,* 1998). This conclusion is further supported by the consistent finding that the skeletal changes that follow ovariectomy are antagonized by pharmacological replacement with E. The physiological role of other ovarian hormones is less clear. The progesterone antagonist RU486 was reported to not alter cancellous bone volume and turnover in female rats whereas administration of androgen and P to animals had variable results (Abe *et al.,* 1992; Barengolts *et al.,* 1991; Kalu *et al.,* 1991; Schmidt *et al.,* 2000).

Alterations in Bone Architecture in Gonadal Hormone-Deficient Laboratory Animals

CORTICAL BONE

The volume of the medullary canal in rat tibiae is enlarged following ovariectomy due to a net increase in bone resorption (Kalu *et al.,* 1989; Turner *et al.,* 1987a). Osteoclast number is increased (Turner *et al.,* 1987b) and bone formation remains unchanged or increased (Turner *et al.,* 1987a,b, 1990c). In contrast, there is an increase in bone formation at the periosteal surface (Turner *et al.,* 1987a). As a result of the opposing changes in radial growth and endocortical modeling, the cortical bone volume changes little in ovariectomized rats (Turner *et al.,* 1987a,b, 1990b,c). It may, in fact, increase in rapidly growing rats because the increase in periosteal addition of bone exceeds the increase in endocortical resorption (Turner *et al.,* 1990b). The cellular mechanism for the differential response of the periosteal and endocortical bone surfaces of the midshaft appears to be related to the different functions and populations of cells that comprise the two bone envelopes in the rat model.

The periosteum is responsible for radial bone growth. Under normal circumstances, bone resorption is associated with developing vascular spaces. Osteoclasts are uncommon at other locations on the periosteum and are not notably increased following ovariectomy. This low level of focal resorption is contrasted with the endocortical surface, which undergoes aggressive bone modeling during growth to increase the volume of the marrow cavity. Estrogen suppresses growth (Turner *et al.,* 1994b) and ovariectomy results in reestablishment of radial bone growth in adult rats. A similar periosteal expansion occurs in aging humans. It is important to note that one of the limitations of rat and mouse studies is that the skeleton of small rodents does not undergo cortical bone remodeling. Ovariectomy increases endocortical bone resorption surface and cortical porosity in dogs (Karambolova *et al.,* 1987). However, no net loss of cortical bone was also reported in this species (Martin *et al.,* 1987). Studies in other large animals with an estrus cycle more similar to the human menstrual cycle (e.g., nonhuman primates) are necessary to adequately characterize the possible effects of sex steroids on Haversian (cortical) bone remodeling.

CANCELLOUS BONE

Ovariectomy results in severe cancellous osteopenia in long bones and vertebrae of rats (Wronski *et al.,* 1989) and vertebrae of monkeys (Jerome *et al.,* 1986; Longcope *et al.,* 1989). The response to ovariectomy in dogs has been less consistent, with no change (Snow and Anderson, 1986) and bone loss (Martin *et al.,* 1987) reported. The rate of bone loss from rat vertebrae occurs more slowly than from long bones (Wronski *et al.,* 1988b, 1989). There appears to be a regional difference within bones. Cancellous osteopenia is more prominent in the proximal tibial metaphysis than the distal metaphysis or proximal epiphysis. It is important to note that the rate of bone loss may be related to differences in bone marrow and prevailing levels of mechanical loading (Westerlind *et al.,* 1997). Additional evidence suggests that there is overlap between mechanical signaling and estrogen action on bone (Damien *et al.,* 1998; Tomkinson *et al.,* 1998).

Ovariectomy results in increases in the osteoblast-lined perimeter, OC-lined perimeter, and OC size in long bones of rats (Wronski *et al.,* 1986; Turner *et al.,* 1988). There are simultaneous increases in the mineral apposition and bone formation rates, suggesting that ovariectomy results in chronic high bone turnover. Cancellous bone turnover remains elevated in rats a year or more after ovariectomy (Wronski *et al.,* 1989; Sibonga *et al.,* 2000). Bone formation is increased in ovariectomized monkeys (Jerome *et al.,* 1986; Longcope *et al.,* 1989), suggesting the bone loss in nonhuman primates is also associated with increased bone turnover. Dempster *et al.* (1995) reported temporal changes in cancellous bone architecture of the distal rat femur after ovariectomy. These investigators demonstrated that the primary mechanism of ovariectomy-induced bone loss is osteoclastic perforation and removal of the trabecular plates. Generalized, gradual trabecular thinning was not observed. Interestingly, a similar process is responsible for removal of the most distal trabeculae during normal bone elongation (Frost, 1963).

Effects of Estrogen Replacement on Bone Metabolism in Ovariectomized Laboratory Animals

Estrogen treatment of ovariectomized rats prevents the increase in medullary area by preventing the increase in OC number (Turner *et al.,* 1987a,b). Similarly, E replacement prevents the increase in periosteal bone formation (Turner *et al.,* 1987a,b). Estrogen reduced the number of preosteoblasts in S phase of the cell cycle, suggesting that the hormone inhibits the production of OBs. At the same time, E decreased steady-state mRNA levels for bone matrix proteins and IGF-I, bone matrix synthesis, reduced OB number, and increased the population of bone-lining cells (Turner *et al.,* 1990a; Turner *et al.,* 1992). E inhibits bone growth by decreasing bone formation, as well as bone resorption, and these changes are associated with a decreased expression of OC survival factors such as IGF-I. E treatment can result in an osteosclerotic response in ovariectomized as well as ovary intact mice. This nonphysiological response can limit the usefulness of the mouse as a model for the analyses and understanding of E action on human bone (Turner, 1999).

Estrogen replacement stabilizes cancellous bone volume in ovariectomized rats by reducing the rate of bone turnover, by reestablishing a neutral or positive balance between bone formation and bone resorption; and by preventing the destruction of trabecular plates (Kalu *et al.,* 1991; Turner *et al.,* 1988; Wronski *et al.,* 1988b, 1993). Estrogen accomplishes these actions in part by reducing the initiation of new bone remodeling. Osteoclast number is reduced in estrogen-treated rats. The hormone probably acts via decreasing the fusion of the OC precursors (Turner *et al.,* 1994b), as well as reducing OC life span (Kameda *et al.,* 1997). The effects of

E on cancellous bone formation are more controversial. There is general agreement that the inhibitory effects of the hormone on initiation of bone remodeling leads to an overall coupled decrease in bone formation as well as improved bone remodeling balance. The latter change may be due to an increased OB life span (Gohel *et al.,* 1999). However, some investigators have reported that E has separate direct stimulatory actions to increase OB number and bone formation (Takano-Yamamoto and Rodan, 1990; Chow *et al.,* 1992). Most investigators, however, have reported that E inhibits indices of bone formation, including OB number and activity (Kalu *et al.,* 1991; Turner *et al.,* 1988, 1993; Wronski *et al.,* 1988b; 1993). Studies to investigate this controversy failed to reveal any evidence for an anabolic action of estrogen on bone formation (Westerlind *et al.,* 1993). Furthermore, time course studies have shown that E results in rapid decreases in mRNA levels for bone matrix proteins and collagen synthesis (Turner *et al.,* 1999). Finally, E does not increase bone volume in adult rats with established bone loss (Wronski *et al.,* 1993).

Skeletal Phenotypes of Mice with Targeted Disruption of ER Genes

The development of mice exhibiting targeted gene deletions (knockout) in ERα, ERβ, or both genes simultaneously has been achieved. Although the complete analysis of the skeletal phenotypes for some of the genotypes has yet to be reported, certain mutants have been examined in detail. As a note of caution, the mutant phenotype of knockout animals may result from multiple influences that occur as a consequence of the targeted mutation, and therefore the mechanisms responsible for the phenotype may not be restricted solely to that mutation. In this regard, disturbing ER signaling in all tissues by ER isoform gene ablation may destabilize the feedback loops regulating the synthesis of other sex steroids in addition to E, thereby leading to changes in the circulating levels of these hormones, some of which also possess major osteotropic activity. For example, the ERαKO female mouse has been reported to have markedly elevated levels of E, whereas the male ERαKO has increased levels of testosterone (Couse and Korach, 1999). As a further complexity, phenotypes in single ER gene mutants may arise as a result of the β isoform being able or unable to either compensate for the loss of the α isoform or, alternatively, suppress ERα activity. In the latter case, loss of ERβ could conceivably enhance rather than suppress E responsiveness in a cell type that normally expresses both ER isoforms.

ERα Knockout Mice

Homozygous deletion of the ERα gene results in reduced cortical bone density and cortical bone formation in both male and female mice, suggesting that E stimulates cortical bone formation via ERα (Korach *et al.,* 1997). Although there is delayed closure of the epiphyseal growth plate, linear bone growth in long bones is generally reduced, which is more pronounced in females (Korach *et al.,* 1997; Schmidt *et al.,* 1999). The effect of ERα knockout on trabecular bone, however, is less certain. Using independently generated animals, trabecular bone has been reported to be either modestly decreased in both sexes (Korach *et al.,* 1997) or unaffected in females and showing an age-related increase as a result of reduced bone turnover in males (Sims *et al.,* 2000). Consequently, the lack of marked trabecular bone loss suggests that ERβ compensates for loss of ERα in this tissue. Female ERαKO mice lose bone following OVX to the same extent as the wild type. However, estrogen responsiveness in the ERαKO female is reduced, as higher estrogen concentrations are required to prevent the bone loss compared to OVX wild-type mice (Ederveen and Kloosterboer, 1999).

Interestingly, both a man with aromatase P450 deficiency (causing complete E deficiency) and a man with a mutation in the ERα gene (causing partial E resistance) exhibited continued longitudinal bone growth due to delayed epiphyseal growth plate ossification in addition to marked trabecular osteopenia (Carani *et al.,* 1997; Smith *et al.,* 1994). From these findings it is therefore apparent that (1) E has opposite effects on longitudinal bone growth in humans and mice and (2) ERα mediates at least some of the effects of E on trabecular bone remodeling in both species.

ERβ Knockout Mice

Deletion of the ERβ gene, as with ERα, results in delayed growth plate closure, but in adult female ERβKO mice, the longitudinal bone growth for some long bones is increased rather than decreased (Windahl *et al.,* 1999). Studies suggest that the cortical and trabecular bone parameters of male ERβKO are not significantly different from wild type (Vidal *et al.,* 2000). In contrast, whereas the trabecular bone of female ERβKO mice is unaffected, there is increased whole body bone mineral content due to increased cortical bone, although this is only observed in postpubertal females (Windahl *et al.,* 1999). The increased cortical bone is a result of elevated periosteal (radial) bone formation, indicating that ERβ is required for pubertal feminization of the skeleton.

Although trabecular bone is unaffected in ERβKO animals at sexual maturity, older (~4 months to 1 year) female ERβKO mice are partially protected against age-related trabecular bone loss, as these animals exhibit higher trabecular bone density and volume than either male ERβKO or wild-type animals (Windahl *et al.,* 2000a; Ederveen *et al.,* 2000). The increase in trabecular bone is thought to be due to enhanced bone formation rather than to changes in growth plate kinetics or altered bone resorption because OB markers and ERα transcript levels are elevated (Windahl *et al.,* 2000a). It has been suggested that increased trabecular bone in female ERβKO mice is caused by the removal of the dominant-negative function of ERβ on ERα activity (Sims *et al.,* 2000). Female ERβKO animals lose cortical and trabecular bone after OVX similarly to wild type (Windahl *et al.,* 1999). Moreover, E treatment prevents OVX-induced bone loss in ERβKO female mice to the

same extent as in wild-type mice in contrast to the weaker E responsiveness of ERαKO females (Ederveen *et al.,* 2000; Ke *et al.,* 2000). Thus, ERβ seems to be nonfunctional in the skeleton of male mice, whereas in females, ERβ mediates the suppression of periosteal bone growth. Further, in female mice, ERβ is not required for the maintenance of trabecular bone in the presence of ERα, but is required in the absence of ERα. In support of the hypothesis that ERβ does not mediate E responses in trabecular bone, at least in males, testosterone, but not E, completely prevents the decrease in bone density at trabecular sites in orchidectomized ERαKO males (Vandenput *et al.,* 2000). An apparent discrepancy, however, is that if ERβ is truly nonfunctional in the male mouse skeleton, then why does the ERαKO male not exhibit E resistance characterized by elevated bone turnover rates and osteopenia rather than the unaltered or slightly increased trabecular bone density that is in fact observed (Sims *et al.,* 2000)?

ERα/β KNOCKOUT MICE

Deletion of both ER isoforms in double knockout mice (ER$\alpha\beta$KO) generates a skeletal phenotype in the male very similar to that caused by ERα single deletion; namely, postpubertal longitudinal and periosteal (cortical) bone growth are reduced, but trabecular bone is unaffected (Vidal *et al.,* 2000). Interestingly, the reduced bone growth in the male has been correlated with decreased serum IGF-1 levels (Vidal *et al.,* 2000). Similarity of the skeletal defects resulting from ERα or ERα plus ERβ gene inactivation in the male knockout is further evidence for the lack of involvement of the β isoform in growth and remodeling of the male skeleton.

In contrast, in females the bone defects in the ER$\alpha\beta$KO mouse are distinct from those produced by deletion of either ER isoform alone, thus supporting a role for both isoforms in the female skeleton. In postpubertal ER$\alpha\beta$KO females, both cortical thickness and trabecular bone density are reduced due to decreased bone formation without effects on osteoclast surface (Sims *et al.,* 2000). Because female ERαKO animals fail to exhibit trabecular bone loss, this finding therefore demonstrates that ERβ in females can compensate for the loss of ERα and that both ER isoforms participate in normal trabecular remodeling.

CONCLUSIONS

Overall it is becoming clear that ERα and ERβ likely perform different functions in cortical and trabecular bone and that the relative importance of the two isoforms at these sites differs between the sexes. However, it is of course questionable whether the ER isoforms perform identical functions during skeletal growth and turnover in humans. Perhaps the most surprising result arising from the ER knockout analysis is that double ER$\alpha\beta$KO mice are viable with grossly normal skeletons, demonstrating that both ER isoforms are dispensable for the normal development of cartilage and bone.

Skeletal Phenotype of Mice with Disruption of PR Genes

Mice have been generated with a homozygous mutation of the PR locus in which expression of both A and B isoforms of PR is prevented. The PR-B has been shown to be the dominant player in gene regulation in P target cells (Giangrande *et al.,* 1999). PRKO mice (i.e., PR-A/B knockouts) exhibit multiple reproductive abnormalities and homozygous females are infertile (Lydon *et al.,* 1995). Female PRKO mice have been analyzed histomorphometrically for possible skeletal defects. Although aged animals had cortical and trabecular bone densities that were not significantly different from age-matched wild-type animals (Bain *et al.,* 1997), subtle abnormalities were evident at earlier ages. Young postpubertal PRKO mice exhibited increased cortical bone thickness with no changes in trabecular bone, whereas rapidly growing prepubertal PRKO mice possessed elevated trabecular bone density and formation without effects on cortical bone (Rickard *et al.,* 1999). Linear bone growth was unaffected at all ages examined. These findings suggest that progesterone may be involved in the early growth and sexual dimorphism of the female skeleton. Studies on PR_AKO female mice showed that progesterone displayed a major effect in reproductive tissue in these PR_AKO mice compared to the wild type (Mulac-Jericevic *et al.,* 2000). This suggests that PR_A may play a dominant-negative role in the actions of PR_B. It will be most interesting to examine the skeleton in these PR_AKO female mice.

Effects of Estrogen on Bone Cells *in Vitro*

Osteoblasts

ESTROGEN REGULATION OF EARLY AND LATE RESPONSE GENES IN OSTEOBLASTS

As outlined briefly earlier, sex steroids regulate the expression of early response genes within 30 min to 2 hr following hormone exposure. The best examples of this are the nuclear protooncogenes, including c-myc, c-jun, jun-B, c-fos, N-myc, and the TGFβ-inducible early gene, TIEG (Spelsberg *et al.,* 1992; Tau *et al.,* 1998). These genes are expressed and regulated by sex steroids in many different reproductive (Murphy *et al.,* 1987; Fink *et al.,* 1988) and bone tissues (Eriksen *et al.,* 1988; Harris *et al.,* 1995; Tau *et al.,* 1998; Oursler *et al.,* 1991, 1994). Thus these genes are excellent markers for steroid actions and interactions. Because these genes are regulated rapidly by steroids in all tissues containing their receptors, and most of these early gene products in turn regulate the gene expression of late genes, a role for nuclear protooncogenes as early response ("regulatory") genes in a cascade model of steroid action in bone and reproductive tissues has been proposed (Landers and Spelsberg, 1992; Spelsberg *et al.,* 1992).

In contrast to these rapidly induced genes, which are universal markers of steroid actions, many late responsive (24–48 hr) structural genes are specific to different tissues and some, like the growth factor/cytokine genes, mediate the effects of E on these tissues. Control of late gene expression by E has been investigated in numerous osteoblast cell culture systems. Unfortunately, despite extensive efforts, only a few putative target genes have shown consistent regulation. The most reliable responses are increases in type I collagen, transforming growth factor (TGF)-β, and insulin-like growth factor (IGF)-1 production (for a review, see Spelsberg *et al.,* 1999). In fact, E has been shown to induce TGFβ production by both OBs and OCs (Oursler, 1998). These factors may mediate some of the anabolic effects of E on bone. For example, TGFβ plays an important role in chondrogenesis and osteogenesis, as both TGFβ and IGF-1 promote the proliferation and differentiation of OB precursors, and TGFβ inhibits the formation of OCs. In addition to regulating IGF-1 synthesis, E may modulate IGF bioactivity by regulating the production of IGF-binding proteins, including IGFBP-2, 3, and 4, which in turn enhance or suppress the activities of IGF-1 and IGF-II. TGFβ and IGF-1 are both sequestered in bone matrix at relatively high concentrations. See Fig. 2 for a detailed list of E responses in OB and OC cells.

Estrogen Regulation of Cytokine Synthesis in Osteoblasts

In bone, a hallmark of E deficiency is an elevated rate of bone turnover in which both OC-mediated bone resorption and, perhaps secondarily, OB-mediated bone formation/activity occurs at an increased rate. See Fig 3 for a model illustrating the known and putative mechanisms of OB–OC interactions. A considerable amount of evidence demonstrates that release of an E-imposed suppression of bone-resorbing cytokine synthesis is responsible for the accelerated bone turnover. The OB and/or monocyte/macrophage lineage release of numerous cytokines that are capable of increasing OC formation, differentiation, or activity has been shown to be inhibited by E. However, which of the cytokines is/are most important for mediating the actions of E remains unresolved. One theory maintains that bone marrow monocyte/macrophage-derived interleukin (IL-1) and tumor necrosis factor (TNF) induce the production of other cytokines, including macrophage colony stimulating factor (M-CSF), granulocyte-macrophage CSF (GM-CSF), and IL-6 by osteoblasts and hematopoietic cells, which together promote osteoclast resorptive activity. Evidence in support of this pathway is as follows: (1) release of IL-1, TNF, and GM-CSF by peripheral blood monocytes from untreated osteoporotic women is higher than from E-treated osteoporotics and nonosteoporotics (Pacifici *et al.,* 1989, 1991), (2) production of M-CSF by osteoblastic/stromal cells is increased by OVX in mice and is dependent on bone marrow synthesis of IL-1 and TNF (Kimble *et al.,* 1996), (3) neutralization of TNF activity either by the inhibitory TNF-binding protein (TNFbp) or by expression of an inhibitory soluble TNF type I receptor in mice prevents OVX-induced bone loss (Ammann *et al.,* 1997), (4) type I IL-1 receptor-null mutant mice fail to lose bone following OVX (Lorenzo *et al.,* 1998), and (5) simultaneous blockade of both IL-1 and TNF activity by IL-1 receptor antagonist (IL-1ra) and TNFbp completely prevents OVX-induced bone loss in rats (Kimble *et al.,* 1995, 1997).

An alternative, but not exclusive, proposed pathway favors IL-6 as the mediator of bone loss in an E-deficient state. Evidence for this pathway is as follows: (1) production

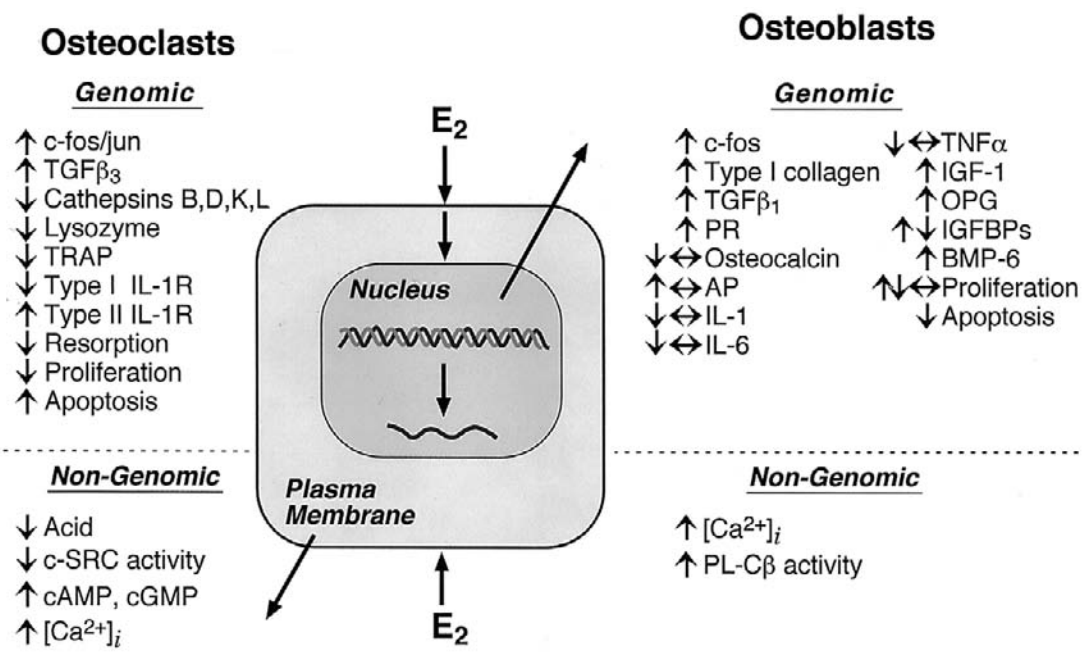

Figure 2 Model outlining all the reported effects of estrogens on osteoblast and osteoclast functions. Arrows indicate the changes induced by estrogen. Double/triple arrows indicate published differences in reported responses.

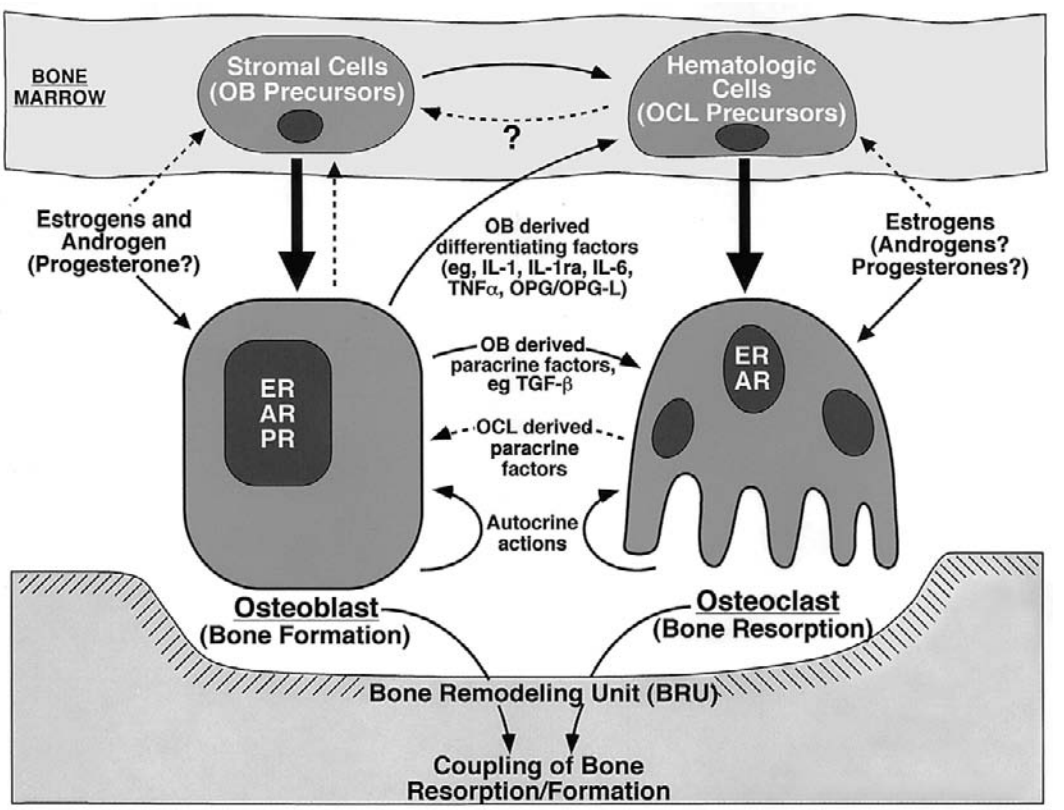

Figure 3 Model of sex steroid actions and interactions with growth factors and cytokines in bone resorption and bone formation. Taken with permission from Spelsberg *et al.* (1999).

of IL-6 is inhibited by E in cells of the stromal/osteoblast lineage *in vitro* (Girasole *et al.,* 1992; Passeri *et al.,* 1993), although this is not a universal finding (Rickard *et al.,* 1992; Rifas *et al.,* 1995), (2) the production of IL-6, as well as the number of OC precursors and osteoclast formation, is increased in *ex vivo* bone marrow cultures from OVX mice compared to sham control animals (Miyaura *et al.,* 1995), (3) all of these effects, as well as the increased population of osteoclasts present in trabecular bone of OVX mice, can be prevented by treatment with E or an anti-IL-6 antibody (Jilka *et al.,* 1992), (4) E may reduce the responsiveness of OB lineage cells and possibly also OCs to IL-6 by decreasing expression of the ligand-binding gp80 and signal-transducing gp130 subunits of the IL-6 receptor (Lin *et al.,* 1997), and (5) IL-6-deficient mice are protected from OVX-induced bone loss (Poli *et al.,* 1994).

Other factors such as osteoprotegerin (OPG), a soluble member of the TNF receptor family, may also mediate the inhibitory effect of E on bone resorption. OPG binds to and inhibits the activity of OPG ligand (OPGL or RANKL), a TNF-related cytokine produced by bone marrow stromal cells and OBs (among other cell types), which has been shown to be essential for OC formation. Among the bone-active cytokines, OPG is unique because deletion of the OPG gene in mice generates severe osteopenia in both cancellous and cortical bone (Bucay *et al.,* 1998), whereas mice overexpressing OPG develop osteopetrosis and do not lose bone as a result of sex hormone deficiency (Simonet *et al.,* 1997). E has been shown to increase OPG expression in OBs overexpressing the ERα gene (Hofbauer *et al.,* 1999), and this effect may be indirect via TGFβ, which has been shown to increase OPG production by bone marrow stromal OB precursor cells (Takai *et al.,* 1998). Assuming these effects also occur *in vivo*, OPG may be a key mediator of the antiresorptive effects of E. A summary of the mechanisms for the control of bone resorption by E is shown in Fig. 4.

Molecular Mechanism of Estrogen Regulation of Cytokine and Other Gene Promoters

The molecular mechanisms involved in the E regulation of several cytokine/late gene promoters have been elucidated. Many of these promoters lack consensus estrogen response elements (EREs) and thus have revealed alternative mechanisms of transcriptional control by the ER, which in some instances does not require direct binding of the ligand-activated ER to DNA. Instead, ER interacts with other transcription factors and coregulators to modulate the binding affinity of these proteins to their cognate regulatory sites in the DNA. For example, repression of the IL-6 promoter by E occurs because protein–protein interactions between the ligand-activated ERα and NF-κB and C/EBPβ prevent binding of the latter factors to their respective sites on DNA (Stein and Yang, 1995; Galien *et al.,* 1996). In contrast, E stimulates transcription from the IGF-1 promoter via enhanced binding of fos/jun heterodimers to AP-1 elements by interaction with activated ERα (Umayahara *et al.,* 1994). Numerous genes, including

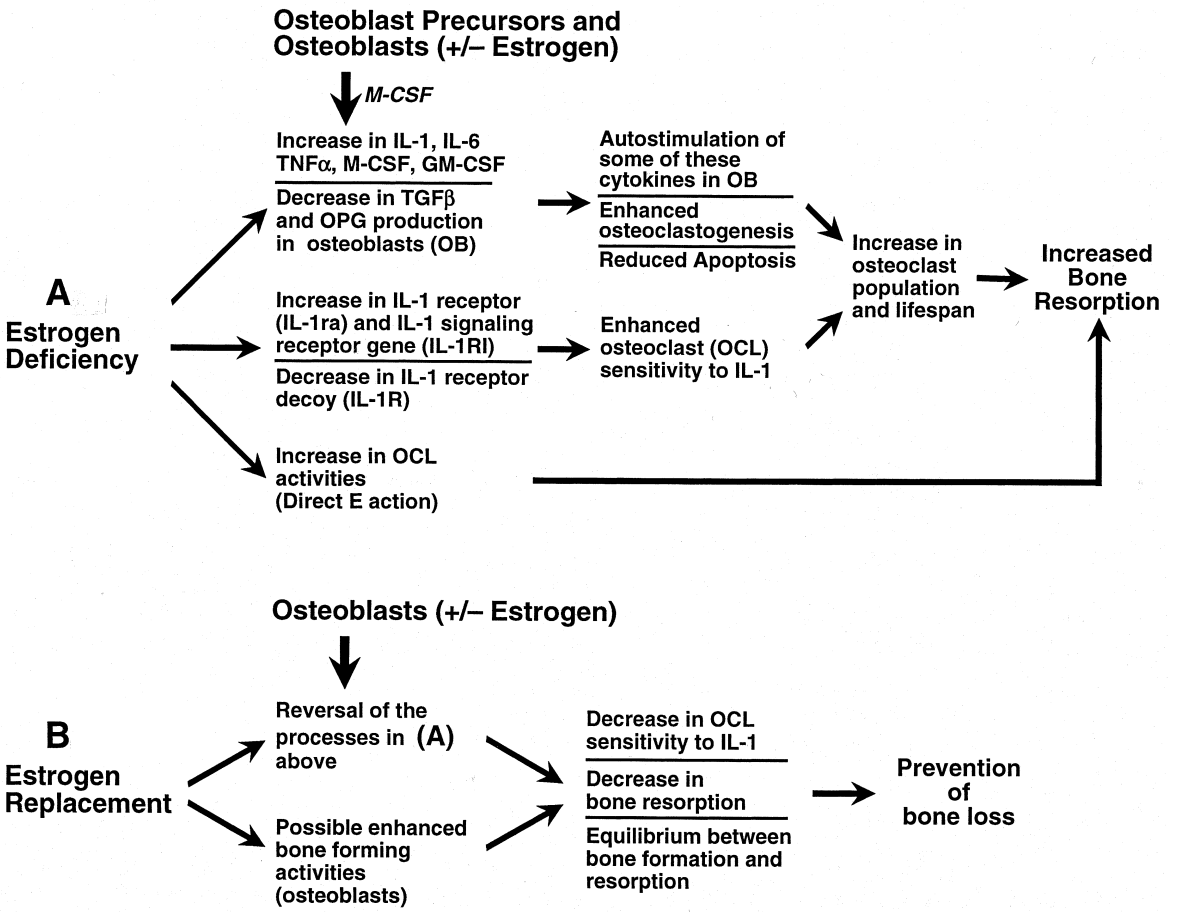

Figure 4 Outline of the potential pathways in estrogen regulation of bone resorption. Reproduced with permission from Spelsberg *et al.* (1999).

retinoic acid receptor-α1 (RAR-α1), c-fos protooncogene, cathepsin D, IGFBP-4, and the epidermal growth factor receptor gene, among others, are modulated by E through the binding of ER/Sp1 transcription factor complexes with either Sp1 sites alone or Sp1/ERE half-sites (Rishi *et al.,* 1995; Krishnan *et al.,* 1994; Salvatori *et al.,* 2000).

Estrogen Regulation of Proliferation, Formation and Apoptosis of Osteoblasts

Osteoblast Proliferation Similar to the conflicting reports on the E regulation of numerous late response genes in bone-forming OBs, there is a lack of a general consensus with regard to the effects of E treatment on OB cell proliferation. While E has been reported to stimulate cell proliferation in primary and immortalized fetal rat calvarial cells (Ernst *et al.,* 1989), normal adult human OBs (Scheven *et al.,* 1992), mouse MC-3T3E1 cells (Majeska *et al.,* 1994), and synchronized human osteosarcoma cells (Ikegami *et al.,* 1994), E has been shown to have no effect on some primary cultures of normal adult OBs (Keeting *et al.,* 1991; Rickard *et al.,* 1993) or to inhibit cell growth in OBs stably transfected with the ERα gene (Watts *et al.,* 1989; Kassem *et al.,* 1996; Robinson *et al.,* 1997).

The effect, if any, of E on the proliferation of OB progenitor cells is also currently unclear. Whereas the proliferation rate of bone marrow-derived OB precursor cells, isolated from OVX rats, was increased compared to cells from sham-operated animals (Modrowski *et al.,* 1993), the *in vitro* treatment of rat bone marrow stromal cells with E showed no effect on cell growth (Rickard *et al.,* 1995).

Osteoblast Formation *In vivo*, OB and OC formation appears to be tightly coupled due, at least in part, to (1) the dependence of OC precursor development on support from cells of the stromal/OB lineage through the provision of cytokines and other stimuli and (2) of OB precursor development on factors released from bone matrix by OC activity. It has been suggested that E deficiency causes not only increased OC formation [as a result of increased production of bone-resorbing cytokines (discussed earlier)], but also stimulates OB formation (Manolagas, 1999). In support of this, ovariectomy of rodents increases the number of bone marrow-derived OB progenitor cells (Scutt *et al.,* 1996; Jilka *et al.,* 1998). In mice, this increase was transient and partially independent of increased bone resorption, suggestive of a possible direct action of E on OB proliferation and/or differentiation (Jilka *et al.,* 1998). Such mechanisms may therefore account for the elevated bone turnover immediately after OVX/menopause. However, the stimulated rates of OC and OB formation due to sex steroid deficiency are only tempo-

rary, and with time after OVX/menopause, bone remodeling declines to levels approximating premenopausal rates.

Apoptosis It is becoming increasingly clear that E also has major effects on the apoptosis of OBs and OCs. E has been shown to have opposite effects on apoptosis in OCs and OBs. In OCs, apoptosis is stimulated by E via a TGFβ-mediated mechanism (Hughes *et al.,* 1996; Kameda *et al.,* 1997). However, apoptosis is prevented by E in osteocytes (Tomkinson *et al.,* 1997, 1998) and OBs (Gohel *et al.,* 1999; Manolagas *et al.,* 1999). The enhancement of OC apoptosis could contribute to the antiresorptive effects of E, and the prevention of OB apoptosis could contribute to the reported anabolic effects of E on the skeleton. As mentioned earlier, the increased formation of OBs and OCs observed after OVX is only temporary, and eventually the opposing effects of E on OB and OC apoptosis are believed to return to dominance. Consequently, an imbalance between bone resorption and formation will be maintained, only now resulting in the net slow rate of bone loss that persists once the initially high bone turnover of the perimenopausal period has subsided.

NONGENOMIC EFFECTS OF ESTROGEN ON OSTEOBLASTS

The cascade model explains many apparent nongenomic actions of steroids, which are, in fact, genomic processes. However, there is a growing body of evidence that steroids also influence the cell surface by nongenomic effects, which involve responses to steroids within minutes and are too rapid to be explained by genomic processes (Moss *et al.,* 1997; Revelli *et al.,* 1998). For instance, a possible cardioprotective effect of E has been shown to occur through the induction of endothelial nitric oxide synthase in human vascular endothelial cells via a ligand-dependent interaction between ERα and phosphatidylinositol-3-OH kinase (Simoncini *et al.,* 2000). In OBs, treatment with E has been shown to cause a rapid (within seconds) increase in intracellular calcium ion concentration due to influx across the plasma membrane and release from intracellular stores (Lieberherr *et al.,* 1993). Additionally, E stimulates the hydrolysis of phosphatidyl inositol phospholipids with the generation of diacylglycerol and inositol 1,4,5-trisphosphate, which in turn activate other second messenger pathways. In female rat OBs, this effect of E has been shown to be mediated by the activation of phospholipase C-β2 via a pertussin toxin-sensitive G protein's $\beta\gamma$ subunits, further supporting the existence of a nonclassical membrane ER (Le Mellay *et al.,* 1999). Activation of MAP kinases and increases in cAMP and cGMP have also been reported in osteosarcoma cells (Endoh *et al.,* 1997).

A nongenomic mechanism involving the *src* tyrosine kinase and MAP kinase signaling pathway has recently been demonstrated to mediate the antiapoptotic effects of E on primary and immortalized OB and osteocytic cells (Kousteni *et al.* 2001). Intriguingly, the antiapoptotic effect of both E and the androgen dihydroxytestosterone were mediated equivalently via both ER (α and β) or by the androgen receptor (AR). Furthermore, only the ligand-binding domain of the receptor protein appeared to be required and the antiapoptotic function was dissociated from the receptors' transcriptional activity (Kousteni *et al* 2001). One possible interpretation of these observations is that ER and/or AR proteins localized at the plasma membrane exhibit unique properties and perform distinct functions to that of the nuclear localized receptor.

The response of the target cell to E may therefore be a superimposition of genomic and nongenomic effects, although the physiological importance of the nongenomic effects is still unclear. The putative membrane receptors for steroid hormones remain unidentified.

Osteoclasts

In addition to indirect effects of E on the formation, differentiation, and activity of OCs via regulation of cytokine synthesis by OBs, E may also have direct actions on osteoclasts and their precursors (see Fig. 2). The bone-resorbing activity of avian, rabbit, and human OCs is decreased by treatment with E, which is due in part to a reduced population of OCs via apoptosis (Kameda *et al.,* 1997; Hughes *et al.,* 1996). E may further inhibit bone resorption by acting directly on OC progenitors to reduce OC formation, as demonstrated by (1) the ability of E to reduce proliferation and increase apoptosis of FLG 29.1 preosteoclastic cells (Zecchi-Orlandini *et al.,* 1999), (2) the antagonism by E of the differentiation of murine hemopoietic blast cells into multinucleated OCs induced by GM-CSF and PTH/PTH-related peptide (Kanatani *et al.,* 1998), and (3) the finding that E suppresses the production of TRAP-positive, multinucleated OCs from primary murine bone marrow myeloid progenitors and the mouse monocytic cell line RAW 264.7 cultured in the presence of soluble RANKL (OPGL) and M-CSF but in the absence of stromal/OB support cells (Shevde *et al.,* 2000). This latter effect of E may be due to downregulation of OPGL-induced JNK activity in OC progenitors (Srivastava *et al.,* 1999).

Additionally, E has been reported to inhibit the expression/secretion of matrix-degrading agents by isolated OCs, including acid and the lysosomal enzymes cathepsin B, D, K, and L, and lysozyme (Kremer *et al.,* 1995; Mano *et al.,* 1996; for a review, see Oursler, 1998). Similar to the studies on OBs, the effects of E on isolated avian OCs *in vitro* appear to be limited by the level of ERα (Pederson *et al.,* 1997). Further, the antiresorptive effects of E on OCs may in part be mediated by reducing the responsiveness of OCs to resorption-stimulatory cytokines through the modulation of cytokine receptor levels and/or postreceptor signaling. For example, in isolated human OCs, E directly reduces mRNA expression of the signaling type I IL-1 receptor while simultaneously increasing levels of the "decoy" type II IL-1 receptor and release of soluble IL-1RII (Sunyer *et al.,* 1999). The IL-1 pathway is a stimulator of OC bone resorption.

Several nongenomic effects of E in OCs have also been reported. Treatment of isolated chick OCs with E inhibited the basal and PTH-stimulated production of acid within 15 min (Gay *et al.,* 1993). This effect appears to be mediated at the plasma membrane because membrane-impermeable conjugates of E produced the same response (Brubaker and Gay,

1994). E treatment of avian OCs is also associated with rapid and transient changes in cell shape and the level of phosphotyrosine proteins at the plasma membrane (Brubaker and Gay, 1999). One of the phosphorylated proteins has been identified as pp60src, a tyrosine kinase required for the bone resorptive function of OCs. Treatment with E affects the degree of phosphorylation, and hence activity, of src as well as its subcellular distribution (Pascoe and Oursler, 2001). However, translocation of the src protein both to (Brubaker and Gay, 1999) and away (Judd and Oursler, 1994) from the cell surface has been reported. Active src kinase phosphorylates many substrates, including ras and the MAP kinases, which have been shown to be stimulated via a nongenomic pathway in E-treated MCF-7 breast cancer cells and rat osteosarcoma cells (Migliaccio *et al.,* 1996; Endoh *et al.,* 1997). Interestingly, constitutive activation of src decreases lysosomal enzyme secretion similarly to treatment with E, further implicating src as an intermediate in the action of E in OCs (Pascoe and Oursler, 2001). Finally, specific cell surface-binding sites for E have been reported in chick OCs and FLG 29.1 human preosteoclastic cells in which treatment with E affected intracellular calcium ion concentrations and pH within seconds to minutes (Brubaker and Gay, 1994; Fiorelli *et al.,* 1996).

Effects of Selective Estrogen Receptor Modulators, Estrogen Metabolites, and Phytoestrogens

SERMs

Despite the success of hormone replacement therapy in the treatment of postmenopausal osteoporosis, prolonged treatment with E, even when supplemented with a progestin, is associated with an increased risk of breast and uterine cancer. Consequently, there has been a tremendous effort to develop synthetic tissue-specific, partial estrogen agonists that possess the desirable estrogen agonist properties in bone and the cardiovascular system without the undesirable growth-promoting effects in reproductive tissue. One such nonsteroidal analog, the benzothiophene derivative, raloxifene (LY139481 HCl), appears to satisfy many/all of these criteria in both laboratory animals and postmenopausal women.

Although raloxifene binds with similar affinity to ERα and ERβ (Kuiper *et al.,* 1998), the compound induces distinctly different conformational changes in ERα (Brzozowski *et al.,* 1997) and ERβ (Paige *et al.,* 1999) when it binds to the ligand-binding domain. Because the conformational differences affect the coregulatory factors recruited by each receptor, this provides the molecular explanation for the divergent effects of E and raloxifene on gene transcription from various E-inducible promoters (eg., AP-1 sites) depending on which ER isoform is present (Paech *et al.,* 1997; Watanabe *et al.,* 1997). Thus, the tissue-selective responses to raloxifene and other partial E agonists may be explained by a combination of the relative level of ERα to ERβ, the repertoire of coregulators present in the cells (described earlier), and the type of DNA response element present in the promoter.

In the OVX rat model of postmenopausal osteoporosis, raloxifene prevented bone loss from sites of both cancellous and cortical bone, reduced serum cholesterol, but did not stimulate uterine hyperplasia (Black *et al.,* 1994; Turner *et al.,* 1994). Interestingly, in growing rats, the effects of E and raloxifene on bone growth and remodeling are not identical. Similar to E, raloxifene prevented the increases in longitudinal and radial bone growth as well as cancellous bone resorption that were induced by OVX, but, in contrast to E, did not prevent the OVX-induced increase in cancellous bone formation (Evans *et al.,* 1994). In adult rats with established osteopenia, raloxifene prevented additional bone loss but, as with estrogen, is unable to replace lost bone (Evans *et al.,* 1996). The prevention of cancellous bone loss by raloxifene occurred by a mechanism mimicking the antiresorptive action of estrogen in which osteoclast numbers and eroded trabecular surfaces were reduced.

More recently, analogs of raloxifene that are more potent antagonists in the uterus have also been shown to prevent OVX-induced bone loss when administered to rats immediately following OVX, as well as block continued bone loss when administered to osteopenic animals (Li *et al.,* 1998). It has also been shown that raloxifene only partially inhibits bone loss during the high turnover phase after OVX, but completely prevents bone loss during the subsequent slower phase of post-OVX bone loss. In clinical trials of postmenopausal women, raloxifene reduced markers of bone resorption and formation and increased bone mineral density relative to patients receiving the placebo, comparable to the effects of conjugated estrogens (Draper *et al.,* 1996; Delmas *et al.,* 1997). Additionally, raloxifene therapy reduced the serum concentrations of total and low-density lipoprotein (LDL) cholesterol but, unlike estrogen, did not stimulate the endometrium.

Although the *in vivo* effects of raloxifene have been studied extensively and in bone appear to closely parallel those of estrogen, comparatively little is known about the cellular and molecular effects of SERMs on bone cells *in vitro* (Bryant *et al.,* 1999). In OB lineage cells, raloxifene has been reported to stimulate creatine kinase activity (Fournier *et al.,* 1996), total protein and type I collagen α-chain synthesis (Qu *et al.,* 1999), and the expression of TGFβ3 mRNA (Yang *et al.,* 1996). To date, the few known actions of raloxifene on OC lineage cells are very much analogous to those of E. Treatment of OVX mice with raloxifene decreased the number of bone marrow-derived preosteoclasts (GM-CFU) (Liu *et al.,* 2000). This effect of raloxifene may be direct given that specific high-affinity-binding sites for raloxifene have been demonstrated in a human leukemic preosteoclastic cell line (Fiorelli *et al.,* 1997). Furthermore, raloxifene suppressed osteoclastic differentiation induced by a combination of M-CSF and OPGL in the osteoclastogenic mouse monocytic cell line RAW264.7 (Shevde *et al.,* 2000).

Estrogen Metabolites

Even though postmenopausal women lose bone at different rates, the levels of serum E do not differ among them (Riis, 1995). However, the serum levels of adrenal androgens

and the conversion of these androgens and estradiol to other estrogen metabolites do differ among individuals and certain races of the population and may play a role in maintaining bone mass in certain individuals.

Studies have shown that the predominant postmenopausal estrogen estrone (E_1) is metabolized through two mutually exclusive hydroxylation pathways (Fishman *et al.,* 1984; Martucci and Fishman, 1993). The catalytic conversions of estrone by 2-hydroxylase or 16α-hydroxylase enzymes results in the formation of either 2-hydroxyestrone (2-OHE_1) or 16α-hydroxyestrone (16α-OHE_1), respectively. The 16α-OHE_1 has been shown to bind covalently and noncovalently to the estrogen receptor (ERα), but with reduced affinity compared to E. However, its lack of binding to the serum-binding globulin makes it more available for E target tissues (Swaneck and Fishman, 1988; Fishman and Martucci, 1980). In addition, 16α-OHE_1 serves as an estrogen agonist in reproductive tissues (Fishman and Martucci, 1980; Schneider *et al.,* 1984). In contrast, 2-OHE_1 also binds very weakly (even less than 16α-OHE_1) to the ER (Fishman and Martucci, 1980) and has been shown to have no estrogenic activity and, in some cases, to act as an antiestrogen (Schneider *et al.,* 1984; Vandewalle and Lefebvre, 1989).

Lim *et al*. (1997) reported that urinary levels of 16α-OHE_1 were lower and 2-OHE_1 levels were higher in postmenopausal osteopenic individuals than in nonosteopenic individuals. Furthermore, the 16α-OHE_1/ 2-OHE_1 ratio was correlated positively with bone mineral density. African-American women, who are at lower risk for developing osteoporosis, reportedly have increased 16α-hydroxylation and therefore have a higher 16α-OHE_1/2-OHE_1 ratio compared to Caucasian women (Coker *et al.,* 1997). When OVX rats were treated with 16α-OHE_1, the cancellous bone turnover associated with ovariectomy was prevented in a similar manner as E (Westerlind *et al.,* 1998). However, 2-OHE_1 displayed no estrogenic activity on trabecular bone turnover in OVX rats. In agreement with these findings *in vivo*, 16α-OHE_1, but not 2-OHE_1, mimicked the effects of E on the regulation of alkaline phosphatase activity and osteocalcin secretion in an E responsive osteoblast cell line stably expressing ERα (Robinson *et al.,* 2000).

Another naturally occurring estrogen metabolite is 2−methoxyestradiol (2−MeO-E_2), produced primarily by the liver. This metabolite has very low affinity for ER (ERα) and because of this its physiological effects are thought not to be mediated through the ER pathway. 2-MeO-E_2 has antitumorigenic activity and may act as a physiological tumor suppressor (Zhu and Conney, 1998). Tumor cell proliferation is inhibited, possibly through the disruption of tubulin polymerization, and angiogenesis is reduced via increased senescence and apoptosis of endothelial cells by 2-MeO-E_2 (Fotsis *et al.,* 1994). The administration of high concentrations of 2-MeO-E_2 to rapidly growing rats has been demonstrated to inhibit longitudinal bone growth but have no effect on either radial bone growth or cancellous bone turnover (Turner and Evans, 2000). This suggests that the metabolite reduces proliferation and/or stimulates apoptosis of rapidly dividing growth plate chondrocytes.

Phytoestrogens

There are three main classes of phytoestrogens: isoflavones, lignans, and coumestans. They are found principally in plants or their seeds, and a single dietary source often contains more than one class of phytoestrogens. Using a competition-binding assay, Kuiper *et al.* (1998) found that although the binding affinity of the phytoestrogens for either ERα or ERβ was lower than that of E, the relative binding affinity of phytoestrogens toward ERβ was significantly greater than that toward ERα. In contrast to phytoestrogens, the relative binding affinity of the SERMs, tamoxifen, and raloxifene, was greater for ERα than for ERβ. Using a reporter gene construct, the phytoestrogens tested were able to activate both ER subtypes, although the overall potency of these compounds was approximately 1/100th to 1/1000th that of E (Kuiper *et al.,* 1998).

The most convincing evidence for phytoestrogen effects on bone comes from animal studies. To date, there have been 13 published studies (11 in rats, 1 in mice, and 1 in macaques) examining effects of various phytoestrogens on bone loss, with generally positive findings. In addition to these animal studies, some preliminary studies in postmenopausal women demonstrate possible efficacy of phytoestrogens in the prevention of bone loss (Potter *et al.,* 1998). The principal issue is whether the actions of phytoestrogens on bone are identical to those of E or the SERMs. In support of this, both genistein in mice and coumestrol in rats prevented post-OVX bone loss by reducing bone resorption and osteoclast number in a manner very similar to E (Ishimi *et al.,* 1999; Draper *et al.,* 1997). However, in contrast, other studies have demonstrated that the reduction of bone loss following OVX in rats by genistein or by soy protein was associated with unchanged parameters of bone resorption but increased bone formation rates (Fanti *et al.,* 1998; Arjmandi *et al.,* 1998; Harrison *et al.,* 1998). These data thus indicate that there may be important differences between phytoestrogen and E effects on bone and, in fact, between the skeletal effects of different phytoestrogens. It has been suggested that some effects of phytoestrogens in different tissues may be due to ER-independent mechanisms, i.e., through protein tyrosine kinases or other mechanisms (Tham *et al.,* 1998).

Progesterone

Introduction

Progesterone is often given in conjunction with E during hormone replacement therapy of postmenopausal women to minimize some of the undesirable effects of E on reproductive tissues. However, the effects of P on bone physiology have not been examined to the large extent that E effects have been studied (for a review, see Prior, 1990). P exerts its effects on cells via mechanisms analogous to E; by binding and activating the progesterone receptor (PR), a member of the steroid nuclear receptor transcription factor family, which subsequently binds to the regulatory regions of target genes,

classically at progesterone response elements (PREs), to modulate gene transcription. Nongenomic effects of P in OBs have also been documented (Le Mellay and Lieberherr, 2000; Grosse *et al.,* 2000).

Progesterone Receptors in Bone Cells

In human, rat, and rabbit, PR exists as two isoforms, A and B (see Fig. 5), that are transcribed from the same gene using different promoters (Kastner *et al.,* 1990). Both PR promoters are estrogen inducible. The PR_B isoform is generally a stronger activator of gene transcription than PR_A, and in certain conditions, PR_A can reduce the transactivation stimulated by PR_B and other steroid receptors, including the ER (Giangrande *et al.,* 1999; Kraus *et al.,* 1995). Because of these important functional distinctions between the two PR isoforms, it is of interest to determine their relative expression level in target cells. The presence of PR mRNA and protein has been demonstrated in primary cultures of human OBs (Eriksen *et al.,* 1988) and in several OB cell lines (HOS TE85, MG-63, and SaOS-2) (Wei *et al.,* 1993; MacNamara *et al.,* 1995), including transcripts for both PR isoforms (MacNamara and Loughrey, 1998). In some cases, but not all, E stimulates the level of PR expression (Eriksen *et al.,* 1988; Harris *et al.,* 1995; MacNamara and Loughrey, 1988). Consequently, some of the effects on bone metabolism that have been attributed to E may in fact be mediated by P.

Effects of Progesterone on OBs and OCs

Progesterone has been reported to affect OB and OB precursor cell proliferation and differentiated functions, but some data are conflicting. Canalis and Raisz (1978) initially demonstrated that P inhibited proliferation and collagen synthesis by fetal rat calvarial cells. In contrast, Slootweg *et al.* (1992) found that whereas P alone did not affect proliferation of SaOS-2 cells, it stimulated cell growth synergistically with E. However, P alone has been found to stimulate the proliferation of TE85 osteosarcoma cells and normal human OBs (Tremollieres *et al.,* 1992). Other studies have shown stimulatory effects of P on the proliferation of normal and transformed osteoblastic cells (Scheven *et al.,* 1992; Verhaar *et al.,* 1994; Manzi *et al.,* 1994). More recently, P has been shown to increase the number of alkaline phosphatase-positive colonies—and hence osteogenic development—of a subclass of osteoprogenitor cells isolated from rat vertebral bone explants (Ishida and Heersche, 1997, 1999). P treatment of primary human OB and TE85 osteosarcoma cells was reported to increase IGF-II secretion (Tremollieres *et al.,* 1992) and inhibit IL-6 secretion in primary human OBs and mouse bone marrow stromal cells (Girasole *et al.,* 1992). P may regulate further aspects of the IGF system in bone because P stimulates expression of IGFBP-5 by a transcription-dependent mechanism in human osteosarcoma cells (Boonyaratanakornkit *et al.,* 1999). IGFBP-5 is unique among the various IGFBPs and *in vitro* it enhances the mitogenic activity of both IGF-1 and IGF-II in OBs.

Effects of Progesterone on Bone Physiology

ANIMALS

A number of experimental animal studies have indicated significant effects of P on bone metabolism. Thus, P has been shown to stimulate mineralization of newly induced bone in rats (Burnett and Reddi, 1983) and to increase the cortical bone formation rate in spayed Beagle dams (Snow and Anderson, 1985). Moreover, P was able to prevent ovariectomy-induced bone loss in rats, with inhibition of resorption indices, but persistently elevated bone formation rates (Barengolts *et al.,* 1990, 1996). In addition, the high circulating levels of P in pseudopregnant rats were associated with preservation of bone mass despite E levels comparable to ovariectomized rats (Bowman and Miller, 1996). However, these studies contrast with histomorphometric analyses, which have failed to detect a beneficial action of

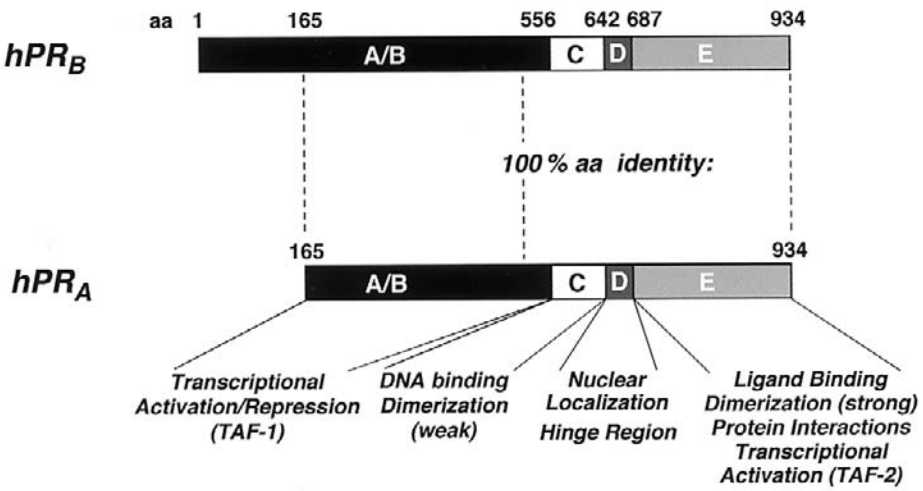

Figure 5 Model of human progesterone receptor isoforms with defined domain functions and homologies.

P on cancellous bone with or without coadministration of E (Kalu *et al.,* 1991; Fujimaki *et al.,* 1995). A more recent study in rats has reported that P has a significant stimulatory effect on bone formation and acts synergistically with estrogen to inhibit bone resorption (Schmidt *et al.,* 2000).

HUMANS

Despite some ongoing controversy (Waller *et al.,* 1996; De Souza *et al.,* 1997), the bulk of evidence from human studies is consistent with a significant role for P in bone metabolism. Thus, women with ovulatory disturbances related to luteal phase defects have been shown to lose bone mass over 1 year compared to women with normal menstrual cycles (Prior *et al.,* 1990) and to have increases in bone mass in response to P therapy during the luteal phase (Prior *et al.,* 1994). Several studies have found that P treatment of postmenopausal women protects against bone loss, particularly at cortical sites (McNeely *et al.,* 1991; Gallagher *et al.,* 1991; Grey *et al.,* 1996). Although some studies have shown that treatment of postmenopausal women with either P alone or combination therapy of E with P is as effective as E alone in the prevention of bone loss (McNeeley *et al.,* 1991; Grey *et al.,* 1996), other trials have demonstrated a lesser effect (PEPI, 1996; Prior *et al.,* 1997). Progesterone reduces postmenopausal bone loss in part by a suppression of bone resorption (Mandel *et al.,* 1982; Lobo *et al.,* 1984).

Conclusions

As described earlier, numerous major advances have occurred in recent years in the area of estrogen action on bone. It is now evident that E affects bone formation and bone resorption at all levels of cellular regulation: progenitor cell proliferation, differentiation, bone metabolism, and bone cell apoptosis. Consequently, the control by E of bone growth and remodeling at the tissue level is now realized to be extremely complex. How E elicits its effects within the cell, as well as the range of possible responses, is also more diverse than previously thought. The latter is due, in part, to the identification of the second ER isoform, ERβ, exhibiting an activity and expression distinct from the classical ERα. In addition, the presence of steroid receptor coregulators adds to the diverse responses. The differential expression and activities of the ER isoforms and the receptor coregulators provide explanations for the cell- and tissue-selective actions of the SERMs.

However, the answers to several important questions remain incomplete. For example, do the two ER isoforms regulate the same target genes? Are the two isoforms coexpressed in OBs and OCs? Does the relative ratio of ERα to ERβ change during differentiation and, if so, is E responsiveness affected? Is E responsiveness in OBs and OCs also determined by alterations in the relative levels of the steroid receptor coactivators and corepressors? Further analysis of the various ER mutant mice should provide answers as to whether the effects of E at particular skeletal sites (such as trabecular surfaces, periosteum, and growth plate) are mediated primarily by one or both ER isoforms. In addition, the generation of bone cell lines from each of the various ER-deficient genotypes should prove valuable in addressing many of the questions just posed.

Advances in our understanding of the actions and importance of P in bone growth and remodeling have, unfortunately, been more modest. The availability of PR-deficient mice will make it possible to examine the interaction between E and P in bone, such as the potential involvement of P and PR as regulators of certain skeletal effects of E. Lastly, and in analogy to studies with ERs, investigation into possible exclusive roles of the A and B isoforms of PR may reveal heretofore unrecognized effects of P on bone.

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CHAPTER 38

Selective Estrogen Receptor Modulators

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Introduction

Selective estrogen receptor modulators (SERMs) act via estrogen receptors to mediate estrogen agonist effects in some systems (e.g., inhibition of bone resorption) while exerting estrogen antagonist effects in other systems (e.g., inhibition of estrogen-dependent endometrial stimulation). Given the recently appreciated complexity of estrogenic mechanisms, it is not surprising that SERMs with differing profiles of target tissue action have been discovered. The major targets demonstrating this heterogeneity of ligand action are nonskeletal, and thus this chapter, while focusing on skeletal effects, discusses both skeletal and selected nonskeletal effects of SERMs in order to illustrate similarities and differences between SERMs. SERMs of primary interest are those that have been released for clinical use.

Mechanism of Action of SERMs

Mechanisms of Estrogen Action

As implied by their name, SERM effects are mediated through specific, high-affinity interactions with estrogen receptors, and thus a current understanding of SERM biology depends on insights developed from the rapidly evolving field of estrogen receptor biology. Estrogen receptors are members of a superfamily of nuclear transcription factors, comprising over 150 members. The superfamily consists of type I receptors responsible for steroid hormone action (estrogens, androgens, progestagens, glucocorticoids, and mineralocorticoids), type II receptors responsible for nonsteriodal hormone action (thyroid hormones, vitamin D receptor ligands, and retinoids), and so-called orphan receptors, which do not have identified cognate ligands (Tsai and O'Malley, 1994).

The "classical" model of estrogen action involves ligand binding to a nuclear estrogen receptor with a resultant conformational change in the receptor that alters its affinity for associated proteins (e.g., heat shock proteins), allowing dimerization of two liganded receptor monomers (Clark *et al.,* 1992). In turn, these dimers interact with transcriptional coactivators or corepressors (McKenna *et al.,* 1999) and then bind directly to specific DNA promoter sequences [estrogen response elements (EREs)], resulting in the modulation of transcription and translation of the corresponding gene products. The ERE promoter is involved in the activation of numerous classical estrogen-responsive genes, including many pathways involved in reproductive processes.

The potential clinical relevance of transcriptional modulators in steroid receptor action has been underscored by the report of a case of androgen insensitivity in a phenotypic female with normal levels of testosterone, as well as normal expression of unmutated androgen receptors. However, the subject's fibroblasts failed to show transcriptional activation in a reporter gene assay using a transfected androgen receptor derived from her own genetic material, from normal subjects, or from a subject with "classical" androgen insensitivity due to an androgen receptor mutation. Conversely, fibroblasts from either normal males or

the female with classical androgen receptor mutation demonstrated normal reporter gene activation using the androgen receptor derived from either normals or the subject with variant androgen insensitivity but not from the subject with androgen receptor mutation. The authors were able to show that the subject's defect likely results from a failure to respond to the transactivation signal from the AF-1 region of the androgen receptor (Adachi *et al.,* 2000). Although not yet described, it is likely that variable target tissue sensitivity to estrogen may also be due to variations in transactivation protein structure or function.

The basic model of steroid hormone action is still applicable to many of the actions of estrogens. However, progress in estrogen receptor biology has provided substantial detail at the molecular level in understanding this process, while also identifying new, alternate pathways of estrogen action. These new insights have provided a framework for understanding the seemingly contradictory abilities of SERMs to act as estrogen agonists in some systems yet to lack estrogen agonism in other systems while partially or completely antagonizing the action of estrogens themselves.

In addition to ERE-dependent pathways, it is now known that estrogens mediate gene transcription through a number of other promoter sequences, including the AP-1 promoter (Paech *et al.,* 1997), the retinoic acid receptor-α1 promoter (Elgort *et al.,* 1996), the transforming growth factor-β (TGF-β) promoter (Yang *et al.,* 1996a), the SF-1 response element (Vanacker *et al.,* 1999), the SP1 response element (Xie *et al.,* 2000), and the NF-κB response element (Inadera *et al.,* 2000). Also complicating the picture was the discovery of ERβ (Kuiper *et al.,* 1996), a distinct ER subtype with homology to the classical estrogen receptor, now known as ERα. Differential activation or inhibition of the various estrogen pathways, depending on which ligands (agonist, antagonist, or SERM), which receptor subtype (ERα or ERβ), and which coactivators and/or corepressors may be present in a given target, provides for a richly complex menu of tissue-specific estrogen responses. In terms of bone, these estrogenic responses lead to modulation of various cytokine levels, which leads to alterations in osteoblast and osteoclast function (see Chapter 39).

Antagonist Actions of SERMs

Antagonist actions of SERMs can now be explained at the molecular level. The key breakthrough which led to this understanding was the elucidation of the crystallographic structure of the ligand-bound estrogen receptor (Brzozowski *et al.,* 1997). The estrogen receptor consists of six sequence domains (A–F), which are associated with various functional correlates, including ligand binding (part of the E domain), DNA binding (the C and D domains), and coactivator/corepressor binding (the A/B domain, which contains the transactivation function-1 or AF-1 region, and the E domain, which contains the AF-2 region). AF-1 and AF-2 regions have been demonstrated via a variety of techniques to be important modulators of estrogen-responsive gene transcription. Thus, various mutagenesis experiments have demonstrated that modifications of the sequence of AF-2 can turn agonists into antagonists and vice versa (Montano *et al.,* 1996; Nichols *et al.,* 1998).

Crystallographic studies have demonstrated that the structures of the various functional regions of the estrogen receptor ligand-binding domain (LBD) are conferred by the three-dimensional conformation of the 12 α-helical segments of the LBD. Helices 1–11 of either the ERα or the ERβ LBD assume similar positions regardless of the ligand (estrogen or SERM) that is bound (Brzozowski *et al.,* 1997; Pike *et al.,* 1999). Of note, the ligand-binding pocket of the receptor is relatively promiscuous (Pike *et al.,* 1999), allowing high-affinity binding of a diverse group of compounds ranging from natural estrogens to several chemical classes of pharmaceutical SERMs (Fig. 1).

The ability of the ligand-binding pocket to accommodate molecules that possess bulky sidechain substituents is key to the antagonist action of many SERMs because binding of these compounds results in a repositioning of helix 12 compared to its position when estradiol is bound. When an estrogen such as diethylstilbesterol (DES) is bound to the receptor, helix 12 assumes a stable position, lying across the entry to the ligand-binding pocket (Fig. 2A, see also color plate). This appears to "lock" the ligand in place and, importantly, results in creation or completion of the AF-2 region of the receptor. As noted earlier, the AF-2 region is an area of the receptor that interacts with certain transcriptional coactivators, which, in turn, modulate estrogen target gene transcription. Thus, the appropriate nuclear receptor interaction domain of the coactivator (e.g., GRIP1 NR box II) makes contact via an LXXLL motif with helices 3, 4, 5, and 12 of the ER LBD (Fig. 2A).

In contrast, when a SERM such as raloxifene (Brzozowski *et al.,* 1997) or 4-hydroxytamoxifen (Shiau *et al.,* 1998) is bound, helix 12 repositions itself to occupy the hydrophobic cleft defined by helices 3, 4, and 5 (Fig. 2B, see also color plate), which alters the conformation of the AF-2 surface and blocks the hydrophobic cleft. An LXXML motif in helix 12 mimics the LXXLL motif of the p160 coactivator (e.g., GRIP1). This reorientation effectively prevents transcriptional modulators, such as the p160 coactivators, from interacting with the AF-2 surface. Conversely, certain corepressors, such as NCo-R, may be recruited to the SERM–ER complex (Jepsen *et al.,* 2000). Given that estrogen and SERMs compete for high-affinity binding to the same ligand-binding domain of the estrogen receptor, the SERM-mediated blockade of AF-2 by helix 12 results in antagonism of estrogen action on target genes that depend predominantly on AF-2.

Additional potential antagonist mechanisms of SERM modulation of estrogen activity have been described. As noted, estrogen receptors depend on dimerization in order for successful stimulation of classical gene targets. The synthetic "pure" estrogen antagonist ICI 164,384 destabilizes ER dimers (Arbuckle *et al.,* 1992) as well as the ER

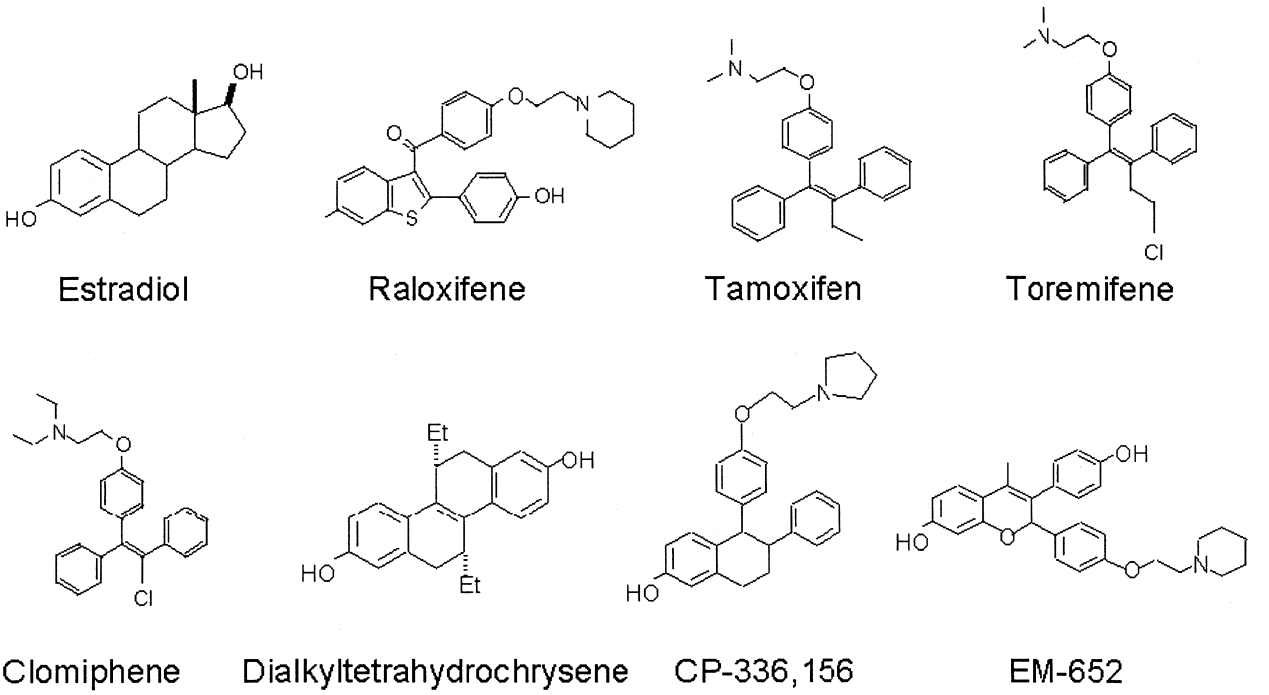

Figure 1 Chemical structures of estradiol and various SERMs representing several chemical classes, including benzothiophenes (raloxifene), triphenylethylenes (tamoxifen, toremifene, and clomiphene), chrysenes (dialkyltetrahyrdochrysene; Meyers *et al.,* 1999; Sun *et al.,* 1999), tetrahydronapthalenes (CP-336,156; Ke *et al.,* 1998), and benzopyrenes (EM-652; Labrie *et al.,* 1999).

protein itself, which may explain its potent antagonist activity *in vivo*. Tamoxifen may also destabilize ER dimers, which have been shown to reduce estrogen-stimulated gene transcription in a yeast model (Wang *et al.,* 1995). Further, ERα and ERβ are capable of forming heterodimers (Pace *et al.,* 1997) in which ERβ can act as a transdominant inhibitor of ERα activity under subsaturating hormone concentrations when both receptor subtypes are cotransfected into a test cell system (Hall and McDonnell, 1999). Given that ER receptor subtypes may respond differently to the

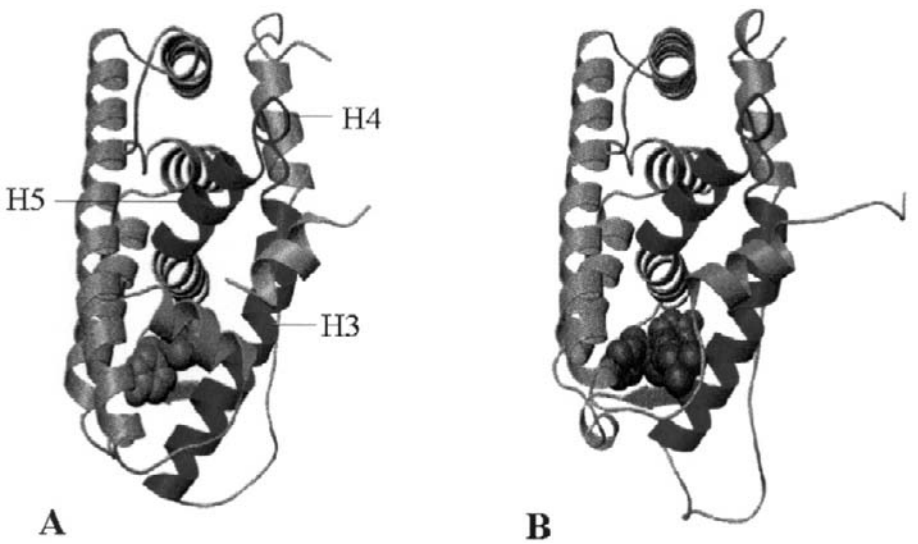

Figure 2 Structures of the DES-ERα LBD-GRIP1 NR box II peptide complex (A) and the OHT-ERα LBD complex (B) [adapted from Shiau *et. al.* (1998), with permission from Elsevier Science]. (A) The GRIP1 coactivator peptide and the DES-ERα LBD are shown as ribbon drawings. The peptide is shown in gold, and helix 12 (residues 538–546) is shown in magenta. Helices 3, 4, and 5 (labeled H3, H4, and H5, respectively) are colored blue. DES, colored green, is shown in space-filling representation. (B) OHT-ERα LBD is depicted as a ribbon drawing. As in A, helix 12 (residues 536–544) is shown in magenta, and helices 3, 4, and 5 are shown in blue. OHT, in red, is shown in space-filling representation. (See also color plate.)

same ligands (Paech *et al.,* 1997), the structural and/or functional interactions between the different ligand-bound receptor subtypes afford a mechanism by which SERMs may alter estrogen action.

Agonist Actions of SERMs

Whereas SERMs may act primarily in classical estrogen pathways as estrogen antagonists, they are known to have intrinsic estrogenic activity in nonclassical pathways. One such pathway (TGF-β) was identified as a candidate gene for estrogen pathways in bone because TGF-β has antiresorptive action and is stimulated *in vivo* by estrogen (Yang *et al.,* 1996b). Because of these properties, Yang *et al.* (1996a) investigated the molecular regulation of TGF-β transcription. Their results showed that whereas estradiol itself had little ability to stimulate TGF-β3 promoter sequence-dependent transcription in a transient transfection assay, raloxifene had potent activity in this regard. Interestingly, a variety of naturally occurring estrogen metabolites were also active, suggesting that this alternate pathway of estrogen action may well have physiological relevance under normal conditions. The lack of effect seen with estradiol itself suggests that the production and distribution of estrogen metabolites may provide regulatory control opportunities for estrogen action in bone (or elsewhere).

Yang *et al.* (1996a) expanded these studies by investigating the effects of using various deletion mutants of the estrogen receptor in the transfection assay. Raloxifene retained transcriptional stimulatory activity even when the DNA-binding domain of the estrogen receptor (but not the ligand binding domain) was deleted. This suggested that the classical ERE consensus sequence was not involved in this estrogen receptor-mediated transcriptional activity and that SERMs may exert some of their agonist activity by stimulating alternate estrogen pathways.

Work investigating the impact of AF-1 and AF-2 truncation mutations of a naturally occurring estrogen receptor mutant (D351Y) that confers estrogen agonist activity to raloxifene or tamoxifen has emphasized the importance of AF-1 in mediating agonist activity of SERMs (Webb *et al.,* 2000). In previous work using receptor chimeras, McInerney *et al.* (1998) demonstrated that whereas SERMs such as tamoxifen were antagonists using wild-type ERβ, these agents would act as agonists when the ERα receptor A/B domain was used to replace the N-terminal sequence of the ERβ receptor, again implicating AF-1 in SERM-mediated estrogen agonism. The extent to which the differences in AF-1 between ERα and ERβ have an impact on SERM activity *in vivo* is uncertain but one potential determinant of the clinical spectrum of activity of a SERM may be distribution of the estrogen receptor subtypes, which is known to vary from tissue to tissue (Couse *et al.,* 1997; Kuiper *et al.,* 1997).

Receptor subtype distribution and function may have specific importance in bone, as a knockout mutation of the two receptor subtypes has different phenotypic effects. ERα knockout female mice exhibit an increase in bone resorption and remodeling, an effect consistent with a role of ERα in the maintenance of skeletal dynamics (Couse and Korach, 1999). ERα knockout mice also demonstrate disproportional body growth, with decreased appendicular, but not axial, growth (Vidal *et al.,* 1999). ERβ knockout female mice have increased adult bone mass, which may be due to lack of maturation-associated inhibition of bone growth during adolescence (Windahl *et al.,* 1999), an effect not seen in ERα knockout female mice (Vidal *et al.,* 1999).

In the male, ERα plays an important role in human skeletal health; an ERα-deficient man was reported to suffer osteoporosis at age 28 (Smith *et al.,* 1994). ERα knockout male mice develop impairment of longitudinal skeletal growth, which is associated with decreased levels of IGF-1, an effect not seen in ERβ knockout male mice (Vidal *et al.,* 2000). The applicability of these mouse model findings to human physiology may be limited because of substantial differences in skeletal homeostasis between mice and men (Couse and Korach, 1999). However, because the effects of SERMs depend on the complement of estrogen receptor subtypes present in a target tissue, there may be clinical implications for SERM action (as yet unrecognized) that are attributable to variation in receptor subtype expression in bone.

Summary of SERM Mechanisms and Further SERM Development

SERMs are now recognized as a specific, new class of pharmacologic agents. Advances in receptor biology now allow coherent, if incomplete, models for how SERMs are able to act simultaneously as agonists, partial agonists, and/or antagonists in different tissues. These insights have been aided greatly by the crystallographic determinations of estrogen receptor structure when bound to one or another ligand (Fig. 2).

These advances may also pave the way for new drug discovery. As an example, coactivators that modulate transcription at the AF-2 region have been shown to depend on the sequence motif LXXLL for AF-2-binding activity (Chang *et al.,* 1999). More recently, Paige and colleagues (1999) demonstrated that peptides containing this motif demonstrate differential receptor-binding patterns ("fingerprints") depending on which ligand is bound to the receptor. This finding supported the previous hypothesis of McDonnell *et al.* (1995) that different SERMs result in ligand-specific differences in receptor conformation (subsequently directly demonstrated via crystallography). The relevance of the differential binding patterns of these peptides was underscored by further work, which demonstrated that cells that are induced to express LXXLL-containing peptides show modulation of estrogen and SERM transcriptional activity (Norris *et al.,* 1999).

These findings have since been extended to demonstrate that flanking sequences of the LXXLL motif also affect the transcriptional response to ligands (Chang *et al.,* 1999). By systematically studying a large panel ($>10^8$) of peptides synthesized by combinatorial methods, these investigators

were able to identify three classes of LXXLL peptides, one of which proved to have specific ERβ antagonist activity (Chang *et al.,* 1999). This approach, as well as others, may lead to the development of estrogen receptor subtype-specific SERMs. Indeed, Sun *et al.* (1999) and Meyers *et al.* (1999) have reported the development of ERα-specific agonist compounds (see Fig. 1 for an example structure). The clinical utility of these ER subtype-specific SERMs is uncertain, but such developments may result in increasingly selective clinical tools.

Physiologic Effects of SERMs

Animal Models

BONE

Tamoxifen was the first SERM to be used on a broad scale in the clinic. Originally identified as an "antiestrogen," it has more recently been recognized as a SERM. Initially developed in the mid-1960s (Harper and Walpole, 1966), tamoxifen was investigated for possible use in contraception. Because of its impact on the hypothalamic/pituitary/ovarian axis, it actually resulted in induction of ovulation (Klopper and Hall, 1971; Williamson and Ellis, 1973), and, in fact, a related compound (clomiphene) is used clinically for induction of ovulation. Following recognition of its antitumor activity in animal models (Jordan, 1974; Nicholson and Golder, 1975), it has been developed for treatment of metastatic breast cancer, for adjuvant treatment of early breast cancer, and for reduction in the incidence of primary breast cancer in high-risk women.

Early preclinical work on tamoxifen in bone models was based on the potential concern that an "antiestrogen" may result in harm to the female skeleton. However, tamoxifen was found to inhibit parathyroid hormone-mediated bone resorption in rat fetal long bones (Stewart and Stern, 1986). Further studies gave conflicting results, and a reduction in bone mass compared to vehicle was seen in a study of ovariectomized rats (Feldmon *et al.,* 1989), although bone mass was maintained in mature and ovariectomized rats in another study (Turner *et al.,* 1987). Additional conflicting data were adduced in studies of bone turnover: a study of calcitriol-induced bone resorption in rats failed to show a beneficial effect of tamoxifen (Goulding *et al.,* 1990), but another study showed reduction of bone resorption by tamoxifen in the ovariectomized rat (Turner *et al.,* 1988). Although mixed results were seen, it was clear that the initial fears that "antiestrogens" would have uniformly unfavorable skeletal effects were not borne out by these studies.

The most extensively studied SERM with respect to skeletal effects is raloxifene. Studies in ovariectomized rats have shown normalization of bone markers by raloxifene (Frolik *et al.,* 1996). Long term studies (up to 12 months) in ovariectomized rats showed raloxifene to be comparable to estrogen in inhibition of cancellous bone loss as determined by densitometry (Black *et al.,* 1994; Sato *et al.,* 1995) and by histomorphometry (Evans *et al.,* 1996). Raloxifene also preserved bone strength both at the femur and at the lumbar spine in ovariectomized rates as effectively as estrogen (Turner *et al.,* 1994). In a primate model (Jerome and Lees, 1996), raloxifene reduced bone turnover and increased bone mass relative to placebo, but the control ovariectomized animals in this study gained bone mass during the course of the experiment, suggesting that the model using recently captured feral animals may not be fully applicable to the human condition.

These preclinical bone results for tamoxifen and raloxifene (as well as results for other SERMs that have been in development) have supported estrogen agonist properties for these SERMs in this target system and, in the case of raloxifene, provided the basis to support a clinical development program for the prevention and treatment of postmenopausal osteoporosis.

CARDIOVASCULAR SYSTEM

Preclinical studies of tamoxifen have focused on effects of lipids, although it has also been characterized as an antioxidant (Wiseman, 1995). Studies of tamoxifen treatment in intact female rats fed an atherogenic diet showed reductions in LDL cholesterol from 29 to 45% (Vinitha *et al.,* 1997). In ovariectomized rats, tamoxifen reduced total cholesterol by approximately 50% (Sato *et al.,* 1996). However, a study of the dilator responses of atherosclerotic epicardial coronary arteries in cynomolgous monkeys to acetylcholine or nitroglycerin showed tamoxifen to be antiestrogenic. Whereas tamoxifen caused a constriction response to acetylcholine, there was no effect of estrogen. Additionally, estrogen caused a relaxation response to nitroglycerin; there was no similar response in tamoxifen-treated animals (Williams *et al.,* 1997).

Raloxifene has been studied systematically for preclinical effects on both lipid and nonlipid cardiovascular effects in animal systems. The results demonstrated a 50–60% reduction in total cholesterol by raloxifene in ovariectomized rats (Sato *et al.,* 1996). Raloxifene has also been shown to have potentially beneficial estrogenic activities in a number of nonlipid animal systems, including increasing rabbit coronary artery nitric oxide activity (Figtree *et al.,* 1999) and reducing neointimal thickening following balloon injury in ovariectomized rats (Kauffman *et al.,* 2000). Further, raloxifene reduced aortic cholesterol accumulation in cholesterol-fed rabbits by a mechanism that likely involved both lipid and nonlipid components (Bjiarnason *et al.,* 2000). In a cholesterol-fed primate model, raloxifene did not show a reduction in coronary atherosclerosis (Clarkson *et al.,* 1998), although a number of explanations related to the experimental conditions may explain this result (Bryant *et al.,* 1998).

The results of preclinical studies of these SERMs are generally consistent with potentially beneficial estrogen agonist activity in both lipid and nonlipid cardiovascular effects.

BREAST

Tamoxifen has been studied very extensively with regard to estrogen antagonist effects in a multitude of breast cancer models, including viral, chemical or radiation-induced carcinogenesis (Jordan *et al.,* 1980, 1993; Gottardis and Jordan, 1987; Welsch *et al.,* 1981). Of interest, long-term tamoxifen exposure may lead to resistance to or dependence on tamoxifen for further growth; the mechanistic basis for this resistance syndrome remains an issue of considerable interest, although comprehensive molecular and biochemical explanations are lacking. At least in a rat model, mutation of the estrogen receptor at the Asp-351 residue has been shown to convert SERMs from antagonists into agonists (Levenson and Jordan, 1998), although mutations of this type are uncommon in clinical cancer (Hopp and Fuqua, 1998). Raloxifene has been shown to markedly reduce the appearance of breast tumors in carcinogen-treated intact rats (Anzano *et al.,* 1996) and to block the proliferation of MCF-7 breast cancer cells, an estrogen-dependent process (Wakeling *et al.,* 1984).

REPRODUCTIVE TRACT

The reproductive tract is a target of particular interest because different SERMs have demonstrated markedly different biological effects in this system. As such, the reproductive tract should offer the possibility to identify, in a clinically relevant way, the mechanisms by which SERMs differ in their *in vivo* actions.

Tamoxifen has demonstrated uterine stimulatory effects in ovariectomized rats (Sato *et al.,* 1996), including modest increased uterine wet weight (25% compared to 300% for estrogen control), endometrial thickness (250%), and myometrial thickness (30%) with changes in endometrial histology consistent with a strong estrogenic effect (pseudostratified columnar epithelium). Similar results were reported by other authors (Ke *et al.,* 1997). Raloxifene demonstrates a substantially different profile (Sato *et al.,* 1996), with a modest increase in uterine wet weight (35%), but only the lowest dose of raloxifene showed a measurable increase of endometrial thickness (60% at a 0.1-mg/kg dose but no increase at 1.0- and 10-mg/kg doses). Also, in contrast to tamoxifen, raloxifene had no effect on myometrial thickness or on eosinophilic infiltratiion (a marker of estrogenic activity in the ovariectomized rat model). These data support that these two SERMs have differential profiles of estrogen receptor-mediated effects, with similar agonist effects in bone and lipid markers along with similar antagonist effects in breast models but divergent effects in the reproductive tract.

Clinical Data

Given the panoply of potential SERM uses, it is not surprising that these compounds have been considered for a variety of clinical indications. Approved clinical uses of SERMs include treatment and prevention of osteoporosis (raloxifene), treatment of breast cancer (tamoxifen, toremifene), reduction in risk of breast cancer in high-risk women (tamoxifen), and induction of ovulation (clomiphene). Many other potential indications for use (e.g., reduction in cardiovascular disease risk in women, treatment of mastalgia, endometriosis or uterine fibroids, multiple uses in men) have been considered or are under study for these or other, newer SERMs.

RALOXIFENE

Bone Studies Of the SERMs approved for human use, raloxifene has been studied to the greatest extent in the skeletal system, including studies of bone remodeling, bone turnover markers, bone histomorphometry, and vertebral fracture risk reduction. Using calcium tracer kinetics in a controlled environment, Heaney and Draper (1997) showed that either cyclic hormone replacement therapy or raloxifene (60 mg/day) led to a positive calcium balance and with reduction in bone resorption at both 4 and 31 weeks compared to untreated controls. The effect of estrogen on bone resorption was greater than that with raloxifene at the 31 week point. Calculated indices of bone formation showed no change with either agent at 4 weeks, but at 31 weeks this was reduced with hormone replacement therapy but not with raloxifene. These results suggested that hormone replacement therapy suppressed bone turnover to a greater extent than raloxifene, although the overall calcium balance was not significantly different between the therapies. Findings consistent with these were reported in a bone biopsy study comparing raloxifene to estrogen treatment (Prestwood *et al.,* 2000).

Several large studies of the effects of raloxifene on both bone turnover markers and bone density have been performed, both in women without osteoporosis and, among women with osteoporosis, in those with or without prevalent vertebral fracture. A study conducted in Europe (Delmas *et al.,* 1997) randomized 601 early postmenopausal women (2–8 years since last menstrual period) without osteoporosis by bone mineral density (BMD) criteria (lumbar spine BMD from 2.0 SD above to 2.5 SD below mean value for young adult normal females) to one of four treatment groups: 30, 60, and 150 mg/day of raloxifene or placebo. All women received 400–600 mg/day of supplemental elemental calcium; vitamin D supplements were not provided. Bone turnover markers were significantly suppressed compared to placebo (Fig. 3), with reductions at 24 months in the range of 15–35% for bone-specific alkaline phosphatase, serum osteocalcin, and urinary C-telopeptide of type I collagen. Raloxifene treatment brought these measurements down to approximately the middle of the normal premenopausal range. At 24 months the BMD in the raloxifene groups was significantly above placebo ($p < 0.001$) at the spine, hip, and total body, with an increment above placebo in the range of approximately 2 to 2.5% at all sites.

Similar results for bone turnover markers and BMD were seen in a study of somewhat older French women (Meunier *et al.,* 1999) with either low bone density or osteoporosis (average BMD T score $= -2.8$) who were

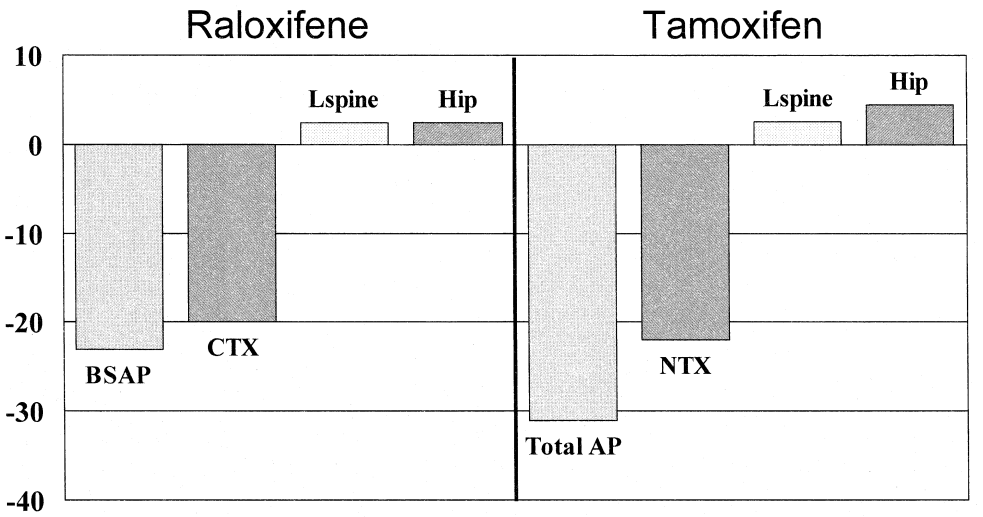

Figure 3 Comparison of the bone effects of 60 mg/day raloxifene (RLX) and 20 mg/day tamoxifen (TAM). Direct comparative data are not available; data presented are percentage difference from placebo at 2 years from separate studies (Delmas *et al.,* 1997, Chang *et al.,* 1996) showing results from similar assays. Generally similar effects are seen for these drugs on bone formation indices (bone-specific alkaline phosphatase shown for RLX, total alkaline phosphatase for TAM), bone resorption indices (C-telopeptide of type 1 collagen/creatinine ratio for RLX and N-telopeptide of type 1 collagen/creatinine ratio for TAM), and BMD response at the hip and spine. Insufficient fracture data for tamoxifen have been reported to allow comparison to raloxifene for this end point.

treated with either 60 or 150 mg raloxifene vs placebo; in this study, all subjects received both calcium and vitamin D supplements.

Two studies of women with osteoporosis have reported on the bone marker and BMD effects of raloxifene. The first (Lufkin *et al.,* 1998) was a small (143 subject) study of 1- year duration that randomized subjects to 60 or 120 mg of raloxifene versus placebo; all subjects received both calcium and vitamin D supplements and had osteoporosis by both BMD (hip or spine BMD below the 10th percentile for premenopausal females) and vertebral fracture criteria (one or more vertebral deformities with >15% height loss compared to adjacent vertebra). As in the previous studies, bone-specific alkaline phosphatase decreased approximately 10–15%, osteocalcin decreased approximately 15–20%, and urinary C-telopeptide of type I collagen/creatinine decreased by 24–30% compared to placebo. BMD at the spine, ultradistal radius, and hip showed modest increases at 1 year of about 1.5–2.5% compared to placebo.

The largest clinical bone study of raloxifene has been the Mulitple Outcomes of Raloxifene Evaluation (MORE), involving 7705 women from 25 countries (Ettinger *et al.,* 1999). The study was stratified into two parts: approximately one-third of women had prevalent vertebral fracture and approximately two-thirds of women had osteoporosis by BMD criteria alone (femoral neck or lumbar spine *T* score less than or equal to −2.5). Subjects were randomized to receive raloxifene 60 or 120 mg/day versus placebo; all subjects received both vitamin D and calcium supplementation. Results of bone markers at 36 months were consistent with the shorter term study findings, with reductions by both doses of raloxifene of about 20% for osteocalcin and 25% for C-telopeptide of type I collagen compared to placebo ($p<0.001$ for all comparisons). Three-year BMD results showed increases compared to placebo at both femoral neck and lumbar spine in the range of approximately 2–3% ($p<0.001$).

The MORE study was designed to evaluate vertebral fracture risk as its primary end point (Ettinger *et al.,* 1999). New vertebral fractures among women without prevalent fracture at study entry were reduced by 50% compared to placebo (2.3% cumulative incident fracture among raloxifene-treated women versus 4.5% for those on placebo); among those with prevalent fracture at study entry the reduction in new fractures was 30% (cumulative incident fracture among raloxifene-treated women was 14.7% for the 60-mg group and 10.7% for the 120-mg group versus 21.2% for those on placebo). These vertebral fracture reductions were statistically significant. A nonsignificant trend was seen for reduction of the secondary end point parameter of all nonvertebral fractures (9.3% cumulative incidence on placebo versus 8.5% cumulative incidence on raloxifene).

Cardiovascular End Point Studies A number of studies have looked at effects of raloxifene on surrogate markers for coronary heart disease. In a phase 1B, 8-week study of raloxifene 200 or 600 mg per day involving 251 women, serum LDL cholesterol decreased by 9.5 and 12.6%, respectively, compared to baseline (Draper *et al.,* 1996). The European osteoporosis prevention study of 601 women (Delmas *et al.,* 1997) in which 30, 60, or 150 mg of raloxifene was provided daily for 2 years showed reductions of total cholesterol (6.4 and 9.7%, respectively) and LDL cholesterol (10.1 and 14.1%) without significant changes in either HDL cholesterol or triglycerides with any dose of raloxifene.

The first focused clinical trial designed specifically to analyze the effects of raloxifene on cardiovascular surrogate markers was a 6-month study of 390 early postmenopausal subjects, comparing hormone replacement therapy, raloxifene 60 or 120 mg/day to placebo. (Walsh *et al.,* 1998). Raloxifene at either dose significantly reduced LDL cholesterol by 12% compared to placebo; a similar fall of 14% was seen with HRT. However, HDL cholesterol rose a significant 11% with HRT but did not change with raloxifene. HDL_2 cholesterol increased significantly by about 16% with raloxifene, compared with a significantly greater increase of 33% with HRT. Similarly, Lp(a) decreased a significant 4% compared to placebo with both doses of raloxifene, but it fell an even greater 16% with HRT. There was a significant (20%) increase in triglycerides with HRT, an undesirable effect, whereas triglycerides fell by 4% with 60 mg raloxifene. Finally, raloxifene lowered fibrinogen (12 and 14% with 60 and 120 mg/day, respectively), in contrast to no effect with HRT. Serum fibrinogen is an epidemiologically identified risk factor for coronary heart disease, although prospective studies demonstrating a clinical benefit of lowering fibrinogen have not been reported. Additional cardiovascular risk factors include homocysteine and C-reactive protein; raloxifene lowered homocysteine levels significantly and similarly to HRT (Walsh *et al.,* 2000), but unlike HRT, it did not increase C-reactive protein levels (Walsh *et al.,* 2000; Blum *et al.,* 2000). Raloxifene was qualitatively similar to estrogen in its modest lowering of several cell adhesion molecules in postmenopausal women (Blum *et al.,* 2000).

These studies of surrogate cardiovascular markers indicate that the SERM raloxifene has effects that are generally similar to estrogens (i.e., agonist actions), effects that would be predicted to have beneficial effects on clinical end points. In some instances, the magnitude of positive effects is less than that seen with estrogen [e.g., HDL, HDL-2, Lp(a)], but in some instances it is similar to or greater than HRT (e.g., LDL, fibrinogen, homocysteine). The ability of these types of studies to predict clinical outcomes is limited, as evidenced by the controversy surrounding the cardiovascular benefits and risks of estrogens themselves. A recent publication has demonstrated that the adverse results seen the Heart Estrogen/Progestin Replacement Study (HERS) of secondary event prevention are entirely compatible with prior observational data, which showed a substantial benefit conferred by estrogen use; this apparent contradiction is explainable if one hypothesizes an early, adverse effect of estrogen use followed by a late, sustained beneficial effect (Blakely, 2000). As in the case of estrogen, it is essential to conduct large-scale clinical end point trials to affirm the clinical effects of SERMs on the cardiovascular system. The ongoing Raloxifene Use in the Heart (RUTH) trial (Barrett-Connor *et al.,* 1998), which recently achieved its planned enrollment of 10,000 subjects, should provide definitive evidence regarding the cardiovascular effects of raloxifene in postmenopausal women at risk for cardiovascular events.

Effects of Raloxifene on the Breast The major osteoporosis trials (Delmas *et al.,* 1997; Meunier *et al.,* 1999; Lufkin *et al.,* 1998; Ettinger *et al.,* 1999) and the study of cardiovascular surrogate markers (Walsh *et al.,* 1998) have shown no increase in reports of mastalgia or other breast abnormalities with raloxifene compared to placebo. In the MORE study, breast cancer incidence was a specified secondary end point, and data reported through a median duration of 40 months (Cummings *et al.,* 1999) showed that raloxifene was associated with a 76% reduction (RR = 0.24; CI = 0.13–0.44). There was no difference in between the two dosage groups (60 and 120 mg/day). As would be expected for an estrogen receptor active agent, this therapy benefit was restricted to cases of estrogen receptor positive tumors (RR = 0.10; CI = 0.04–0.24); there was no increase in estrogen receptor negative tumors either (RR = 0.88; CI = 0.26–3.00). Patients enrolled in the MORE trial are being followed for an additional 4 years beyond its initial 4 year duration in the Continuing Outcomes for Raloxifene Evaluation (CORE) trial for the primary purpose of continuing to monitor breast cancer incidence with long-term raloxifene versus placebo. Additionally, a large (22,000 subject) study known as the Study of Tamoxifen and Raloxifene (STAR) comparing the breast cancer incidence in high risk postmenopausal women is currently enrolling.

Effects of Raloxifene on the Uterus Because of the differences noted between different SERMs in the preclinical model effects on the reproductive tract, it is critical to review the clinical effects of these agents on the uterus. The raloxifene clinical trials completed to date have enrolled well over 10,000 postmenopausal women, more than two-thirds of whom had an intact uterus at study entry. The results of several raloxifene clinical trials that employed systematic uterine monitoring (Delmas *et al.,* 1997; Lufkin *et al.,* 1998; Walsh *et al.,* 1998; Cohen *et al.,* 2000) were summarized (Cohen *et al.,* 2000; Davies *et al.,* 1999) to review its effects on the uterus in approximately 1500 postmenopausal women. There were no differences between raloxifene and placebo in relation to reports of vaginal bleeding or change in endometrial thickness as assessed by endometrial ultrasonography after 12, 24, or 36 months of treatment. Another study conducted in 415 postmenopausal women for 12 months (Goldstein *et al.,* 2000) also found no differences between raloxifene and placebo using the additional monitoring methods of saline-infused sonohysterography and scheduled, routine endometrial histologic sampling.

Systematic uterine monitoring was undertaken in a large subset of women in the MORE trial (Cummings *et al.,* 1999). Transvaginal ultrasound results showed a clinically insignificant (0.3 mm) increase in endometrial thickness in women treated with raloxifene compared to placebo. Importantly, there has not been an increase in reports of endometrial carcinoma in the large cohort (5957 subjects with an intact uterus at randomization); at 40 months of follow-up there had been four cases of endometrial cancer

reported in the placebo group versus six cases in the combined raloxifene groups for a RR = 0.8 (CI = 0.2–2.7).

Other Effects of Raloxifene Like estrogen (Daly *et al.,* 1996; Jick *et al.,* 1996; Grodstein *et al.,* 1996), raloxifene is associated with an approximate three-fold increased risk of venous thromboembolic disease (Cummings *et al.,* 1999). Although the mechanism of this risk is not fully understood, it would appear to be an estrogen agonist action shared by the major SERMs, including tamoxifen (Lipton *et al.,* 1984; Fisher *et al.,* 1998; Fisher and Redmond, 1992). In contrast to estrogen but similar to tamoxifen, raloxifene increases the risk of hot flashes (Delmas *et al.,* 1997; Ettinger *et al.,* 1999; Cohen and Lu, 2000), presumably as an estrogen antagonist effect at the hypothalamic/pituitary level.

In summary, raloxifene demostrates a SERM profile in the human, with major estrogen agonist actions in the bone and cardiovascular systems but major estrogen antagonist actions in the breast and uterus.

TAMOXIFEN

Bone Studies Because tamoxifen was originally identified as an "antiestrogen," it was initially assumed that it may have adverse effects on estrogen target tissues beyond the breast. However, cross-sectional and small prospective studies of tamoxifen (Ward *et al.,* 1993; Kristensen *et al.,* 1994; Gotfredson *et al.,* 1984; Love *et al.,* 1988; Fornander *et al.,* 1990; Fentiman *et al.,* 1989; Turken *et al.,* 1989; Ryan *et al.,* 1991) were reassuring in that no adverse effects on bone were demonstrated. The first sizable (140 postmenopausal women with node negative breast cancer) prospective, randomized, placebo-controlled 2-year-long clinical showed significant improvement in spinal bone density with tamoxifen, (10 mg twice daily) vs placebo (Love *et al.,* 1992a). Consistent with an estrogen agonist effect, markers of bone turnover, including serum osteocalcin and total alkaline phosphatase, decreased significantly ($p<0.001$) in response to tamoxifen. Lumbar spine BMD increased by 0.61% per year with tamoxifen compared with a decrease of 1.00% per year with placebo ($p<0.001$). Follow-up of this same cohort at 5 years showed the bone benefit of tamoxifen to be durable (Love *et al.,* 1994).

Further support for the beneficial effects of tamoxifen on the bone of postmenopausal women with breast cancer came from a study utilizing histomorphometry (Wright *et al.,* 1994). In this study, 21 women who had received at least 15 months of tamoxifen underwent transiliac bone biopsy, and these results were compared to 19 untreated controls. A significantly lower tissue-based formation rate and a longer remodeling period were noted in the treated women, consistent with an estrogen agonist action resulting in reduced bone turnover.

In addition to studies of women with breast cancer, tamoxifen has also been prospectively evaluated in women without breast cancer (Grey *et al.,* 1995a; Powles *et al.,* 1996). Among 57 women randomly assigned to 20 mg/day of tamoxifen versus placebo (Grey *et al.,* 1995a), bone turnover markers (serum alkaline phosphatase and urinary hydroxyproline, N-telopeptide of type I collagen and calcium) all declined significantly on tamoxifen compared to placebo. Also, lumbar spine BMD increased with tamoxifen treatment by 2.1% over placebo at 2 years, although there was no statistically significant difference between treatment groups at the proximal femur. In another study, 54 healthy postmenopausal women were randomized to 20 mg/day tamoxifen or placebo for 3 years; tamoxifen-treated women experienced 2–3% improvements in BMD at spine and hip by 3 years compared to small losses in women receiving placebo ($p<0.002$, spine; $p<0.05$, hip) (Powles *et al.,* 1996). This study also examined the effect of tamoxifen on the BMD of premenopausal women, which declined at both the hip and the spine compared to placebo. This latter effect is compatible with a partial estrogen agonist effect of tamoxifen on BMD.

As shown in Fig. 3, the bone metabolic effects of raloxifene and tamoxifen are generally similar (subject to the understanding that raloxifene has been systematically studied to a far greater degree in bone).

Effects of Tamoxifen on Fracture Risk A reduction in osteoporotic fracture has not been the primary end point of any tamoxifen studies to date. However, two studies have examined the effect of tamoxifen on fracture risk as a secondary end point. In the Danish Breast Cancer Cooperative Group (Kristensen *et al.,* 1996), 1716 high-risk women were randomized to no treatment or to radiation therapy and tamoxifen (30 mg per day). The study examined the occurrence of hip fractures during the first year of treatment; no data were obtained on vertebral deformities or other nonvertebral fractures. Overall, there was no difference in the rates of hip fracture between treatment groups, although a subset analysis showed an increase in intertrochanteric fracture on tamoxifen versus placebo during the first year (RR = 2.12; CI = 1.12–4.01). Clinical fracture occurrence was a secondary end point of the large Breast Cancer Prevention Trial (Fisher *et al.,* 1998) conducted by the National Surgical Adjuvant Breast and Bowel Group (NSABP). This trial randomized 13,388 high-risk women to either tamoxifen (20 mg/day) or placebo. During the 36-month follow-up of the trial there was no difference in the occurrence of all reported clinical fractures (483 subjects on placebo and 472 on tamoxifen). The treatment group difference approached statistical significance when only those fractures likely to be osteoporotic (hip, distal radius, and clinical spine fractures) were included in the analysis (RR=0.81; CI=0.63–1.05).

Effects of Tamoxifen on Cardiovascular End Points As in the case of bone studies, most data on lipid and cardiovascular end points with tamoxifen derive from breast cancer studies. Tamoxifen has been associated with reductions in total cholesterol and LDL cholesterol in the ranges of approximately

5–15% and 5–30%, respectively (Ilanchezhian *et al.,* 1995; Grey *et al.,* 1995b; Saarto *et al.,* 1996; Thangaraju *et al.,* 1994). Similar to raloxifene, the effect of tamoxifen on HDL has been neutral (Saarto *et al.,* 1996; Shewmon *et al.,* 1994; Biloma and Jordan 1996). Also similar to raloxifene, reductions in both fibrinogen (Love *et al.,* 1992b) and Lp (a) (Shewmon *et al.,* 1994) have been reported. Tamoxifen has either no effect (Grey *et al.,* 1995b) or increases triglycerides (Love *et al.,* 1990). This profile of activities is generally estrogen agonist and would be predicted to be generally favorable.

Data regarding tamoxifen effects on myocardial infarction come from breast cancer treatment and prevention trials, including the Scottish Cancer Trials Breast Group (McDonald and Stewar 1991), the Stockholm Breast Cancer Study Group (Rutqvist and Mattson, 1993), the NSABP B-14 trial (Costantino *et al.,* 1997), and NSABP BCPT (Fisher *et al.,* 1998). In the Scottish studies the risk of myocardial infarction was reduced significantly and there was a trend toward reduction of other ischemic events in tamoxifen users vs nonusers (McDonald and Stewart, 1991). The Swedish study also reported a significant reduction in cardiac disease with tamoxifen (Rutqvist and Mattson, 1993). The NSABP B-14 breast cancer treatment study showed a similar, but not statistically significant, 34% reduction in fatal heart disease (Costantino *et al.,* 1997). These breast cancer treatment studies are, however, confounded by a high death rate from breast cancer. The BCPT prevention trial did not show a reduced incidence of ischemic heart disease events, but this study was conducted in a younger population and the overall number of cardiac events was very low (Fisher *et al.,* 1998). As for both estrogens and other SERMs, definitive data supporting a cardiovascular benefit for tamoxifen have not been presented.

Effects of Tamoxifen on the Breast Tamoxifen has a widely studied and well recognized role in the treatment of breast cancer. This experience was summarized in an overview analysis (Early Breast Cancer Trialists' Collaborative Group, 1998) covering data on 37,000 women enrolled in 55 randomized clinical trials evaluating the effects of tamoxifen in patients with breast cancer. From the perspective of SERM biology, a key finding was the observation that tamoxifen benefits in breast cancer are restricted to cases of estrogen receptor-positive disease. Tamoxifen has been shown to have an optimum treatment duration for breast cancer treatment of 5 years; continuing the therapy longer results in a slight increase in recurrent tumor development compared to stopping after 5 years. This may be due to the emergence of tamoxifen-resistant (or, perhaps, tamoxifen-dependent) cell clones; the mechanism of this effect has not been clarified but may, at least in some cases, be attributable to somatic mutation of the estrogen receptor conferring an estrogen agonist profile to an antagonist drug.

Tamoxifen was the active treatment arm for the large NSABP BCPT study (Fisher *et al.,* 1998). This study showed that tamoxifen reduced the risk of invasive breast cancers by 49% and noninvasive cancers by 50% after 3 years of follow-up. Interestingly, this effect was approximately the same in magnitude for both pre- and postmenopausal women enrolled in this trial; this suggests a modulating role in the appearance of clinical breast cancers in both estrogen-replete and estrogen-deficient women. As seen in the MORE trial for raloxifene, there was no difference between placebo and tamoxifen in the incidence of estrogen receptor-negative tumors.

Results that conflict with the North American BCPT trial results have been reported from two smaller European breast cancer prevention studies (Powles *et al.,* 1998; Veronesi *et al.,* 1998). Many methodologic and study group differences between the trials (such as family history, use of HRT during trial, frequency of hysterectomy, loss to follow-up during study) could explain these differences, and the results of the North American study are generally considered to be sound. One area of much discussion is whether the results from studies like the MORE trial and the BCPT trial represent early treatment of preexisting lesions versus true "prevention" of cancer. Although the clinical implications of a substantial delay in progression of a preexisting lesion may well be beneficial, a true "prevention" effect would have a greater long-term health impact. A recent publication used modeling techniques to simulate tumor growth rate and thus to estimate the clinical appearance time of "new" versus "preexisting" tumors based on the BCPT clinical results (Radmacher and Simon, 2000), and the results suggested that the duration of the BCPT was sufficient for a substantial portion of the tumors that had been prevented from appearing in the tamoxifen arm had, indeed, truly been prevented from forming in the first place. As noted earlier, longer term observations of SERM treatment, as being conducted in the CORE trial, will provide further evidence to support or refute a true "prevention" effect of SERMs in breast cancer.

Effects of Tamoxifen on the Uterus Many years elapsed between the clinical introduction of tamoxifen and recognition of its potentially harmful effects in the uterus, largely due to a lack of systematic surveillance in early studies. It is now known that tamoxifen increases the risk of uterine bleeding and of both benign and malignant disease of the uterus (Fisher *et al.,* 1994, 1998; Cohen *et al.,* 1993; Cook *et al.,* 1995). Abnormalities such as adenomyosis, endometrial hyperplasia, and benign polyp formation are more common with tamoxifen treatment than with placebo treatment. Most importantly, the relative risk of endometrial cancer with tamoxifen treatment increases between 2.5- and 7.5-fold (Cosman and Lindsay, 1999). In the BCPT (Fisher *et al.,* 1998), the relative risk for uterine cancer was 2.53 ($p < 0.05$). In this study, the excess incidence of these cancers was only in women over age 50 (i.e., postmenopausal women). The endometrial cancers associated with tamoxifen use generally cause symptoms early (i.e., vaginal bleeding), and thus all of the endometrial cancers seen in the tamoxifen treatment arm in the BCPT were identified at an early stage of disease.

Other Effects of Tamoxifen Like raloxifene and estrogen, tamoxifen is associated with an approximate three-fold increase in the incidence of venous thromboembolism (Fisher and Redmand, 1992; McDonald *et al.,* 1995). Tamoxifen also increases the occurrence of hot flashes in both pre- and postmenopausal women (Fisher *et al.,* 1998). There also appears to be increased risk (RR 1.14, 95% CI 1.01–1.29) of cataract development with tamoxifen (Fisher *et al.,* 1998); the relation of this finding to the SERM profile is uncertain and may be an unrelated pharmacologic effect of tamoxifen.

TOREMIFENE

Toremifene is another clinically available SERM that has been characterized with respect to its estrogen target tissue pharmacologic profile. Toremifene is a tamoxifen analog (Fig. 1) that has been approved for treatment of breast cancer. Compared to tamoxifen, toremifene is less prone to DNA adduct formation (Hellmann-Blumberg *et al.,* 1998); in clinical use it demonstrates breast cancer efficacy similar to that seen for tamoxifen (Gershanovich *et al.,* 1997). Very limited bone and lipid testing has been published (Saarto *et al.,* 1996; Marttunen *et al.,* 1998), showing that toremifene exhibits expected SERM effects to reduce total and LDL cholesterol and to increase BMD.

Summary and Conclusions

SERMs represent a recently recognized pharmacologic class of compounds that share some of the agonist actions of estrogen but antagonize estrogen in other contexts. SERMs have been shown to be useful alternatives in the treatment of a variety of health conditions, including infertility, osteoporosis, and breast cancer. SERMs that have been introduced into clinical use show some heterogeneity in their SERM profile of action, with the most marked differences noted in the effects on the reproductive tract (Table I). These differences, coupled with insights into SERM action at the mechanistic level, provide assurance that this area will be a fertile field for the development of new compounds with newly targeted profiles of SERM action in the future.

Table I Clinical Safety of Raloxifene and Tamoxifen

	Raloxifene	Tamoxifen
Hot flashes	↑	↑
VTE risk	↑	↑
Endometrial thickness	↔	↑
Endometrial cancer risk	↔	↑
Leg cramps	↑	↑
Cataract risk	0	↑

Comparison of the safety of raloxifene and tamoxifen. Data are summarized from various studies and do not represent direct comparison (see text).

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CHAPTER 39

Mechanisms of Estrogen Action in Bone

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Introduction

Postmenopausal osteoporosis is a heterogeneous disorder characterized by a progressive loss of bone tissue that begins after natural or surgical menopause and leads to fracture within 15–20 years from the cessation of the ovarian function.

Although suboptimal skeletal development ("low peak bone mass") and age-related bone loss may be contributing factors, a hormone-dependent increase in bone resorption and an accelerated loss of bone mass in the first 5 or 10 years after menopause appear to be the main pathogenetic factors (Riggs and Melton, 1986a,b) of this condition. That estrogen deficiency plays a major role in postmenopausal bone loss is strongly supported by the higher prevalence of osteoporosis in women than in men (Nilas and Christiansen, 1987), the increase in the rate of bone mineral loss detectable by bone densitometry after artificial or natural menopause (Genant *et al.,* 1982; Riggs *et al.,* 1981; Slemenda *et al.,* 1987), the existence of a relationship between estrogen levels and rates of bone loss (Johnston *et al.,* 1985; Ohta *et al.,* 1993; Ohta *et al.,* 1992), and the protective effect of estrogen replacement with respect to both bone mass loss and fracture incidence (Ettinger *et al.,* 1985; Horsman *et al.,* 1983; Lindsay *et al.,* 1980). The potential fracture risk for any postmenopausal female depends on the degree of bone turnover, the rate and extent of bone loss, associated disease processes that induce bone loss, age of menarche and menopause, and bone mass content achieved at maturity. The latter depends on the extent of estrogen exposure, habitual physical activity, quantity of calcium intake, and genetic predisposition. The manner with which the genetic "signal" conditions those biological mechanisms that are essential to achieve peak bone mass in adolescence is still unknown, although evidence shows that low peak bone mass may be linked to a particular vitamin D receptor phenotype (Morrison *et al.,* 1994).

The bone-sparing effect of estrogen is mainly related to its ability to block bone resorption (Manolagas and Jilka, 1995), although stimulation of bone formation is likely to play a contributory role (Bain *et al.,* 1993; Chow *et al.,* 1992). Estrogen-dependent inhibition of bone resorption is, in turn, due to both decreased osteoclastogenesis and diminished resorptive activity of mature osteoclasts. However, inhibition of osteoclast formation is currently regarded as the main mechanisms by which E_2 prevents bone loss (Manolagas and Jilka, 1995; Pacifici, 1996).

Cells and Cytokines That Regulate Osteoclast Formation

Osteoclasts arise by cytokine-driven proliferation and differentiation of hematopoietic precursors of the monocytic lineage. This process is facilitated by bone marrow stromal cells (Fig. 1), a population that provides physical support for nascent osteoclasts (OC) and produces soluble and membrane-associated factors essential for the proliferation and/or the differentiation of osteoclast precursors (Roodman, 1996). Lymphocytes of both T and B-cell lineages also contribute to the regulation of osteoclastogenesis, especially in stimulated conditions. For example, during inflammation, activated T cells assume a key role in stimulating osteoclast formation and do so by producing potent membrane-bound

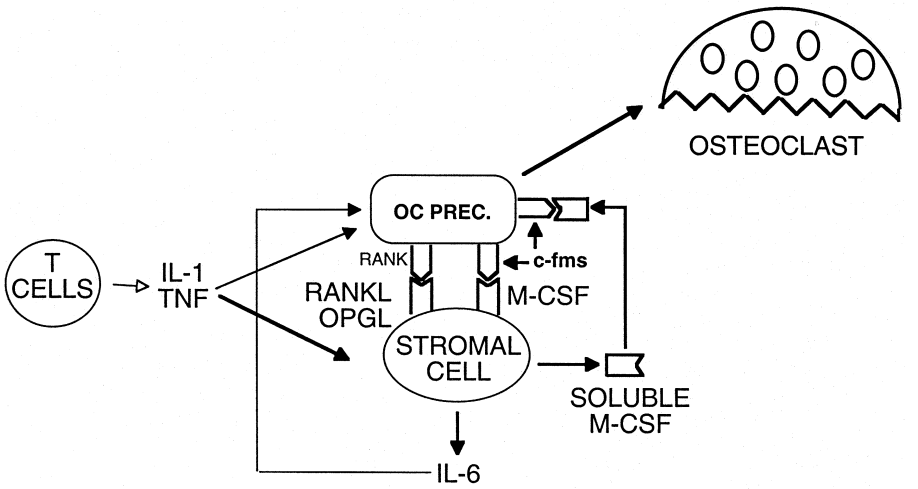

Figure 1 Cells and cytokines critical for osteoclast formation. Estrogen decreases osteoclast formation by downregulating the monocytic production of IL-1 and TNF and the stromal cell production of M-CSF and IL-6.

and soluble cytokines (Kong *et al.,* 1999a). B cells have been reported to have complex and controversial effects on osteoclastogenesis. For example, B-cell-deficient mice have been found to display decreased trabecular area and increased bone resorption, as compared to B-replete mice of the same strain (Dissanayake *et al.,* 1997), suggesting that B cells inhibit bone resorption and osteoclastogenesis. In contrast, other studies have shown that estrogen deficiency upregulates B lymphopoiesis in the bone marrow (Erben *et al.,* 1998; Masuzawa *et al.,* 1994), suggesting that cells of the B lineage may contribute to the increased OC production characteristic of estrogen-deficient animals. In humans, B cells inhibit OC formation, as they are an important source of TGFβ, a factor that inhibits osteoclast formation by inducing apoptosis of early and late osteoclast precursors and mature osteoclasts (Weitzmann *et al.,* 2000).

Among the cytokines involved in the regulation of osteoclast formation are receptor activator NFκB ligand (RANKL) (also known as OPGL, TRANCE, or ODF) and M-CSF (Kong *et al.,* 1999b; Lacey *et al.,* 1998; Macdonald *et al.,* 1986; Suda *et al.,* 1992; Tanaka *et al.,* 1993; Yasuda *et al.,* 1998). These factors are produced primarily by stromal cells, osteoblasts, and activated T cells (Kong *et al.,* 1999a).

RANKL is a member of the TNF family, which exists in a membrane-bound and in a soluble form. RANKL binds to the transmembrane receptor RANK, which is expressed on the surface of osteoclasts and osteoclast precursors of the monocytic lineage (Lacey *et al.,* 1998). RANKL binds also to OPG, a soluble decoy receptor produced by numerous hematopoietic cells. Thus, OPG, by sequestering RANKL and preventing its binding to RANK, functions as a potent antiosteoclastogenic cytokine (Simonet *et al.,* 1997).

In the presence of M-CSF, RANKL induces the differentiation of monocytic cells into osteoclasts (Lacey *et al.,* 1998) by activating the MAP kinase JNK, an enzyme that enhances the production of two essential osteoclastogenic transcription factors: c-*Fos* and c-*Jun* (Hsu *et al.,* 1999). RANKL binding to RANK also activates NF-κB, a family of transcription factors essential for osteoclast formation and survival.

In physiological conditions, M-CSF and RANKL are the only factors produced in the bone marrow in an amount sufficient to induce osteoclast formation. Thus, M-CSF and RANKL are regarded as true essential physiologic osteoclastogenic cytokines. The critical role of each of these cytokines in the osteoclastogenic process is demonstrated by the finding that deletion of either gene prompts osteopetrosis due to the absence of osteoclasts, a circumstance reversed by administration of the relevant cytokine (Felix *et al.,* 1990; Kodama *et al.,* 1991; Kong *et al.,* 1999b).

M-CSF induces the proliferation of early osteoclast precursors, the differentiation of more mature osteoclasts, and the fusion of mononucleated preosteoclasts and increases the survival of mature osteoclasts (Fuller *et al.,* 1993; Sarma and Flanagan, 1996; Suda *et al.,* 1999). RANKL does not induce cell proliferation, but promotes the differentiation of osteoclast precursors from an early stage of maturation to fully mature multinucleated osteoclasts. RANKL is also capable of activating mature osteoclasts, thus rendering these cells capable of resorbing bone.

While consensus exists that RANKL stimulates bone resorption in organ cultures, the effect of M-CSF on bone resorption is controversial, as both inhibitory and stimulatory effects on bone resorption have been reported (Edwards *et al.,* 1998; Fuller *et al.,* 1993; Hattersley *et al.,* 1988; Lees and Heersche, 1999; Sarma and Flanagan, 1996; Suda *et al.,* 1999).

Monocytes, stromal cells, osteoblasts, and lymphocyte produce inflammatory cytokines, which have direct proosteoclastogenic effects. Among these factors are IL-1, IL-6, IL-11, and TNF (Bertolini *et al.,* 1986; Canalis, 1986; Girasole *et al.,* 1992; Gowen *et al.,* 1983, 1985; Jilka *et al.,* 1992; Lorenzo *et al.,* 1987; Passeri *et al.,* 1993; Stashenko

et al., 1987; Thomson *et al.,* 1987). These factors stimulate osteoclast formation by increasing the stromal cell production of RANKL (Hofbauer *et al.,* 1999b; O'Brien *et al.,* 1999; Yasuda *et al.,* 1998) and M-CSF (Fibbe *et al.,* 1988; Thery *et al.,* 1992). Another factor relevant for osteoclastogenesis is TGFβ. This cytokine stimulates OPG production (Takai *et al.,* 1998), thus inhibiting osteoclast formation.

While in physiological conditions, IL-1, IL-6, and TNF are produced in the bone marrow at low concentration and their bone marrow levels increase both during inflammation and in conditions of estrogen deficiency (Manolagas and Jilka, 1995; Pacifici, 1996). Thus, IL-1, IL-6, and TNF play a critical role in enhancing osteoclast production, survival, and activity in pathological conditions.

Kobayasi *et al.* (2000) demonstrated that TNF, in the presence of M-CSF, induces the differentiation of monocytes into mature multinucleated osteoclasts (through NF-κB and JNK activation), which are, however, incapable of resorbing bone. Neither IL-1 nor IL-6 is capable of directly promoting the differentiation of osteoclast precursors into multinucleated osteoclasts. However, the addition of IL-1 to cultures of osteoclasts generated using TNF and M-CSF induces the capacity of resorbing bone and increases their survival.

It has been demonstrated that T cells from ovariectomized animals release increased amounts of TNF and that T cell-produced TNF synergizes with RANKL, thus potentiating osteoclast formation (Cenci *et al.,* 2000b).

Thus, TNF is a true osteoclastogenic cytokine, which can induce osteoclast formation via a direct effect on osteoclast precursors and by synergizing with RANKL. In contrast, IL-1 is incapable of inducing osteoclast formation, although it promotes osteoclast activation and survival.

Because M-CSF and RANKL are present in the bone marrow in physiological conditions, osteopetrosis is not a feature of transgenic mice lacking the capacity of producing and/or responding to IL-1, IL-6, or TNF (Ammann *et al.,* 1997; Lorenzo *et al.,* 1998; Poli *et al.,* 1994). Thus, IL-1, IL-6, and TNF stimulate osteoclastogenesis in pathological conditions, but are not essential for baseline osteoclastogenesis.

In summary, accumulated data demonstrate that RANKL and M-CSF are the only two factors known at the present time that are absolutely critical for osteoclast formation in physiological conditions. In contrast, the inflammatory cytokines IL-1, IL-6, and TNF are not essential for the maintenance of baseline osteoclastogenesis, although they are key for enhancing osteoclast formation and osteoclast activity during inflammation (Isomaki and Punnonen, 1997; Suda *et al.,* 1999) and in conditions of E_2 deficiency (Manolagas and Jilka, 1995; Pacifici, 1996).

Effects of Estrogen on the Production of Osteclastogenic Cytokines

It is now recognized that estrogen downregulates the production of several proosteoclastogenic factors, including IL-1, IL-6, TNF, M-CSF, and PGE_2. In addition, estrogen stimulates the production of important antiosteoclastogenic factors, including IL-1ra (Pacifici *et al.,* 1993), OPG (Hofbauer *et al.,* 1999a), and TGFβ (Oursler *et al.,* 1991).

The cytokines first recognized to be regulated by estrogen were IL-1 and TNF. This observation was prompted by the finding that monocytes of patients with "high turnover" osteoporosis, the histological hallmark of postmenopausal osteoporosis, secrete increased amounts of IL-1 (Pacifici *et al.,* 1987). Cross-sectional and prospective comparisons of pre- and postmenopausal women revealed that monocytic production of IL-1 and TNF increases after natural and surgical menopause and is decreased by treatment with estrogen and progesterone (Pacifici *et al.,* 1989, 1990). Subsequent observations showed that the postmenopausal increase in IL-1 activity results from an effect of estrogen on the production of both IL-1β and IL-1ra (Pacifici *et al.,* 1993). Studies in normal women undergoing ovariectomy (ovx) (Fiore *et al.,* 1993; Pacifici *et al.,* 1991) revealed that estrogen withdrawal is associated not only with an increased production of IL-1 and TNF, but also of GM-CSF. Changes in these cytokine levels occur in a temporal sequence consistent with a causal role of IL-1, TNF, and GM-CSF in the pathogenesis of ovx induced bone loss (Pacifici *et al.,* 1991).

Estrogen and progesterone have been shown to decrease the secretion of IL-1 from peripheral blood and bone marrow monocytes and to decrease the steady-state expression of IL-1 mRNA in monocytes (Polan *et al.,* 1988). However, the exact molecular mechanism by which E_2 decreases IL-1 production remains to be determined. Estrogen has been shown to increase the expression of the decoy type II, IL-1 receptor in bone marrow cells and osteoclasts (Sunyer *et al.,* 1997). Thus, upregulation of cell responsiveness to IL-1 via downregulation of IL-1RII is also likely to be a key mechanism by which estrogen deficiency induces bone loss.

The mechanism by which estrogen represses TNF gene expression has been found to involve ERβ and AP-1 (Fig. 2). ERα and ERβ respond differently to ligands, leading to opposite effects on AP-1-induced gene expression (Paech *et al.,* 1997). Specifically, whereas ERα-mediated effects of E_2 lead to stimulation of AP-1-induced gene expression, E_2 acts as a repressor of AP-1-induced transcription when bound to ERβ (Paech *et al.,* 1997).

Cells of the monocytic lineage are known to express both ERα and ERβ. Estrogen binding to ERβ leads to decreased activation of the Jun terminal kinase (JNK), a phenomenon that leads to decreased production of c-Jun and JunD, two members of the AP-1 family of transcription factors (Srivastava *et al.,* 1999). Decreased AP-1-production results in decreased AP-1-induced TNF gene expression and lower TNF production (An *et al.,* 1999; Srivastava *et al.,* 1999)

Studies conducted to determine if estrogen regulates the production of IL-6 revealed that in murine stromal and osteoblastic cells, IL-6 production is inhibited by the addition of estrogen (Girasole *et al.,* 1992) and is stimulated by estrogen withdrawal (Passeri *et al.,* 1993).

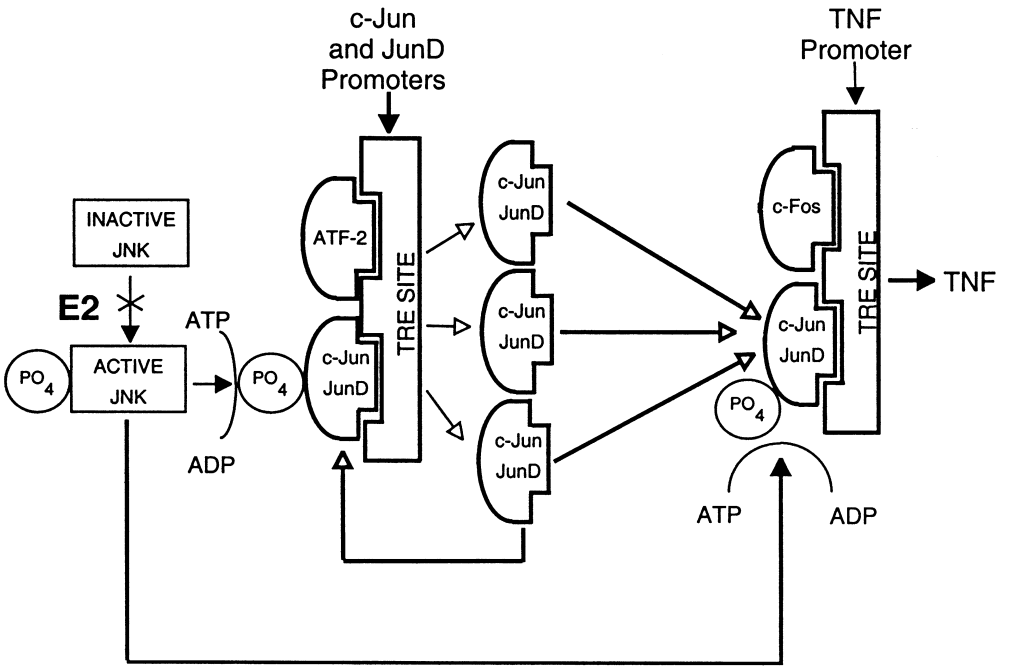

Figure 2 Mechanism by which estrogen represses TNF gene expression. By blocking JNK activation, estrogen blunts the autostimulation of the Jun promoter, thus decreasing the production of Jun. JNK inhibitions also decrease the phosphorylation in the N terminus of Jun bound to the TNF promoter, thus repressing its transcriptional activity.

In vivo studies also revealed that the production of IL-6 is increased in cultures of bone marrow cells from ovx mice (Jilka *et al.,* 1992). This effect is mediated, at least in the mouse, by an indirect effect of estrogen on the transcription activity of the proximal 225-bp sequence of the IL-6 promoter (Pottratz *et al.,* 1994; Ray *et al.,* 1994)

Interestingly, although studies with human cell lines demonstrated inhibitory effects of estrogen on the human IL-6 promoter (Stein and Yang, 1995), three independent groups have failed to demonstrate an inhibitory effect of estrogen on IL-6 production from human bone cells and stromal cells expressing functional estrogen receptors (Chaudhary *et al.,* 1992; Rickard *et al.,* 1992; Rifas *et al.,* 1995). These data raise the possibility that the production of human IL-6 protein does not increase in conditions of estrogen deficiency. This is further supported by a report that surgical menopause in humans is not followed by an increase in IL-6, although it causes an increase in the soluble IL-6 receptor (Girasole *et al.,* 1995).

Studies have unveiled that one of the key mechanism by which estrogen regulates osteoclastogenesis is by modulating the stromal cell production of M-CSF.

In conditions of E_2 deficiency, the high bone marrow levels of IL-1 and TNF lead to the expansion of a stromal cell population that produces larger amounts of soluble M-CSF (Kimble *et al.,* 1996). These high M-CSF-producing stromal cells have an increased capacity to support osteoclastogenesis (Fig. 3). Interestingly, estrogen has no direct regulatory effects on the production of soluble M-CSF as it regulates M-CSF secretion exclusively by conditioning the differentiation of stromal toward a phenotype characterized by a lower production of M-CSF. The high M-CSF-producing stromal cells found in estrogen-deficient mice are characterized by increased phosphorylation of the transcription factor Egr-1.

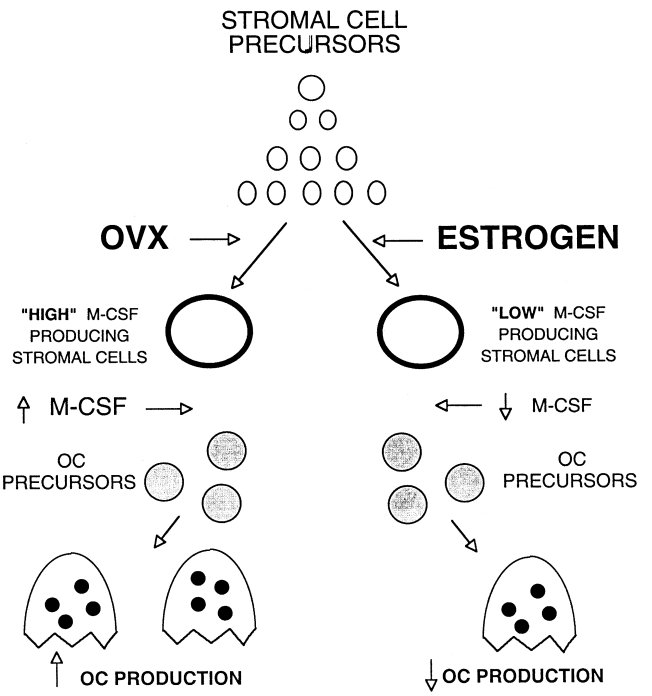

Figure 3 Estrogen regulates the differentiation of stromal cell precursors and leads to the formation of "low" M-CSF-producing stromal cells.

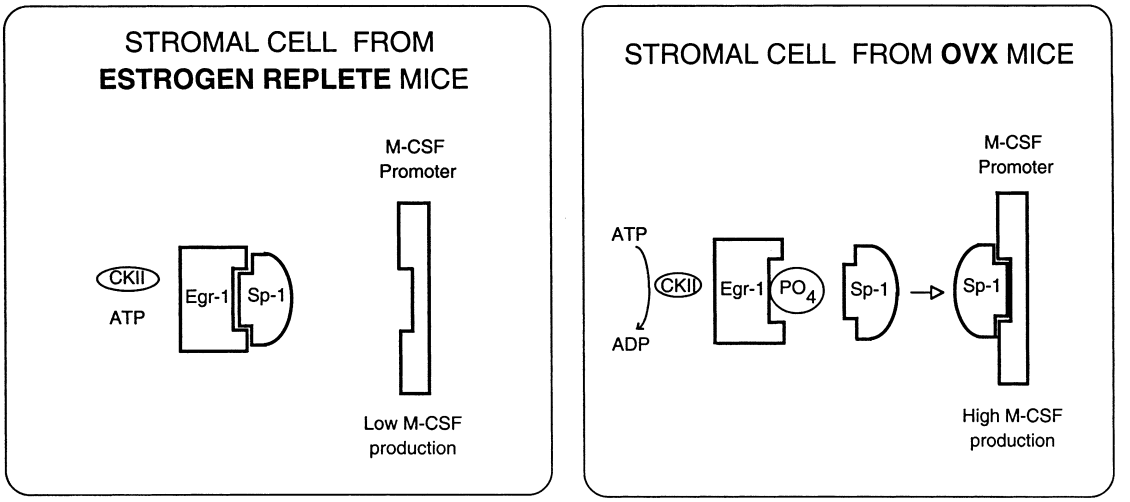

Figure 4 Mechanism by which stromal cells from estrogen-deficient mice produce low levels of M-CSF. Stromal cells from estrogen-deficient mice exhibit increased CKII-dependent phosphorylation of the nuclear protein Egr-1. Phosphorylated Egr-1 binds less avidly to the transcriptional activator Sp-1, and the resulting higher levels of free Sp-1 stimulate M-CSF gene expression.

While Egr-1 binds and sequesters the nuclear protein Sp-1, phosphorylated Egr-1 does not bind to Sp-1. As a result, cells from estrogen-deficient mice are characterized by increased levels of free Sp-1. This protein binds to the M-CSF promoter and stimulates M-CSF gene expression (Srivastava *et al.,* 1998) (Fig. 4).

In addition to an indirect effect on soluble M-CSF, E_2 has been shown to decrease the production of membrane-bound M-CSF via a direct effect on bone marrow cells (Lea *et al.,* 1999; Sarma *et al.,* 1998). However, the source of membrane-bound M-CSF under estrogen regulation remains to be defined. Regardless of the specific cell involved, estrogen regulates this key osteoclastogenic cytokine by at least two distinct mechanisms.

Little information on the effects of menopause on the production of RANKL is currently available. However, the promoter region of the RANKL gene does not contain regions known to be repressed (directly or indirectly) by estrogen (Kitazawa *et al.,* 1999). Therefore it is likely that future studies will confirm the preliminary observation available at the moment, which indicates that estrogen does not regulate RANKL. In contrast, estrogen has been shown to increase the production of OPG in osteoblastic cells (Hofbauer *et al.,* 1999a). Thus, estrogen enhancement of OPG secretion by osteoblastic cells is likely to represent another major mechanism in explaining the antiresorptive action of estrogen on bone.

Another possible intermediary in estrogen action is transforming growth factor β (TGFβ). This growth factor is a multifunctional protein that is produced by many mammalian cells, including osteoblasts, and has a wide range of biological activities. TGFβ is a potent osteoblast mitogen (Oursler, 1994). In specific experimental conditions, TGFβ decreases both osteoclastic resorptive activity and osteoclast recruitment.

Oursler *et al.* (1991) have reported that estrogen increases the steady-state level of TGFβ mRNA and the release of TGFβ protein. This mechanism provides the first example of "positive" effects of estrogen in bone, which may result in decreased bone turnover.

Effects of Menopause on the Production of Bone-Resorbing Cytokines

The abundance of *in vitro* studies that demonstrated the potent effects of IL-1, TNF, and IL-6 on bone prompted a series of investigations on the relationship among bone remodeling, cytokine production, and osteoporosis. These studies were conducted using cultures of peripheral blood monocytes because these cells, when cultured in polystyrene plates with ordinary tissue culture medium (which contains small amounts of LPS), express IL-1 and TNF mRNA and secrete small quantities of IL-1 and TNF protein (Dinarello, 1989; Fuhlbrigge *et al.,* 1987; Kitazawa *et al.,* 1994).

Another reason that prompted investigators to select this model is that the secretion of cytokines from peripheral blood monocytes reflects the secretory activity of bone marrow mononuclear cells (Kitazawa *et al.,* 1994). This is not surprising because the *in vitro* production of cytokines from cultured monocytes is a reflection of phenotypic characteristics acquired in response to local stimuli during their maturation in the bone marrow, and these characteristics are maintained after release into the circulation (Witsell and Schook, 1991). This phenomenon is thought to play an important role in providing the basis for tissue and functional specificity. Consequently, monocyte cytokine secretion is relevant to postmenopausal bone loss, as it mirrors cytokine secretion from marrow resident cells of the

monocytemacrophage lineage or monocytes that have homed to bone (Horowitz, 1993).

It is also important to recognize that monocytes are the major source of IL-1 and TNF in bone marrow (Dinarello, 1989). Moreover, the anatomical proximity of mononuclear cells to remodeling loci, the capacity to secrete numerous products all recognized for their effects in bone remodeling, and the expression of integrin receptors (Hynes, 1992), which make these cells capable of adhering to the bone matrix, make them likely candidates as participants in skeletal remodeling.

Investigations of monocyte production of IL-1 led to the discovery that monocytes of patients with "high turnover" osteoporosis secrete higher IL-1 activity than those from patients with "low turnover" osteoporosis and, indeed, those from normal subjects (Pacifici *et al.,* 1987). Because increased bone turnover is characteristic of the early postmenopausal period, these data suggested the hypothesis that the bone-sparing effect of estrogen is related to its ability to block the production of IL-1 from cells of the monocytic lineage. This hypothesis was first tested on studies designed to investigate the effect of natural menopause and estrogen/progesterone replacement on the monocytic production of IL-1. Results showed that IL-1 activity increases after menopause in both normal and osteoporotic women. However, whereas IL-1 activity in normal women returned spontaneously to premenopausal levels within 7 years after menopause, in osteoporotic subjects the increase in IL-1 activity lasted up to 15 years after menopause (Pacifici *et al.,* 1989). As a result, the finding of increased IL-1 activity 8–15 years after menopause is characteristic of women with postmenopausal osteoporosis. Data also showed that treatment of women in both the early and the late postmenopausal periods with estrogen and progesterone normalizes IL-1 activity within the first month of treatment. Similar effects of menopause have also been documented for TNF and GM-CSF. The latter is a cytokine recognized as a potent stimulator of osteoclastogenesis (Pacifici *et al.,* 1990).

The increased production of cytokines associated with estrogen withdrawal occurs in a time fashion consistent with a direct causal role of these factors in postmenopausal bone loss. This was demonstrated by analyzing the time course of changes in cytokine secretion and markers of bone turnover in normal women undergoing bilateral ovariectomy. Using this strategy, it was demonstrated that the monocytic secretion of GM-CSF increases within 1 week after ovariectomy. This is followed by a marked increase in TNF and IL-1 at 2 weeks post surgery. The increase in the latter two cytokines is associated with a concurrent increase in biochemical indices of bone resorption (Pacifici *et al.,* 1991). Initiation of estrogen replacement therapy at 1 month after ovariectomy results in the rapid normalization of cytokine production (Pacifici *et al.,* 1991). Subsequent studies confirmed that natural and surgical menopause are associated with an increased production of IL-1 and TNF from peripheral blood and bone marrow monocytes (Fiore *et al.,* 1993; Kimble *et al.,* 1994; Matsuda *et al.,* 1991; Pioli *et al.,* 1992). An increased mononuclear cell production of IL-6 has also been reported after ovariectomy (Pioli *et al.,* 1992). Because IL-1 and TNF are powerful stimulators of IL-6 production (Chaudhary *et al.,* 1992; Lacey *et al.,* 1993), the latter is likely to reflect the impact of the higher levels of IL-1 and TNF induced by ovariectomy.

That the increased monocytic production of cytokines plays a direct role in inducing bone resorption was later demonstrated by Cohen-Solal *et al.* (1993), who examined the bone resorption activity of monocyte supernatants obtained from pre- and postmenopausal women. Using this approach, it was found that culture media of monocytes obtained from postmenopausal women have a higher *in vitro* bone resorption activity than that from either premenopausal women or estrogen-treated postmenopausal women. The increased bone resorption activity of media from postmenopausal subjects is blocked by the addition of IL-1ra and anti-TNF antibody.

Studies by Suda and co-workers (1999) of bone marrow supernatants from estrogen-deficient mice have also indicated that IL-1 plays a dominant role in mediating the impact of estrogen withdrawal on bone resorption. Antibodies against IL-1α (the dominant IL-1 species in mice) but not antibodies against many other cytokines completely blocked the bone resorption activity of the monocyte-conditioned medium. Antibodies IL-1β, IL-6, and IL-6 receptor resulted in a partial neutralization of bone resorption activity.

Thus, it is likely that IL-1 and TNF account for most of the resorption activity produced by cultured monocytes. Yet undetermined is whether this effect is direct or mediated by other factors produced in response to IL-1 and TNF.

More direct evidence in favor of a cause–effect relationship between increased production of IL-1 and TNF (and IL-6) and postmenopausal osteoporosis is also provided by findings that IL-1, TNF, and IL-6 mRNAs are expressed more frequently in bone cells from untreated postmenopausal women than in those from women on estrogen replacement.

Effect of Menopause on the Production of IL-1 Receptor Antagonist

IL-1 bioassays are based on the measurement of the proliferation of IL-1-dependent cell lines. Thus, IL-1 bioactivity is stimulated by IL-1 and inhibited by IL-1ra. Therefore, IL-1 bioactivity reflects closely the IL-1/IL-1ra ratio. Because mammalian cells secrete IL-1ra along with IL-1, the regulatory effects of ovarian steroid on IL-1 bioactivity may involve both IL-1 (α or β) and IL-1ra. Studies have addressed this issue and revealed that estrogen and progesterone downregulate the production of both IL-1β and IL-1ra (Pacifici *et al.,* 1993). In contrast, estrogen and progesterone have no inhibitory effects on the secretion of

IL-1α. Interestingly, in normal women, the decrease in IL-1 bioactivity that accompanies the passage of time since menopause is associated with a parallel increase in the secretion of IL-1ra. Thus, in normal women, the increasing production of IL-1ra that accompanies the passage of time since menopause is likely to help restore normal monocytic IL-1 bioactivity after menopause. IL-1 is a powerful autocrine factor. In fact, IL-1 produced by monocytes binds to IL-1 receptors expressed on the monocyte surface and further stimulates IL-1 secretion (Dinarello *et al.,* 1991). Because this process is inhibited by IL-1ra, the progressive postmenopausal increase in IL-1ra secretion observed in nonosteoporotic women may also help explain the parallel decrease in the secretion of IL-1β observed in these subjects as time elapses from menopause.

As discussed earlier, in osteoporotic women the production of IL-1 bioactivity is increased for a length of time twice as long as in normal women. This is associated with an increased secretion of IL-1β, which persists as long as the increase in IL-1 bioactivity. Interestingly, the levels of IL-1ra measured in osteoporotic women are higher than those of normal women, but do not change with the passage of time since menopause (Pacifici *et al.,* 1993). Thus, in osteoporotic women, IL-1 bioactivity appears to be regulated primarily by changes in the production of IL-1β.

Because only a small fraction of the cytokines released into the bone microenvironment escape into the systemic circulation, studies based on the measurement of serum cytokine levels have been, for the most part, unrewarding. However, the development of supersensitive cytokine assays has made it possible to document that the serum IL-1/IL-1ra ratio is significantly higher in women with postmenopausal osteoporosis than in their normal counterparts (Khosla *et al.,* 1994). The use of these sensitive assays has also led to the demonstration that the rate of bone loss in osteoporotic women correlates inversely with serum IL-1ra levels (Hannon *et al.,* 1993). Taken together, these data indicate that a modulatory action of estrogen and progesterone on the secretion of IL-1ra contributes to the events of the menopause and the effects of hormone replacement on IL-1 bioactivity.

The molecular mechanism by which estrogen and menopause regulate the monocytic production of IL-1ra remains to be defined. The local microenvironment is known to condition the production of IL-1ra. For example, alveolar macrophages from patients with interstitial lung disease produce more IL-1ra than those from normal controls (Galve-de Rochemonteix *et al.,* 1992). It is likely, therefore, that the increased bone resorption induced by IL-1 and other cytokines after menopause may lead to the release of factors in the bone microenvironment that, in turn, stimulate the secretion of IL-1ra. One such a factor is TGFβ (Arend, 1991), a constituent of the bone matrix released locally upon activation of osteoclastic bone resorption (Oreffo *et al.,* 1989; Pfeilschifter *et al.,* 1988). Differences in the secretory pattern of IL-1ra observed between normal and osteoporotic women could, indeed, result from the more intense bone resorption and the resulting higher release of TGFβ that characterize the postmenopausal period of women with osteoporosis (Delmas, 1988).

Since an altered T4/T8 lymphocyte ratio (Imai *et al.,* 1990; Rosen *et al.,* 1990) and abnormal mixed leukocyte reactions have been reported in osteoporotic patients (Duke-Cohan *et al.,* 1985), it is conceivable that specific monocyte phenotypes characterized by the ability to produce constitutively high amounts of IL-1ra may be expressed preferentially in osteoporotic patients. Should this be the case, the difference in IL-1ra levels observed between normal and osteoporotic patients could be related to intrinsic differences in the prevailing monocyte population.

Effects of Ovariectomy on the Response of Maturing Osteoclasts to Osteclastogenic Cytokines

Osteoclastic differentiation of monocytic cells is driven by engagement of the receptors c-fms and RANK by M-CSF and RANKL, respectively. Thus one additional mechanism by which estrogen may repress osteoclast formation is by diminishing the responsiveness of maturing osteoclasts to stimulatory cytokines.

While no effects of estrogen have been described on the expression of c-fms or M-CSF signaling, estrogen appears to be capable of decreasing the responsiveness of maturing osteoclasts to RANKL. In fact, in vitro estrogen treatment decreases RANKL-induced osteoclast formation by about 50% (Shevde *et al.,* 2000). This phenomenon is a result of the ability of estrogen to repress RANKL-induced JNK activation and the consequent diminished production of c-Jun and c-Fos. However, it should be emphasized that the contribution of this mechanism to the regulation of osteoclast formation *in vivo* remains to be determined.

Cytokine Inhibitors and Transgenic Mice: Tools for Investigating the Contribution of Candidate Factors to Ovariectomy-Induced Bone Loss

Because several cytokines are under hormonal control and exhibit overlapping biological effects, analysis of cytokine expression and secretion in bone and bone marrow cells is unlikely to provide definite evidence in favor of a cause–effect relationship between increased cytokine production and postmenopausal bone loss. However, direct demonstration that cytokines mediate the impact of estrogen deficiency on bone can be achieved with the use of genetic models and specific cytokine antagonists, such as the IL-1 antagonist, IL-1ra, and the TNF antagonist, TNF-binding protein (TNFbp).

Lorenzo *et al.* have shown that mice insensitive to IL-1 due to the lack of IL-1 receptor type I are protected against ovx-induced bone loss. These findings confirmed earlier studies conducted by treating ovariectomized rats with IL-1ra beginning either at the time of surgery (early

postovariectomy period) or 4 weeks later (late postovariectomy period) (Kimble *et al.,* 1994). These experiments revealed that the functional block of IL-1 has distinct effects in both periods. In fact, in the second month after ovariectomy, treatment with IL-1ra completely blocked bone loss, duplicating the effect of estrogen. In contrast, in the first month after ovariectomy, bone loss was completely prevented by estrogen replacement therapy and decreased IL-1ra treatment by about 40%. These findings indicated that cytokines produced independently of IL-1 contribute to induce bone loss in the early postovariectomy period. Because IL-1 and TNF have powerful additive and synergistic effects in many systems, TNF appeared to be the most likely candidate factor. That TNF contributes to bone loss in the early postovariectomy period was demonstrated by treating ovariectomized rats with IL-1ra, TNFbp, and a combination of the two inhibitors for 2 weeks starting at the time of surgery (Kimble *et al.,* 1995). This critical experiment demonstrated that while treatment with either IL-1ra or TNFbp alone partially prevented ovariectomy-induced bone loss, complete bone sparing was achieved when ovariectomized rats were treated simultaneously with IL-1ra and TNFbp.

Histomorphometric studies also demonstrated important effects of IL-1ra and TNFbp on bone formation (Kimble *et al.,* 1994, 1997). Two weeks after surgery there were no significant differences in trabecular and cortical bone formation rate between ovariectomy and sham-operated rats. Interestingly, however, treatment with either IL-1ra or TNFbp induced a marked increase in bone formation rate in ovariectomized but not in sham-operated rats. This suggests that inhibition of endocortical bone formation resulting from high levels of IL-1 and TNF (characteristic of the early postovariectomy period) counteracts and masks direct stimulatory effects of ovariectomy on bone formation. In contrast, in the late postovariectomy period, bone formation is increased in the trabecular but not in the cortical bone, and in this time period neither IL-1ra nor TNFbp has significant effects on this index.

Thus, when taken together, data support the hypothesis that estrogen deficiency modulates bone resorption via an IL-1/TNF-dependent pathway and bone formation via a complex mechanism that involves an IL-1/TNF-independent stimulatory effect and an IL-1/TNF-mediated inhibitory effect. Early after ovariectomy the dominant phenomena mediated by IL-1 and TNF are the stimulation of osteoclast activity and the inhibition of bone formation. As time progresses from ovariectomy, the IL-1- and TNF-dependent inhibition of bone formation subsides while the most important effect of these factors become the induction of osteoclastogenesis.

These initial observations about the causal role of TNF were confirmed by Ammann *et al.* (1997), who reported that transgenic mice insensitive to TNF due to the overexpression of soluble TNF receptor are also protected against ovx-induced bone loss (Ammann *et al.,* 1997). Finally, an orally active inhibitor of IL-1 and TNF production was also shown to completely prevent bone loss in ovx rats (Bradbeer *et al.,* 1996).

Although the finding that functional block of either IL-1 or TNF is sufficient to prevent ovx-induced bone loss may appear to be difficult to explain, it should be emphasized that in most biological systems, IL-1 and TNF have potent synergistic effects. Thus, the functional block of one of these two cytokines elicits biological effects identical to those induced by the block of both IL-1 and TNF. The long-term stimulation of bone resorption that follows ovx is sustained primarily by an expansion of the osteoclastic pool. Because OC formation is stimulated synergistically by IL-1 and TNF (Kitazawa *et al.,* 1994), it is not surprising that long-term inhibition of either IL-1 or TNF results in complete prevention of ovx-induced bone loss.

Although evidence has accumulated that demonstrates that TNF plays a key role in the pathogenesis of ovx-induced bone loss, cells producing TNF have been scarcely defined. We have found that T cells in ovx mice are the major source of TNF. Ovariectomy enhances the T-cell production of TNF, which, acting through the TNF receptor p55, augments M-CSF and RANKL-induced osteoclastogenesis (Cenci *et al.,* 2000b). Attesting to the relevance of this phenomenon *in vivo*, ovariectomy fails to induce bone loss and stimulate bone resorption (Fig. 5) in T-cell-deficient mice. These data establish T cells as essential mediators of the bone-wasting effects of estrogen deficiency *in vivo* (Cenci *et al.,* 2000b). TNF potentiates the response of maturing osteoclasts to RANKL because TNF and RANKL synergistically activate the NF-κB and JNK pathways (Lam *et al.,* 2000).

While studies with transgenic mice and inhibitors of IL-1 and TNF have consistently demonstrated that IL-1 and TNF are key inducers of bone loss in ovx animals, investigations aimed at assessing the contribution of IL-6 to ovx-induced bone loss have yielded conflicting results. In favor of a causal role for IL-6 in ovx-induced bone loss is the report of Poli *et al.* (1994) indicating that IL-6 knockout mice are protected against the loss of trabecular bone induced by ovx. Against a significant pathogenetic role of IL-6 are studies demonstrating that osteoporosis is not a feature of transgenic mice overexpressing IL-6 (Kitamura *et al.,* 1995). Studies have also been conducted by injecting an antibody-neutralizing IL-6 in ovx mice. Neutralizing IL-6 prevents the increase in OC formation induced by estrogen deficiency (Jilka *et al.,* 1992; Kimble *et al.,* 1997), but does prevent ovx-induced bone loss and does not decrease *in vivo* bone resorption (Kimble *et al.,* 1997). These findings confirm that IL-6 contributes to the expansion of the osteoclastic pool induced by ovx. However, this cytokine does not appear to be the dominant factor in inducing bone loss in estrogen-deficient mice.

Studies have been conducted to elucidate the relevance of M-CSF in the pathogenesis of ovx-induced bone loss *in vivo*. In agreement with the key role of M-CSF in osteoclastogenesis, these studies have demonstrated that the functional block of M-CSF by the anti-M-CSF antibody 5A1

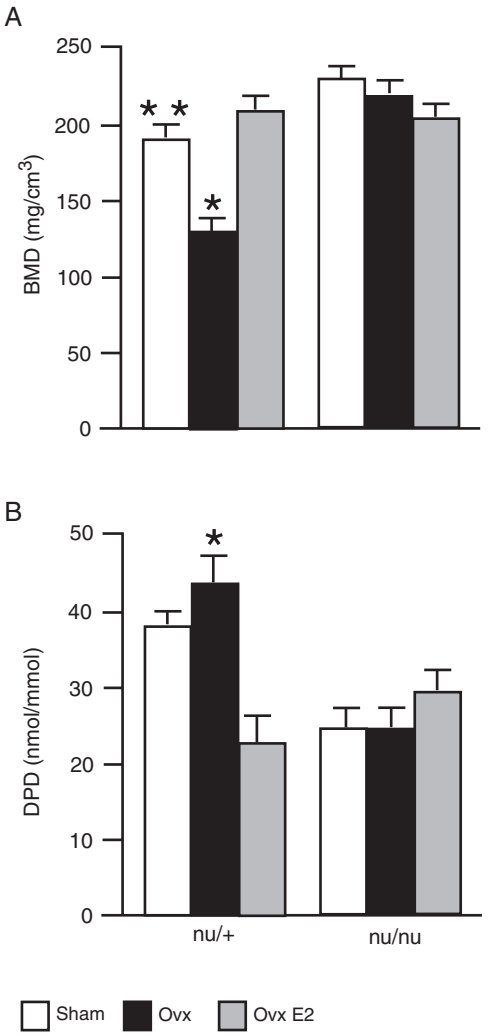

Figure 5 Ovariectomy fails to induce bone loss and upregulate DPD excretion in nude mice ($n = 6$ per group). $^{*}p < 0.05$ compared to all other groups. $^{*}p < 0.05$ compared to sham nu/nu. (A) Trabecular BMD (mean ± SEM) of the tibia was measured 4 weeks after surgery in excised tibiae by pQCT. (B) DPD excretion (mean ± SEM) 4 weeks after surgery.

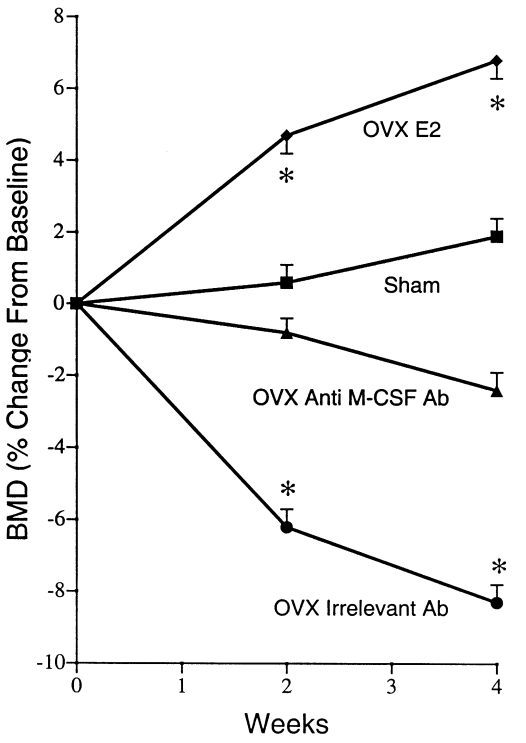

Figure 6 Treatment with anti-M-CSF Ab 5A1 Ab prevents ovx-induced bone loss. Results (mean ± SEM) are expressed as percentage change from baseline. $^{*}p < 0.05$ compared to baseline and to any other group.

completely prevents ovx-induced bone loss in mice (Fig. 6) (Cenci *et al.,* 2000a). That M-CSF is another cytokine that plays a key role in ovx-induced bone loss was further demonstrated by examining mice lacking the transcription factor Egr-1 (Wilson *et al.,* 1992). Egr-1-deficient mice produce maximal amounts of M-CSF both in the presence and in the absence of estrogen (Srivastava *et al.,* 1998). Thus, Egr-1-deficient mice have high M-CSF stromal cell production, increased osteoclastic bone resorption, and low bone density (Cenci *et al.,* 2000a). Furthermore, ovx does not further stimulate osteoclast formation in these mice, as it fails to further enhance M-CSF production. Importantly, Egr-1-deficient mice are completely protected against ovx-induced bone loss, a finding that confirms the relevance of M-CSF (Cenci *et al.,* 2000a).

No studies have been conducted to determine the effects of ovx in mice insensitive to RANKL, although one would predict that these animal will sustain significant bone loss due to the stimulated production of TNF.

The role of IL-1, IL-6, TNF, M-CSF, and RANKL in osteoclastogenesis has been directly investigated using murine bone marrow cultures and RANKL-stimulated monocytes obtained from ovariectomized mice. Ovariectomy not only increases the number of bone marrow cells, but also increases the number of osteoclasts generated by *ex vivo* cultures of bone marrow cells (Kalu, 1990). IL-1ra and TNFbp both completely prevent the increase in osteoclastogenesis induced by ovariectomy (Kimble *et al.,* 1997). *In vivo* treatment of ovx mice with the anti-M-CSF antibody prevents the effects of ovx on *ex vivo* osteoclast formation and bone resorption (Cenci *et al.,* 2000a) in a manner similar to treatment with IL-1 and TNF antagonists. These data are consistent with the notion that estrogen deficiency increases M-CSF production indirectly via an IL-1- and TNF-mediated mechanism (Kimble *et al.,* 1996; Srivastava *et al.,* 1998).

Osteoclast formation is also decreased, in part, by the anti-IL-6 antibody 20F3. However, the anti-IL-6 antibody is less effective than IL-1ra and TNFbp (Kimble *et al.,* 1997). Another important difference between these inhibitors is that *in vivo* treatment with IL-1ra and TNFbp also decreases the urinary excretion of DPD in a manner similar to estrogen, whereas the anti-IL-6 antibody does not. In contrast, when *in vitro* bone resorption is evaluated by examining the effects of the three inhibitors on the formation of resorption

lacunae, it appears that IL-1ra, anti-IL-6 antibody, and TNFbp all inhibit the formation of resorption pits (Kitazawa *et al.,* 1994).

Because the regulatory role of IL-6 is limited to the initial steps of the osteoclast differentiation process (Roodman, 1992), it could be that the block of IL-6 *in vivo* is insufficient to prevent the complete maturation and activation of those cells that are downstream with respect to the IL-6-dependent steps. According to this hypothesis, the lack of change in DPD excretion with anti-IL-6 antibody treatment would reflect the maintenance of an unaltered pool of active, mature osteoclasts. Conversely, the decreased pit formation observed with the IL-6 block is likely to reflect the decreased bone marrow content of osteoclast precursors and the resulting decrease in the number of cells that reach functional maturity *in vitro* (Suda *et al.,* 1992). From these data it appears reasonable to hypothesize that inhibition of IL-1 and TNF blocks bone resorption *in vivo* and *in vitro* because, at least in rodent, these cytokines regulate early and late steps of osteoclast maturation.

Studies have demonstrated that the presence of severe osteopetrosis is due to the complete lack of osteoclasts in mice lacking either RANKL or the RANKL receptor RANK. However, the effects of ovariectomy and/or estrogen deficiency in these animals remain to be investigated.

Summary and Conclusions

The mechanism(s) of the bone-sparing effects of estrogen appears to be particularly complex as it involve the regulated production of cytokines from hematopoietic cells and bone cells (Horowitz, 1993; Turner *et al.,* 1994) and the responsiveness of stromal cells to these cytokines. In addition, the contribution of specific factors to postmenopausal bone loss appears to vary as the system adapts over time to the hormonal withdrawal. Although many details of this process remain to be defined, it is now clearly established that estrogen downregulates the production of proosteoclastogenic and antiosteoclastogenic factors by targeting several bone and bone marrow cells. Estrogen represses the monocytic production of IL-1 and IL-6 and the proliferation of T cells in the bone marrow, thus leading to decreased T-cell TNF production. Sex steroids also regulate the production of IL-6, OPG, and TGFβ by stromal cells and osteoblasts.

At the present time, stromal cell-produced RANKL and M-CSF should be regarded as the factors responsible for osteoclast renewal in unstimulated conditions. The enhanced osteoclastogenesis and the increased osteoclastic bone resorption leading to postmenopausal bone loss result from the stimulated production of inflammatory cytokines. Among them, monocytic IL-1 and T-cell-produced TNF appear to play a particularly important role. First, the increase in bone marrow levels of IL-1 and TNF induced by estrogen deficiency leads to the selection of a population of stromal cells that exhibit increased Egr-1 phosphorylation, decreased binding of Egr-1 to Sp-1, and enhanced free Sp-1 levels. This, in turn, results in increased Sp-1-induced M-CSF production. Second, IL-1 and TNF increase the production of M-CSF and RANKL by stromal cells and osteoblasts. Finally, T-cell-produced TNF augments the capacity of RANKL to stimulate the differentiation of osteoclast precursors into mature osteoclasts.

Uncertainty remains on the exact contribution of IL-6 to the pathogenesis of ovariectomy-induced bone loss because of insufficient data demonstrating that the block of IL-6 decreases bone resorption and bone loss *in vivo*. However, the exact role of IL-6 is likely to be defined in the near future.

Remarkable progress has been accomplished in clarifying the mechanism of the bone-sparing effect of estrogen in animal models. A more challenging task will be to demonstrate the relevance of the mechanisms described earlier in human subjects.

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CHAPTER 40

Thyroid Hormone and Bone

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Thyroid hormone has profound effects on skeletal development and differentiation and also modulates the activities of mature bone. Both beneficial and deleterious effects of thyroid hormone on the skeleton are seen, depending on the stage of development and the concentration of hormone presented to the cells. The roles of specific thyroid hormone receptor (TR) isoforms and of other factors present in the bone microenvironment in determining thyroid hormone effects are only beginning to be understood. This chapter focuses on the relationship of the *in vitro* effects of thyroid hormone on bone cells to the observed effects of the hormone on the skeleton *in vivo* in both experimental animals and clinical studies.

Mechanism of Thyroid Hormone Action on the Skeleton

Nuclear Receptors

Structural and Genetic Studies

Thyroid hormone receptors are members of the steroid receptor superfamily (Evans, 1988). All of these receptors share a common modular structure with a centrally located DNA-binding domain composed of two zinc fingers and a carboxy-terminal ligand-binding domain that is also involved in receptor dimerization and interactions with coactivators and corepressors. The receptors are nuclear proteins capable of binding to cognate DNA elements in the absence of their ligands. Binding of the ligand to the receptor alters the receptor conformation and subsequently enables the activation or repression of specific genes. Most commonly, the unliganded thyroid hormone receptor represses gene transcription. Interaction of the unliganded receptor with a corepressor complex, including histone deacetylases, results in the condensation of chromatin structure and repression of transcription through decreased access of transcription factors (Koenig, 1998; Wu and Koenig, 2000). Interaction of the receptor with the active thyroid hormone triiodothyronine (T_3) results in a conformational change that leads to dissociation from the repressor complex and interaction with an activation complex containing histone acetylase. Other mechanisms of transcriptional regulation, independent of histone acetylation, have also been described (Fondell *et al.,* 1996).

Thyroid hormone receptors are encoded by two genes: one found on chromosome 17 encoding TRα and one on chromosome 3 encoding TRβ. Alternative splicing of TRα transcripts results in the generation of several carboxy-terminal products (Izumo and Mahdavi, 1988; Chassande *et al.,* 1997). The TRα1 isoform is a commonly expressed active isoform of the receptor. TRα2, which is homologous to the v-erb A oncogene, is a nonbinding isoform resulting from alternative splicing of the TRα primary transcript. TRα2 fails to heterodimerize with retinoic acid receptors (RXR) (Reginato *et al.,* 1996) and may act as a dominant-negative repressor (Koenig *et al.,* 1989; Sap *et al.,* 1986). The TRβ1 and the amino-terminal splice variant, TRβ2, are both active. Tissue expression of TRβ2 is limited, and this isoform is expressed most significantly in the hypothalamus and pituitary (Lazar, 1993), although TRβ2 mRNA has been found in osteoblasts (Abu, 2000). Differential cell and tissue expression of the α1 and β1 isoforms could lead to different responses to thyroid hormone. It could also allow for the development of thyroid hormone analogs that have tissue specificity due to their preferential interaction with one receptor isoform. An example of such an analog is the TRβ-selective agonist GC-1, which had greater effects on lipid metabolism and less on cardiac activity (Trost *et al.,* 2000). The effects of this compound on the skeleton have not been reported, although another agonist, tiratricol (3,5,3′-triiodothyroacetic acid), showed enhanced effects on hepatic lipids and skeletal metabolism (Sherman *et al.,* 1997).

TR isoforms have been found in skeletal tissues. mRNAs for TRα1, TRα2 and TRβ have been detected in MG63, ROS 17/2.8, and UMR-106 cell lines (Williams *et al.,* 1994; Allain *et al.,* 1996). Immunohistochemical staining with antibodies recognizing a TRα epitope or specific TRα2 and TRβ revealed the presence of receptor protein in osteoblast cell lines and in osteoclasts in tissue smears from a human osteoclastoma (Allain *et al.,* 1996). TRα1, TRα2, TRβ1 and TRβ2 mRNAs were expressed in chondrocytes at all stages of differentiation; TRα1, TRα2, and TRβ1 mRNAs were highly expressed in osteoblasts at bone-remodeling sites; and mRNA for all of the isoforms was present and highly expressed in multinucleated osteoclastic cells from an osteoclastoma (Abu *et al.,* 1997). In contrast to mRNA expression, TRα1 protein expression was not seen in the osteoclastoma cells and was limited to osteoblasts at sites of remodeling and undifferentiated chondrocytes (Abu *et al.,* 2000). TRα1, TRα2, and TRβ1 mRNA have also been detected in rat femurs and vertebrae (Milne *et al.,* 1999).

To determine the role(s) of specific TR isoforms, TR-deficient mouse strains have been generated by homologous recombination. TR$\alpha1^{-/-}$ mice did not show bone defects (Wikstrom *et al.,* 1998), whereas mice in which both TRα isoforms were deleted showed growth retardation and impaired development of epiphyseal bone, with disorganization of chondrocyte columns, decreased hypertrophic chondrocytes, and low ossification (Fraichard *et al.,* 1997). The animals died shortly after weaning. TRβ knockout mice failed to show bone defects (Gauthier *et al.,* 1999; Gothe *et al.,* 1999), suggesting that the TRβ isoform is not essential for bone development in the mice. The TR$\alpha1^{-/-}$ TR$\beta^{-/-}$ double knockout produced viable mice, the majority of which survived at least through 12 months, although there was increased mortality compared to wild-type mice (Gothe *et al.,* 1999). TR$\alpha1^{-/-}$ TR$\beta^{-/-}$ double knockout mice exhibited retarded growth and significantly reduced levels of growth hormone (GH) and insulin-like growth factor I (IGF-I). Bone length was decreased significantly in limbs and vertebrae, with the effect being most marked in the femur. Growth plates were disorganized and epiphyseal ossification was delayed. Dual X-ray absorptiometry showed decreased bone area and bone mineral content but no significant effect on bone mineral density. Middiaphyseal peripheral quantitative computed tomography (pQCT) scans of the femurs revealed decreased cortical density, cortical area, bone mineral content, and periosteal circumference. Cross-sectional moment of inertia and moment of resistance were decreased significantly. It was noted that the phenotype was less severe than that resulting from thyroidectomy, and this was postulated to be a reflection of the fact that in the case of thyroidectomy, T_3 would not be available to alleviate the transcriptional repression effected by the TRs (Gothe *et al.,* 1999). It is also possible that novel, previously unrecognized thyroid hormone receptor isoforms may be present that could compensate for the loss of the deleted receptors.

Skeletal alterations associated with mutations in the TRβ1 gene have been described in patients with resistance to thyroid hormones (RTH). In most reported cases, the defect shows an autosomal dominant pattern of inheritance. The mutations are clustered and largely located within domains in the carboxy-terminal region. They are mainly nucleotide substitutions that result in single amino acid changes (Refetoff, 1993). The mutant alleles may act by a dominant-negative mechanism to inhibit the ability of the normal allele to elicit normal receptor function (Chatterjee *et al.,* 1991; Sakurai *et al.,* 1990). The dominant-negative action appears to be at the level of DNA binding (Kopp *et al.,* 1996). The mutant phenotypes are heterogeneous, but some patients have shown evidence of retarded bone age and stippled epiphyses, similar to characteristics of hypothyroidism, with resulting short stature. In other patients there is accelerated bone age, accelerated chondrocyte maturation, and early epiphyseal closure, again resulting in short stature (Behr *et al.,* 1997). The target sites at which resistance occurs (pituitary or peripheral) may determine the phenotype. It is possible that mutations in TRα may be lethal and thus are not seen.

Similar to the pattern for retinoid and vitamin D receptors, DNA-binding sites for thyroid hormone receptors include monomeric, palindromic, inverted repeat, and direct repeat response elements derived from a common AGGTCA motif. Multiple functional forms exist for thyroid hormone receptors, including monomers, homodimers, heterodimers between thyroid hormone isoforms, and heterodimers with retinoid and vitamin D receptors. In bone (Williams *et al.,* 1994; Williams *et al.,* 1995), as in other tissues (Glass, 1994; Brent *et al.,* 1991), DNA binding and transcriptional activation are enhanced when the thyroid hormone receptor isoforms are present as heterodimers with retinoid or vitamin D receptors. In osteoblast cell lines, interactions among the retinoid, vitamin D, and thyroid hormone ligands appeared to mediate specific responses (Williams *et al.,* 1994, 1995). Studies on the effects of treatment combinations on the expression of osteoblast phenotypic genes in the cell lines revealed complex responses that indicated the importance of dose, treatment duration, and degree of confluence in dictating the magnitude of the response (Williams *et al.,* 1995). However, in primary rat osteoblastic cells, alteration of the ligand combinations did not influence the responses (Bland *et al.,* 1997).

Competitive Binding Studies

Two studies of T_3 binding to nuclei from ROS 17/2.8 cells gave the following parameters: K_d 5 n*M*, B_{max} 0.13 ng/mg DNA, with incubation for 60 min at 37°C (Rizzoli *et al.,* 1986) and K_d 150 p*M*, B_{max} 24 fmol/100 μg DNA, with incubation for 2.5 hr at 37°C (Sato *et al.,* 1987). In UMR-106 cells, two nuclear-binding sites were identified: one with K_d 260 p*M*, B_{max} 7.7 pg/mg DNA, and one of lower affinity, K_d 1.8 n*M* (LeBron *et al.,* 1989). In MC3T3-E1 cells, K_d for T_3 binding was 120 p*M* (Kasono *et al.,* 1988). T_3 receptors were also found in cell lines (ROS 25/1, ROS 17/2.8-3) that did not show an alkaline phosphatase response to T_3, suggesting that there is a postreceptor defect in these cell lines (Sato *et al.,* 1987). There was good agreement between the relative affinity of different ligands [T_3 = 1, thyroxin (T_4) = 0.1, 3,3′-

diiodothyronine = 0.013, reverse T_3 (rT_3) = 0.002, monoiodotyrosine = 0, diiodotyrosine = 0] and their ability to increase alkaline phosphatase in ROS 17/2.8 cells (Sato *et al.,* 1987). In another study, T_4 had a 20-fold lower and rT_3 a 400-fold lower affinity compared with T_3 in ROS 17/2.8 cells (Rizzoli *et al.,* 1986). In binding studies with a nuclear fraction from neonatal mouse calvaria, carried out for 60 min at 22°C, K_d for T_3 was 3 n*M* and the B_{max} 1.9 pmol/mg DNA (Krieger *et al.,* 1988). The cardiotonic agent milrinone, which has structural homology to T_4 (Mylotte *et al.,* 1985), did not compete for binding to the calvarial receptors. Both time and temperature dependence were observed in the binding studies, with binding being more rapid at 37°C than at 22°C (Krieger *et al.,* 1988; Sato *et al.,* 1987). Kinetic analysis of normal nuclear receptors gave K_a of $9 \times 10^8\ M^{-1}\ min^{-1}$ and K_d of 0.016 min^{-1} with a $t_{1/2}$ of approximately 36 min (Krieger *et al.,* 1988). For binding studies in the cell lines, the method of Samuels *et al.* (1979) was used to remove thyroid hormones from serum used in the growth medium. The procedure involves treatment of the serum with AG1-X8 resin and removes more than 90% of the T_4 and 95% of the T_3.

Relevance of *in Vitro* Concentrations and *in Vivo* Dosages of Thyroid Hormone to Physiological and Pathophysiological Concentrations

It has been reported that the normal range for serum-free T_3 is similar in rats and humans, and is 3.3–8.2 p*M* (Jurney *et al.,* 1983). Wide concentration ranges of thyroid hormones have been used in experimental studies, especially *in vitro,* and often markedly different dosages are required to obtain the same response in a different cell line, model system, or laboratory. The differentiation state and the production of modulating factors are potential variables that can affect the response in a given system. In addition, the presence of thyroid hormone in the added sera or the presence of binding sites in stripped sera can dramatically influence the free hormone available to the cells or tissue. Several studies have estimated the amount of free hormone available under the experimental conditions used (Sato *et al.,* 1987; Allain *et al.,* 1992). In one study, an equilibrium dialysis method was used to determine free T_4 and T_3 after treating fetal calf serum with AG1-X8 resin (Sato *et al.,* 1987).T_4 and T_3 concentrations in the fetal calf serum prior to extraction were 11.1 μg/dl and 157 ng/dl, respectively. It was determined that addition of 10 n*M* T_4 to the stripped serum provided 80 p*M* free T_4 and that addition of 1 n*M* T_3 provided 40 p*M* free T_3. In the other study, in which 10% neonatal calf serum was used, the free T_3 was measured by radioimmunoassay (Allain *et al.,* 1992). It was determined that the addition of 10 p*M* T_3 yielded a free T_3 concentration of 2.1 p*M,* that 0.1 n*M* yielded 4 p*M,* that 1 n*M* yielded 11.8 p*M* and that 10 n*M* yielded >39 p*M* (Allain *et al.,* 1992). Although the type and percentage of serum would influence the final values, these measurements and calculations are of value in comparing studies and in relating *in vitro* concentrations to the concentrations of thyroid hormones in normal serum.

Membrane Actions

Signal Transduction Pathways

Thyroid hormones interact with several signal transduction pathways in bone cells. These results suggest that extranuclear actions could initiate some of the thyroid hormone effects on bone. Rapid (within 30 sec) increases in inositol mono-, bis-, and trisphosphates are elicited by treatment of fetal rat limb bones with 100 n*M* and 1 μM T_3 (Lakatos and Stern, 1991). The inactive analogs diiodothyronine and rT_3 did not increase inositol phosphates. This effect of T_3 was inhibited by indomethacin and could represent an initiation pathway for the prostaglandin-dependent effects of thyroid hormones on bone resorption, discussed later. Thyroid hormones at high doses inhibit cyclic AMP phosphodiesterase (Marcus, 1975). T_3 at 0.1 and 1 n*M* increased ornithine decarboxylase and potentiated the responses of this enzyme to parathyroid hormone (PTH) (Schmid *et al.,* 1986). Specific cellular functions associated with membrane receptors have not been identified, although it has been proposed that nongenomic actions of thyroid hormones serve homeostatic functions for membrane transport and may modulate genomic actions of the hormones (Davis and Davis, 1996).

Gene Products

Thyroid hormone promotes the proliferation and differentiation of osteoblastic cells (Ohishi *et al.,* 1994; Ishida *et al.,* 1995). This is reflected in the increased expression of a number of markers.

Osteoblast Phenotypic Markers

Alkaline Phosphatase T_4 (10 n*M*) and 1 n*M* T_3 increased alkaline phosphatase in ROS 17/2.8 cells (Sato *et al.,* 1987). The effect was seen within 4 days of culture. Responses were more robust in subconfluent cells and were inhibited by 1 μg/ml cycloheximide or 0.1 μg/ml actinomycin D. T_3 modulated the stimulatory effect of hydrocortisone on alkaline phosphatase. At low hydrocortisone concentrations (1 n*M,* 10 n*M*), 1 μ*M* T_4 resulted in an additive effect, whereas at higher hydrocortisone concentrations (0.1 μ*M,* 1 μ*M*), coincubation with T_4 decreased the stimulatory effect (Sato *et al.,* 1987). T_3 also increased alkaline phosphatase in MC3T3-E1 cells (Kasono *et al.,* 1988; Klaushofer *et al.,* 1995). In one study, significant responses were elicited with 0.1 n*M* T_3 and 10 n*M* T_4; the effect of T_4 was further increased by concentrations up to 1 μ*M* and was less at 10 μ*M*; the effect of T_3, however, was maximal at 1 n*M,* and no increase was observed at 100 n*M* (Kasono *et al.,* 1988). T_3 failed to affect alkaline phosphatase in UMR-106 cells (LeBron *et al.,* 1989; Huang *et al.,* 2000), possibly due to the high basal expression of the enzyme in this cell line. T_3 had biphasic effects on alkaline phosphatase in normal rat osteoblastic cells (Ernst and Froesch, 1987), stimulating at concentrations of 0.01 and 0.1 n*M* and inhibiting at a concentration of 10 n*M.* In neonatal rat calvarial cells, mRNA for alkaline phosphatase was

decreased by 1 or 4 days exposure to 1 n*M* T_3 (Schmid *et al.,* 1992). In cells derived from human trabecular bone explants and cultured in medium containing charcoal-stripped serum, alkaline phosphatase in the cell layer was increased by T_3 at concentrations up to 200 n*M* (Kassem *et al.,* 1993). Alkaline phosphatase was also increased by thyroid hormone in isolated tibiae (Stracke *et al.,* 1986) and in primary human (Kassem *et al.,* 1993) and rodent (Egrise *et al.,* 1990) osteoblasts. Thus, most but not all osteoblastic cells respond to thyroid hormones with an increase in alkaline phosphatase. Effects are seen at concentrations in the physiologic range; however, the dose dependence of the effect is quite variable and may be dependent on cell type and culture conditions.

Osteocalcin T_3 increased osteocalcin in a dose-dependent manner in ROS 17/2.8 cells (Rizzoli *et al.,* 1986; Sato *et al.,* 1987). In medium containing 2% T_3-depleted serum, a significant effect was seen at 1 n*M,* with a half-maximal effect at 2.5 n*M*; the osteocalcin concentration in the control medium was 9 ng/10^6 cells and was increased to 12.3 ng/10^6 cells by 1 n*M* T_3 (Rizzoli *et al.,* 1986). A striking difference in the response of osteocalcin mRNA to T_3 was observed between cells derived from femoral and vertebral bone marrow, cultured under conditions leading to osteogenic differentiation (Milne *et al.,* 1998). In cultures from femoral marrow, T_3 supplementation (10 or 100 n*M*) prevented the time-dependent decrease in osteocalcin mRNA observed in untreated cells. In cultures from the vertebral marrow, osteocalcin mRNA expression was maintained over time in untreated cells, and T_3 failed to augment the response.

Collagen In cultured rat osteoblastic cells, decreases in collagen and noncollagenous protein synthesis were noted with 0.01 and 0.1 n*M* T_3, but not with higher concentrations (Ernst and Froesch, 1987). Thyroid hormones did not inhibit collagen synthesis in rat calvaria (Canalis, 1980). In neonatal mouse calvaria precultured with indomethacin to inhibit prostaglandin synthesis, both collagen and noncollagenous protein synthesis were stimulated by T_3 and by triiodothyroacetic acid at concentrations in the 0.01 – 10 n*M* range (Kawaguchi *et al.,* 1994a). Cells from human trabecular bone explants showed decreased type I procollagen carboxy-terminal propeptide production when treated with T_3 (Kassem *et al.,* 1993). The synthesis of collagen thus appears to be regulated by T_3 in a complex manner and may be influenced by T_3 stimulation of other cellular products, such as prostaglandins. Collagen type I gene expression was regulated differentially by T_3 in marrow cultures from femoral and vertebral bones, with a more marked stimulatory effect in the femoral bones (Milne *et al.,* 1998).

Other Phenotypic Responses A series of studies has characterized other changes elicited by T_3 in MC3T3-E1 osteoblastic cells (Luegmayr *et al.,* 1996; Franzl-Zelman *et al.,* 1997; Varga *et al.,* 1997, 1999; Luegmayer *et al.,* 1998, 2000). In addition to alkaline phosphatase, expression of c-fos, c-jun, and an osteocalcin-related protein were increased in T_3-treated cells. Morphological changes were also observed. T_3-treated cells ceased proliferation and became flattened, enlarged, and polygonal. The amount of F-actin increased and the patterns of actin expression were altered. Pancadherin/β catenin immunoprecipitation was increased by T_3, which could reflect the organization of adherens junctions. Apoptosis was also accelerated. In ROS 17/2.8 cells, treatment with T_3 increased expression of receptors for PTH: conversely, PTH increased binding of T_3 (Gu *et al.,* 2001).

Insulin-like Growth Factors and IGF-Binding Proteins

IGF-I has significant anabolic effects on bone, increasing cell replication and both collagen and noncollagen protein synthesis (Canalis, 1980; Hock *et al.,* 1986; McCarthy *et al.,* 1989; Centrella *et al.,* 1990; Pirskanen *et al.,* 1993). IGF-I is increased in fetal rat bones treated with thyroid hormone (Schmid *et al.,* 1992; Lakatos *et al.,* 1993; Varga *et al.,* 1994; Klaushofer *et al.,* 1995). At 1 n*M,* T_3 stimulates IGF-I production in neonatal rat calvarial osteoblasts (Schmid *et al.,* 1992). There is a dose-dependent, biphasic effect of T_3 and T_4 on IGF-I production in UMR-106 cells and fetal rat bone organ cultures (Lakatos *et al.,* 1993). IGF-I mRNA is increased by T_3 treatment in MC3T3-E1 cells (Varga *et al.,* 1994; Klaushofer *et al.,* 1995). T_3 increased IGF-I expression more markedly in cells from vertebral marrow than in cells from femoral marrow (Milne *et al.,* 1998). Interference with IGF-I action by decreasing expression or function of the IGF-I receptor by the use of antisense oligonucleotides, antibodies, and antagonist peptide decreased the anabolic effects of T_3 on MC3T3-E1 cells and primary mouse calvarial osteoblasts, including effects on alkaline phosphatase, osteocalcin, and collagen synthesis (Huang *et al.,* 2000). The effects of thyroid hormones on IGFs may be modulated by changes in IGF-binding proteins (IGFBPs). The physiological role of the binding proteins is not fully understood; however, they can influence the cellular uptake and turnover of IGF-I. The binding proteins may represent a mechanism for retention of IGFs in the bone matrix (Bautista *et al.,* 1991). IGFBPs can also modulate IGF action in osteoblastic cells. IGFBP-2 and IGFBP-4 can inhibit IGF-I actions (Mohan *et al.,* 1989; Feyen *et al.,* 1991). Both enhancing and inhibitory (Schmid *et al.,* 1995) effects are produced by IGFBP-3 (Ernst and Rodan, 1990; Schmid *et al.,* 1991). In rat osteoblasts, T_3 stimulates the production of IGFBP-2 and IGFBP-3 (Schmid *et al.,* 1992). T_3 increases IGFBP-4 expression in MC3T3-E1 cells (Glantschnig *et al.,* 1996), which could regulate the response to T_3 and contribute to the decreased anabolic effects observed at higher concentrations. Alterations in thyroid status *in vivo* influence the expression of IGFBPs in a complex manner. In hyperthyroid rats, IGFBP-3 gene expression in liver is decreased; however, in hypothyroid (propylthiouracil-treated) animals, IGFBP-1 and IGFBP-2 gene expression are increased and IGFBP-3 mRNA is decreased (Rodriguezarnao *et al.,* 1993). In hyperthyroid patients, serum IGFBP-3 and IGFBP-4, but not IGFBP-5, are increased (Lakatos *et al.,* 2000).

Cell and Tissue Phenotypic Responses

Osteoblast Proliferation

T_3 can increase proliferation of rodent and human osteoblastic cells (Ernst and Froesch, 1987; Kassem *et al.,* 1993). In the rodent cell cultures, 0.01 and 1 n*M* were stimulatory, and 10 n*M* was inhibitory in longer term cultures (Ernst and Froesch, 1987). Cell number was decreased after 8 days of incubation with T_4 in MC3T3-E1 cells; inhibition was observed with 10 n*M* T_3 and was maximal at 1 μM (Kasono *et al.,* 1988). In other investigations, T_3 did not significantly affect growth of ROS 25/1, UMR-106, or ROS 17/2.8 cells (Sato *et al.,* 1987; LeBron *et al.,* 1989; Williams *et al.,* 1994). The diversity of the responses obtained suggests that in addition to thyroid hormone dose, the cell type, passage number, degree of confluence, and the presence or production of other factors can determine the particular outcome that is observed. In explanted neonatal mouse calvaria, T_3 stimulated thymidine incorporation in a dose-dependent manner and was significant at 10 p*M* (Kawaguchi *et al.,* 1994a). A preculture period was required to demonstrate the effect, as high levels of prostaglandin production from untreated tissues appeared to mask treatment effects.

Nodule Formation

Bone nodule formation has been used as a parameter of bone cell differentiation, presumably representing the capability of the cell to generate a mineralized matrix. T_3 or T_4, at concentrations of 1 n*M*–0.1 μM, decreased nodule formation by 21-day fetal rat calvarial cells cultured in medium containing 15% heat-inactivated fetal bovine serum, and lower concentrations, starting at 1 p*M,* had no effect (Ishida *et al.,* 1995). When serum was depleted of T_3 with AG-1X-10 resin (Samuels *et al.,* 1979), basal bone nodule formation was increased (Ishida *et al.,* 1995). Dexamethasone (10 n*M*), enhanced bone nodule formation markedly. This was promoted by low concentrations of T_3 (1 and 10 p*M* and inhibited by higher concentrations (10 and 100 n*M*). Although the results suggest that high concentrations of thyroid hormones can inhibit mineralization, the authors point out that the procedure to strip serum of thyroid hormone could remove other inhibitory factors as well.

Osteoclast Activation

Two studies indicate that the resorptive effects of thyroid hormones on bone are mediated indirectly through the stimulation of osteoblasts or other cell types present in bone. T_3 failed to activate isolated osteoclasts; however, when mixed bone cells were added to the cultures, a significant response was observed with 1 μM T_3, although not with lower concentrations (Allain *et al.,* 1992). UMR-106 cells failed to activate the osteoclasts in the presence of T_3, suggesting that a different cell type or a different osteoclast stage might be responsible for the activation observed with the mixed bone cells. In another study, either UMR-106-01 cells or rat calvarial cells were able to activate the osteoclasts (Britto *et al.,* 1994). Responses were detected at lower T_3 concentrations in the latter study, perhaps due to the use of stripped serum.

Resorption

T_3 stimulates resorption in bone organ cultures. Fetal rat limb bones (Mundy *et al.,* 1979; Hoffmann *et al.,* 1986; Lakatos and Stern, 1992) and neonatal mouse calvaria (Krieger *et al.,* 1988; Klaushofer *et al.,* 1989; Kawaguchi *et al.,* 1994) are the models that have been studied most extensively. In both the limb bones and calvaria, responses to T_3 are slower to develop than the effects of PTH (Mundy *et al.,* 1979; Klaushofer *et al.,* 1989; Kawaguchi *et al.,* 1994b) and the dose–response curves are generally shallow (Mundy *et al.,* 1979; Hoffmann *et al.,* 1986; Krieger *et al.,* 1988). Higher doses of thyroid hormones *in vitro* can have inhibitory effects on resorption (Orbai and Gazariu, 1982). One of the most striking differences from the effects of PTH is that the maximal responses to thyroid hormones are lower (Mundy *et al.,* 1979; Lakatos and Stern, 1992; Kawaguchi *et al.,* 1994b), sometimes only about 50% of those elicited with maximal concentrations of PTH. The slower responses and lower efficacy of thyroid hormones compared with PTH may be the basis for the observation that thyroxin does not exhibit "escape" from the inhibitory effects of calcitonin (Krieger *et al.,* 1987; Klaushofer *et al.,* 1989). Alternatively, this may reflect a different mechanism for the direct effect of thyroid hormones compared with PTH. Further evidence for such a difference between the mechanism of T_3 and PTH responses is the contrast in their interaction with TGFβ (Lakatos and Stern,1992). TGFβ enhanced the early responses to PTH and inhibited the later effects, whereas the interaction with T_3 displayed a somewhat reverse time course. A range of threshold concentrations was observed for both T_3 and T_4 in the different studies, with no clear basis in terms of the composition of the medium. 3,5,3′-Triiodothyroacetic acid, an analog that binds to nuclear receptors, especially β forms, with higher affinity than T_3, was a more potent stimulator of resorption than T_3 (Kawaguchi *et al.,*1994). In cultured fetal bones, T_3 increases collagen degradation (Halme *et al.,* 1972). T_3 increased mRNA for the metalloproteinases collagenase-3 and gelatinase B in cultures of osteoblastic cells, effects that were not inhibited by indomethacin (Pereira *et al.,* 1999).

Several mechanisms may mediate thyroid hormone-stimulated resorption. In neonatal mouse calvaria, resorption was inhibited by indomethacin, implicating a prostaglandin-dependent pathway (Krieger *et al.,* 1988; Klaushofer *et al.,* 1989; Kawaguchi *et al.,* 1994b). Other studies have shown prostaglandin-independent effects on the calvaria (Conaway *et al.,* 1998). In fetal rat limb bones, the T_3 effect is not affected by indomethacin. However, in limb bone cultures, T_3 potentiates the bone-resorbing effect of IL-1 (Tarjan and Stern, 1995), and the effect of IL-1 and the IL-1/T_3 combination is sensitive to indomethacin. T_3 also potentiates the IL-1-

mediated production of IL-6 in this model (Tarjan and Stern, 1995), as well as in MC3T3-E1 osteoblastic cells (Tokuda *et al.,* 1998) and in bone marrow stromal cells (Kim *et al.,* 1999). In contrast, in MC3T3-E1 cells, T_3 reduced the IL-6 production elicited by prostaglandin, cholera toxin, and forskolin, possibly reflecting cross-talk through effects on a cAMP pathway (Tokuda *et al.,* 1998). In mouse bone marrow cultures, T_3 promoted calcitriol-induced osteoclast formation through an IL-6-dependent pathway (Schiller *et al.,*1998). T_3 also increased IL-6 production in MG-63 cells and human bone marrow stromal cells (Siddiqi *et al.,* 1998). These findings suggest that the indirect stimulation of osteoclast differentiation by IL-6 may be a component of the resorptive effect of thyroid hormone. The IL-1 receptor antagonist protein did not prevent the resorptive effect of thyroid hormones in limb bone cultures (Kawaguchi *et al.,* 1994b). Thyroid hormone effects on resorption were blocked by aphidicolin or cortisol (Kawaguchi *et al.,* 1994b) and by hydroxyurea (Conaway *et al.,* 1998), indicating the involvement of cell replication. Immunosuppressive cyclosporins blocked the thyroid hormone effects in limb bone cultures (Lakatos and Stern, 1992), and interferon-γ (Klaushofer *et al.,* 1989) and the antibody to TGFβ (Klaushofer *et al.,* 1995) blocked the thyroid hormone effects in calvaria, consistent with the participation of other local factors in the resorptive response to thyroid hormone.

Remodeling

Most *in vitro* studies have focused on either anabolic or catabolic effects of thyroid hormone, under conditions designed to optimize the study of the particular response. However, because there are dose-dependent biphasic effects on formation parameters and delayed (Klaushofer *et al.,* 1989) and submaximal (Mundy *et al.,* 1979; Krieger *et al.,* 1988; Lakatos and Stern, 1992) effects on resorption, it may be that neither effect can be studied to the exclusion of the other, and the net effects on bone remodeling may be accessible to *in vitro* investigation. A model system designed to study growth, mineralization, and resorption in radii and ulnae of 16-day fetal mice (Soskolne *et al.,* 1990) revealed interesting differences between effects of T_3 and PTH. Effects of T_3 were studied over a $0.1\ nM - 10\ \mu M$ dose range. T_3 concentrations in the $10\ nM - 0.3\ \mu M$ range resulted in increases in diaphyseal length, in calcium, phosphate, and hydroxyproline content, and in decreases in ^{45}Ca release. At higher concentrations (1 and 10 μM), T_3 stimulated ^{45}Ca release. In contrast, when PTH was studied over a $1\ pM - 0.1\ \mu M$ range, only resorptive effects were observed, these being at concentrations of 1 n*M* and higher.

Chondrocyte Responses

Thyroid hormones block clonal expansion of the proliferative cell layer of the epiphyseal growth plate and promote chondrocyte maturation (Nilsson *et al.,* 1994). In earlier studies on isolated cells, T_3 was found to inhibit chondrocyte proliferation (Burch *et al.,* 1987). T_3 suppressed the synthesis of DNA, protein, and type II collagen when added to rapidly proliferating chicken growth plate chondrocytes cultured in serum-free media (Ishikawa *et al.,* 1998). When T_4 was added to chemically defined medium containing insulin and growth hormone, there were dose-dependent increases in type X collagen and in alkaline phosphatase (Ballock and Reddi, 1994). T_3 was approximately 50 times more potent than T_4 in promoting expression of the hypertrophic markers in prehypertrophic chondrocytes in cells cultured with insulin/transferrin/selenium (Alini *et al.,* 1996). There was a biphasic dose dependency of the effects of T_3 and T_4 to stimulate the synthesis of type II collagen and chondroitin sulfate-rich proteoglycans in cultured rabbit articular chondrocytes (Glade *et al.,* 1994). In an *in vitro* model of cartilage formation from a chondrocyte pellet, the developing cartilage assumed the structural architecture of the normal epiphysis if thyroid hormones were present, whereas the structure was random in their absence (Ballock and Reddi, 1994). Findings from cocultures of vascular endothelial cells and chondrocytes suggest that vascular endothelial cells may also produce factors that act synergistically with thyroid hormone to derepress the late differentiation of resting chrondrocytes and permit them to become hypertrophic and express type X collagen and alkaline phosphatase (Bittner *et al.,* 1998), leading to mineralization.

In Vivo Responses of the Skeleton to Thyroid Hormones: Animal Studies

When thyroid hormones are administered to young rats, bone growth is enhanced (Glasscock and Nicoll, 1981). This response is not seen in older rats, suggesting that the stage of cellular differentiation or the environment in terms of other hormones and local factors can influence the manifestation of thyroid hormone responses. T_3 treatment of neonatal rats elicited a narrowing of the sagittal suture and increased mineral apposition rates at the osseous edges of the sutures (Akita *et al.,* 1994). Histomorphometric analysis was consistent with the conclusion that T_3 is critical for bone remodeling (Allain *et al.,* 1995).

Hypothyroidism

Animal models of hypothyroidism include the use of the antithyroid agents propylthiouracil or methimazole to block the synthesis of thyroid hormones. Treatment of young rats with methimazole for 7 weeks resulted in a marked increase in trabecular bone volume of the subchondral spongiosa of the mandibular condyles and a decrease in cartilage cellularity (Lewinson *et al.,* 1994). IGF-I was present in the condyles of control rats, but lacking in hypothyroid rats. Replacement of T_4 during the last 2 weeks of treatment restored the parameters to normal (Lewinson *et al.,* 1994).

Histomorphometric studies in iliac crest biopsies of young rats made hypothyroid by a 12-week treatment with propylthiouracil showed that both osteoid surfaces and eroded surfaces were reduced and cancellous bone volume was increased (Allain *et al.,* 1995). In a study in which 21-day rats were made hypothyroid by administration of methimazole, T_4 given daily at doses of 2 to 64 μg/kg/day for 21 days elicited biphasic effects on epiphyseal growth plate width and logitudinal growth rate (Ren *et al.,* 1990). The dose–response curve paralleled that of serum IGF-I concentrations, which were postulated to contribute to the growth responses (Ren *et al.,* 1990).

An interesting animal model for hypothyroidism utilizes transgenic mice (line TG66-19) in which the bovine thyroglobulin promoter drives the expression of the herpes simplex type I virus thymidine kinase gene in thyrocytes. This enzyme converts ganciclovir to ganciclovir-5′-phosphate, which inhibits DNA replication, resulting in loss of thyrocytes, loss of follicles, and undetectable T_3 and T_4; levels of PTH and CT are unaffected (Wallace *et al.,* 1991, 1995). In this transgenic mouse model, administration of 15 or 50 μg of ganciclovir to mouse dams during days 14–18 of gestation resulted in growth delay in pups carrying the transgene (Wallace *et al.,* 1995). The authors point out that the reason their effects were more dramatic than those obtained with the *hyt/hyt* mouse, a strain that has an inactivating mutation in the TSH receptor, is that in the latter model, circulating T_4 is still 10–20% of normal (Adams *et al.,* 1989). Effects of mutations in thyroid hormone receptors in mouse models were discussed earlier.

Hyperthyroidism

A range of T_4 regimens has been used to elicit hyperthyroidism in animal models. The duration of treatment is generally at least 3 weeks and dosages range from 200 μg to 1 g/day. Lower concentrations have been used in animals previously made hypothyroid with antithyroid drugs (Lewinson *et al.,* 1994). When the animals were rendered hyperthyroid by treatment with T_4 (200 μg/day for 12 weeks), the mineral apposition rate and the mineral formation rate were increased markedly, with a smaller increase in eroded surfaces (Allain *et al.,* 1995). A greater sensitivity of cortical bone (femur) than trabecular bone (spine) to thyroid hormone-induced bone loss has been noted in animal models of hyperthyroidism (Ongphiphadhanakul *et al.,* 1993; Suwanwalaikorn *et al.,* 1996, 1997; Gouveia *et al.,* 1997; Zeni *et al.,* 2000). Tooth movement was greater in T_3-treated rats undergoing orthodontic procedures than in control untreated animals, probably reflecting greater root resorption (Shirazi *et al.,* 1999). Ten-day-old rats treated with 100 μg/kg/day for up to 60 days displayed altered parameters of cranial width, narrowing of the suture gap of the sagittal suture, and intense immunohistochemical staining for IGF-I along the suture margins, consistent with the possibility that local IGF-I is involved in the effect of thyroid hormone to cause premature suture closure (Akita *et al.,* 1996).

Ovariectomized rats treated with a low dose of T_4 (30 μg/kg/day for 12 weeks) showed increased bone turnover and decreased bone density compared with controls; however, in the presence of 17β-estradiol, their bone mass and mineral apposition rate were greater than those of controls (Yamaura *et al.,* 1994). T_4, (250 μg/kg/day for 5 weeks) increased serum osteocalcin and urinary pyridinolines and produced a greater loss of bone mineral compared with either ovariectomy alone or T_4 alone (Zeni *et al.,* 2000). In contrast to the effects of these high doses of T_4, administration of a more physiological concentration (10 μg/kg/day) to ovariectomized rats resulted in a generalized increase in bone mineral density at both lumbar and vertebral sites (Gouveia *et al.,* 1997). Estradiol prevented T_3-stimulated decreases in bone mineral density in ovariectomized thyroidectomized rats, but had no effect in animals that were not treated with T_3 (DiPippo *et al.,* 1995). These results raise the possibility of cross-talk at the level of binding of estradiol and T_3 receptors to DNA target sites.

Pathophysiological Effects of Altered Thyroid Hormone Status in Humans

Hypothyroidism

Bone turnover is decreased in hypothyroidism (Mosekilde and Melson, 1978). In juvenile hypothyroidism, there is delayed skeletal maturation and epiphysial dysgenesis. In a study of children with congenital hypothyroidism treated with T_4, the bone age at 1.5 years was correlated positively with the dose of T_4 administered during the first year and with the concentrations of serum T_4 (Heyerdahl *et al.,* 1994). As discussed previously, multiple skeletal abnormalities have been described in syndromes of RTH, including short stature, delayed skeletal maturation, and stippled epiphyses (Refetoff *et al.,* 1993). Serum IGF-I is lower in hypothyroid patients (Lakatos *et al.,* 2000). Bone resorption is decreased in patients with hypothyroidism, as indicated by reduced urinary pyridinium cross-links (Nakamura *et al.,* 1996).

Hyperthyroidism

Since the initial description of bone loss in thyrotoxicosis by von Recklinghausen more than a century ago (von Recklinghausen, 1891), substantial additional evidence has shown that excessive thyroid hormone production can lead to bone loss. In patients with hyperthyroidism, markers of bone turnover are increased. Pyridinoline and hydroxypyridinoline cross-link excretion are elevated (Harvey *et al.,* 1991; Garnero *et al.,* 1994; Nagasaka *et al.,* 1997; Engler *et al.,* 1999), as are urinary N-terminal telopeptide of type I collagen (NTX) (Mora *et al.,*1999; Pantazi *et al.,* 2000) and serum carboxy-terminal-1-telopeptide (ICTP) (Loviselli *et al.,* 1997; Miyakawa *et al.,* 1996; Nagasaka *et al.,* 1997). Evidence of activation of osteoblasts in hyperthyroidism is the elevation of alkaline phosphatase (Mosekilde and Christesen,

1977; Cooper *et al.,* 1979; Martinez *et al.,* 1986; Nagasaka *et al.,* 1997; Pantazi *et al.* 2000), osteocalcin (Martinez *et al.,* 1986; Lee *et al.,* 1990; Mosekilde *et al.,* 1990; Nagasaka *et al.,* 1997; Loviselli *et al.,* 1997; Pantazi *et al.,* 2000), and carboxy-terminal propeptide of type I procollagen (PICP) (Nagasaka *et al.,* 1997). Osteocalcin showed a better correlation than alkaline phosphatase with thyroid hormone concentrations (Martinez *et al.,* 1986; Garnero *et al.,* 1994). Greater increases in the resorption markers than the formation markers suggest an imbalance between resorption and formation, leading to bone loss (Garnero *et al.,* 1994; Miyakawa *et al.,* 1996). Histomorphometric analyses show increased osteoclast numbers and resorbing surfaces, with loss of trabecular bone volume (Meunier *et al.,* 1972; Mosekilde and Melsen, 1978). Histomorphometric data yield a kinetic model demonstrating accelerated bone remodeling, with a disproportionately greater increase in resorption and a net loss of bone with each cycle of remodeling (Eriksen, 1986). Decreased bone mineral content in hyperthyroidism is well documented (Fraser *et al.,* 1971; Krolner *et al.,* 1983; Toh *et al.,* 1985), and fracture risk is increased in hyperthyroidism (Fraser *et al.,* 1971; Cummings *et al.,* 1995; Wejda *et al.,* 1995; Vestergaard *et al.,* 2000a). Mild hyperthyroidism may increase bone loss in postmenopausal women (Lakatos *et al.,* 1986). In children, however, thyrotoxicosis can lead to acceleration of growth and skeletal development (Schlesinger and Fisher, 1951; Saggese *et al.,* 1990).

T_4 Therapy and Bone Loss

A particularly critical issue regarding the effects of thyroid hormone on the skeleton is the question of what amounts of exogenously administered thyroid hormones increase the risk of bone loss, especially among individuals already at risk for osteoporotic fractures from other causes. Thyroid hormones are given as replacement therapy for hypothyroidism after thyroidectomy, as well as in other states where patients may have inadequate thyroid hormone secretion and goiter, such as autoimmune thyroiditis. Thyroid hormones are also used as suppressive therapy for toxic nodular goiter or for thyroid cancer. There may be patients who used excess thyroid hormones in the past for weight loss or as a tonic. Decreased bone density, accelerated bone turnover, and increased risk of fracture in patients treated with T_4 have been documented extensively (Fallon *et al.,* 1983; Coindre *et al.,* 1986; Ross *et al.,* 1987; Paul *et al.,* 1988; Stall *et al.,* 1990; Taelman *et al.,* 1990; Adlin *et al.,* 1991; Diamond *et al.,* 1991; Greenspan *et al.,* 1991; Lehmke *et al.,* 1992; Frevert *et al.,* 1994; Garton *et al.,* 1994; Grant *et al.,* 1995; McDermott *et al.,* 1995; Campbell *et al.,* 1996; Affinito *et al.* 1996; Jodar *et al.,* 1998; Hadji *et al.,* 2000). Several studies and a meta-analysis (Marcocci *et al.,* 1994; Uzzan *et al.,* 1996; Greenspan and Greenspan, 1999; Campbell *et al.,* 1996; Affinito *et al.,* 1996) concluded that the dose of T_4 and duration of treatment are major determinants of the occurrence of bone loss. Other factors that appear to amplify the risk include a previous history of hyperthyroidism (Grant *et al.,* 1995), age (Duncan *et al.,* 1994), and postmenopausal status (Greenspan *et al.,* 1991; Stepan and Limanova, 1992; Franklyn *et al.,* 1994; Garton *et al.,* 1994; Affinito *et al.,* 1996; Jodar *et al.,* 1998). It has been suggested that a low dietary calcium intake can contribute to the risk of T_4-induced bone loss (Kung *et al.,* 1983). Estrogen and HRT protected against the bone loss associated with T_4 treatment (Schneider *et al.,* 1994; Franklyn *et al.,* 1995). Consistent with the importance of dose and duration, TSH has been a useful predictive marker for bone loss (Wartofsky, 1991). A meta-analysis of 13 publications in which TSH was suppressed by thyroid hormone treatment projected that a premenopausal woman at an average age of 39.6 years, treated with L-T_4, (164 μg/day for 8.5 years), would have an excess annual bone loss of 0.31% and 2.67% less bone mass than a control (Faber and Galloe, 1994).

In contrast with these findings, a number of studies report that T_4 treatment failed to produce bone loss (Toh and Brown, 1990; Ribot *et al.,* 1990; Franklyn and Sheppard, 1992; Grant *et al.,* 1993; Fujiyama *et al.,* 1995; Hawkins *et al.,* 1994; Schneider *et al.,* 1995; DeRosa *et al.,* 1995; Saggase *et al.,* 1996, 1997; Marcocci *et al.,* 1997; Gurlek and Gedik, 1999; Rachedi *et al.,* 1999; Nuzzo *et al.,* 1998; Knudsen *et al.,* 1998; Hanna *et al.* 1998; Langdahl *et al.,* 1996a). One explanation for this apparent disparity, in the case of patients receiving T_4 replacement therapy, is that their cortical bone density was initially higher due to their hypothyroidism and that T_4 replacement resulted in a normalization (Ross, 2000). A longitudinal study would indicate bone loss, whereas a cross-sectional study would not reveal a significant difference from the control group. Another possible basis for some of the diversity of findings is that the accelerated bone turnover and increased fracture risk with T_4 treatment can be a transient phenomenon. In one report, correlations between serum-free T_4 and serum procollagen III peptide, which had been noted after 6 months of T_4 treatment, were not found in patients treated chronically (Nystrom *et al.,* 1989). There may be an initial increase in cortical width and bone porosity that results in increased fracture risk until a new steady-state condition is established (Coindre *et al.,* 1986). Another study found a temporary increase in fracture risk in previously hypothyroid patients, which was most prevalent in patients over 50 years of age and limited to forearm fractures (Vestergaard *et al.,* 2000b)

Stimulation of the production of local factors by thyroid hormone, which was observed *in vitro* and animal studies, is also seen in humans. Thyroid hormone increases circulating IL-6 (Lakatos *et al.,* 1997; Siddiqi *et al.,* 1999) and IGF-I (Brixen *et al.,* 1995; Kassem *et al.,* 1998; Foldes *et al.,* 1999; Lakatos *et al.,* 2000). One can speculate that the greater rate of production of IGF-I in children could explain the findings of studies in which large doses of thyroid hormone were not deleterious to bone in children (Kooh *et al.,* 1996; Verrotti *et al.,* 1998; Dickerman *et al.,* 1997; Leger *et al.,* 1997; Van Vleit *et al.,* 1999; Tumer *et al.,* 1999). The anabolic effect of the increased IGF-I could compensate for or override the bone breakdown.

Reversibility/Treatment/Prevention of Thyroid Hormone-Stimulated Bone Loss

Recovery of bone loss in hyperthyroid patients following antithyroid treatment has been inconsistent (Fraser *et al.,* 1971; Toh *et al.,* 1985; Saggese *et al.,* 1990; Diamond *et al.,* 1994; Mudde *et al.,* 1994), but may be achieved more readily in younger individuals (Fraser *et al.,* 1971; Saggese *et al.,* 1990). Studies have documented protective effects of methimazole (Langdahl *et al.,* 1996b; Nagasaka *et al.,* 1997; Mora *et al.,* 1999). Surgery and radioactive iodine also prevented bone loss in hyperthyroid patients (Langdahl *et al.,* 1996c; Arata *et al.* 1997), but were less protective than methimazole (Vestergaard *et al.,* 2000a). The protective effects of estrogen during treatment with T_4 were noted earlier, and androgen may also be beneficial (Lakatos *et al.,* 1989). Bisphosphonates may also protect against thyroid hormone-induced bone loss. Both animal (Ongphiphadhanakul *et al.,* 1993; Rosen *et al.,* 1993a; Yamamoto *et al.,* 1993; Kung and Ng, 1994) and human (Rosen *et al.,* 1993b; Lupoli *et al.,* 1996) studies have demonstrated that bisphosphonates are effective in preventing thyroid hormone-stimulated bone loss. Etidronate (0.5 mg/100 g administered twice weekly) prevented decreased bone mineral density and increased mRNA for alkaline phosphatase, tartrate-resistant acid phosphatase, and histone H4 in femurs of rats treated with L-T_4 for 20 days (Ongphiphadhanakul *et al.,* 1993). The combination of L-T_4 and etidronate resulted in lower expression of mRNA for type I collagen, osteocalcin, and osteopontin, which was lower than that of controls, although neither L-T_4 nor etidronate alone affected these parameters. Alendronate (1.75 mg/kg orally twice weekly) prevented increased bone turnover resulting from the administration of excess T_4 for 3 weeks (Lupoli *et al.,* 1996). The preventive effect was assessed by histomorphometry and measurement of osteocalcin. Bone volume was above control in all alendronate-treated groups in the study. Pretreatment of rats with pamidronate (5 μmol/kg/day subcutaneously for 1 week prior to T_3) prevented increases in alkaline phosphatase and osteocalcin at 1 week and losses of bone mineral density at 3 weeks in the femur and spine (Rosen *et al.,* 1993b). Pamidronate pretreatement (30 mg iv, daily for 2 days) prevented increases in urinary calcium/creatinine ratio, urinary hydroxyproline, and urinary pyridinoline cross-links in normal male subjects treated with T_3 (Rosen *et al.,* 1993a). Calcium and calcitonin were also found to offer some protective benefit (Kung and Yeung, 1996).

Overview, Speculations, and Future Directions

Thyroid hormones interact with both nuclear receptors and membrane-binding sites in bone and influence many of the phenotypic responses of bone cells. In intact organisms, deficiency or excess thyroid hormone can alter skeletal development and maintenance, indicating the importance of physiological concentrations of the hormones for normal skeletal physiology. Effects of thyroid hormones to amplify bone turnover are evident from clinical studies, *in vivo* investigations in animals, and *in vitro* models. There is a dose dependence to the effects, with anabolic effects declining and catabolic effects becoming more prominent with the higher concentrations of thyroid hormone present in hyperthyroidism and with the higher doses that are used for suppressive therapy. Determination of TSH to guide thyroid hormone dosage can decrease the occurrence of bone loss resulting from the therapeutic use of thyroid hormone. However, factors other than dosage can influence the response to thyroid hormone. The anabolic effects of thyroid hormone on bone are more apparent in younger animals and children, consistent with the possibility that growth factors can have significant mediating or modulating effects. Thyroid hormones increase IGF-I in osteoblasts and experimental animals; elevated circulating thyroid hormones are associated with increases in IGF-I and IGFBPs in patients. Other physiological factors modulate the skeletal effects of thyroid hormones; e.g., estrogens can decrease the deleterious effects of excess thyroid hormone. The pharmacological inhibition of bone resorption with bisphosphonates can also diminish thyroid hormone-stimulated bone loss. The biphasic effects of thyroid hormones observed *in vivo* are also seen in *in vitro* models at physiologically and pathophysiologically relevant concentrations. Thyroid hormones stimulate osteoblast proliferation, promote the differentiation of this cell type, and stimulate differentiated functions, as shown by increases in alkaline phosphatase activity, osteocalcin expression, and stimulation of collagen synthesis. The variation in the responses when different model systems are used indicates that there are additional modulating factors that are not yet understood. The osteoblast is the target cell for thyroid hormone activation of mature osteoclasts. The promotion of resorption by thyroid hormones may be mediated through the activation of cytokine pathways that lead to osteoclast differentiation. Thus, thyroid hormones act at receptor, cellular, and organismal levels to modulate or interact with many of the other factors and pathways that determine the status of the skeleton. The elucidation of thyroid hormone receptor diversity and its consequences for the skeleton is clearly an important goal for the near future.

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CHAPTER 41

Clinical and Basic Aspects of Glucocorticoid Action in Bone

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General Introduction

Glucocorticoid-induced osteoporosis was first reported by Cushing (1932) when he described osteoporosis in patients with high levels of cortisol due to an adrenocorticotrophin-producing tumor of the pituitary gland. The problem became clinically significant in 1949 when pharmacological doses of glucocorticoids were introduced for therapeutic use because of their potent anti-inflammatory and immunosuppressive effects. It became clear that treatment with glucocorticoids causes a loss of bone mass and pathologic fractures. Since then, efforts have been made to elucidate the cause of steroid-induced bone loss.

Cortisol, the glucocorticoid secreted by the adrenal gland, is essential in physiologic doses for the differentiation and function of osteoblasts and osteoclasts, and modulates the effects of other hormones and mediators of cell function, whereas supraphysiologic doses inhibit bone formation. These direct effects on bone, combined with effects on other systems that indirectly regulate bone metabolism, cause rapid bone loss in patients treated with glucocorticoids. The mechanisms involved and the resulting clinical picture are the subjects of this chapter.

Pharmacology of Glucocorticoids

Synthetic derivatives of cortisol with less mineralocorticoid effect have been developed. The compounds prescribed most frequently are prednisone, prednisolone, methylprednisolone, betamethasone, dexamethasone, and triamcinolone. Prednisone is metabolized to prednisolone. The 4,5 double bond and the 3-ketone structures are both necessary for typical adrenocorticoid activity. Introduction of the 1,2 double bond, as in prednisone or prednisolone, enhances the ratio of carbohydrate regulating potency to sodium-retaining potency. 6α-methylation of the B ring (6α-methylprednisolone) increases anti-inflammatory potency while reducing electrolyte-retaining properties. 9α-fluorination enhances all biologic activities, whereas 16-methylation eliminates the sodium-retaining effect, but only slightly alters other effects on metabolism or inflammation. Substitution in the 17α-ester position produces a group of extremely potent steroids, beclomethasone diproprionate and budesonide, which are effective when applied topically to skin or administered by inhalation (Gilman *et al.,* 1990). The absorption of inhaled steroids is virtually equivalent to that of oral administration, and absorption from skin is significant if applied over a large surface or under plastic film. Despite this, inhaled or topical steroids reduce side effects because the drugs are targeted to the site of the disease and lower doses can be used. Deflazacort, an oxazoline derivative of prednisone, has been developed with the hope of reducing the catabolic effects of glucocorticoids while maintaining anti-inflammatory effects (Gennari *et al.,* 1984), but the results have been disappointing. The development of synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression and yet exhibit anti-inflammatory activity holds

promise for the development of glucocorticoid-based drugs that separate beneficial from deleterious effects.

Glucocorticoids are widely used in the treatment of asthma, collagen-vascular disease, inflammatory bowel disease, granulomatous, and skin diseases. The skeletal response to glucocorticoids is not disease specific, and accelerated bone loss has been described in patients with each of these diseases when they are treated with steroids (de Deuxchaisnes *et al.,* 1984; Reid *et al.,* 1986a; Rizzato *et al.,* 1988).

Characteristics of Bone Loss

Bone loss, measured by dual-energy X-ray absorptiometry in patients receiving glucocorticoids for more than a year, has been reported to average 0.6–6% per year (Sambrook *et al.,* 1990; Lukert *et al.,* 1992; Laan *et al.,* 1993). Trabecular bone and the cortical rim of the vertebral body are more susceptible to the effects of glucocorticoids than the cortical bone of the extremities year (Seeman *et al.,* 1982; Laan *et al.,* 1993). Consequently, compression fractures of the spine are frequently the first sign of glucocorticoid-induced bone loss, and the proximal femur becomes more fragile. Although bone loss appears to be most rapid during the first 6–12 months of treatment, loss remains above average for the duration of treatment (Gennari, 1985; Lukert *et al.,* 1992). Approximately 30–50% of patients taking glucocorticoids long term and 50% of patients with Cushing's disease (excessive endogenous production of steroids) have at least one atraumatic fracture (Ross *et al.,* 1982; Adinoff *et al.,* 1983). The risk for hip fracture is doubled and the risk for vertebral fracture is increased five-fold by oral doses of prednisone exceeding 7.5 mg/day (Van Staa *et al.,* 2000). The risk for fracture increases within the first 3 months after the initiation of glucocorticoid therapy and decreases within 3 months after discontinuation.

One study showed that the fracture threshold for vertebral fractures may be higher for patients taking steroids than for those with involutional osteoporosis (Luengo *et al.,* 1990); i.e., fractures occur at a higher bone density in steroid-treated patients. However, a more recent study found no increase in risk of clinical fracture in corticosteroid-treated patients whose bone densities were matched to controls; i.e., patients on steroids tended to fracture at bone densities similar to patients with involutional osteoporosis.

Glucocorticoid-induced bone loss is partially reversible after cessation of prednisone administration or removal of the cause of excessive endogenous production of cortisol (Manning *et al.,* 1992; Laan *et al.,* 1993; Rizzato *et al.,* 1993; Van Staa *et al.,* 2000). Bone loss is also partially reversible during treatment with estrogen/progesterone therapy, bisphosphonates, calcitonin, parathyroid hormone (PTH), or sodium fluoride while prednisone is continued (Meunier *et al.,* 1987; Luengo *et al.,* 1990; Lukert *et al.,* 1992; Struys *et al.,* 1995; Saag *et al.,* 1998; Lane *et al.,* 2000; Reid *et al.,* 2000).

Histomorphometric studies on bone from glucocorticoid-treated patients show that glucocorticoids cause apoptosis of osteoblasts and osteocytes and depress osteoblastic function, while simultaneously, the frequency of activation of bone remodeling units is increased. Thus, there is an increase in the number of sites at which bone is being resorbed, and the ability of osteoblasts to replace bone at each site is decreased. This results in a reduced wall thickness of cancellous bone packets and, eventually, to perforation and removal of trabecular plates (Bressot *et al.,* 1979; Meunier *et al.,* 1982; Dempster, 1989; Weinstein *et al.,* 1998; Plotkin *et al.,* 1999).

Serum levels of osteocalcin, the most abundant noncollagen bone matrix protein and a biochemical marker of bone formation, are suppressed in patients receiving either oral or inhaled glucocorticoids (Lukert *et al.,* 1986; Puolijoki *et al.,* 1992). Surprisingly, urinary hydroxyproline and pyridinium cross-links, markers of bone resorption, are not increased by glucocorticoids (Cosman *et al.,* 1994; Lukert *et al.,* 1995). Conversely, serum tartrate-resistant acid phosphatase was elevated during short-term steroid therapy. It was felt that the high doses used in this study could have been toxic to osteoclasts, causing cell death and liberation of cytoplasmic TRAP into serum in the absence of increased bone resorption (Cosman *et al.,* 1994). The finding of a 96% increase in osteoclast perimeter observed in vertebrae taken from mice receiving prednisolone for 7 days makes it more likely that osteoclastic bone resorption is indeed increased early in the course of glucocorticoid administration (Weinstein *et al.,* 1998).

Risk Factors for Glucocorticoid-Induced Bone Loss

The usual risk factors for involutional osteoporosis (age, race, sex, weight, and parity) do not apply to the same extent to glucocorticoid-induced bone loss (Dykman *et al.,* 1985). Everyone taking high doses (greater than 10 mg/day of prednisone) loses significant amounts of bone (Garton *et al.,* 1993). Postmenopausal women receiving equivalent doses of steroids are more at risk for fractures than premenopausal women or men, presumably because they also have age and menopause-related bone loss. It is unlikely that there is a threshold dose of glucocorticoid below which bone loss does not occur. A retrospective cohort study showed that the risk for fracture is increased even for doses below 7.5 mg/day and increases further with increasing daily and cumulative doses (Van Staa *et al.,* 2000). Even high doses of some inhaled steroids (Ip *et al.,* 1994), but not others (Medici *et al.,* 2000), cause bone loss.

Indirect Mechanisms for the Pathogenesis of Glucocorticoid-Induced Bone Loss

Glucocorticoids affect nearly every system in the body. We will first discuss the effects of glucocorticoids on systems that modulate bone metabolism indirectly (Table I) to set the stage for a discussion of the direct effects of glucocorticoids on bone.

Table I Effect of Glucocorticoids on Systems That Modulate Bone Remodeling

Pituitary
Inhibition of secretion of growth hormone, FSH/LH and ACTH
Cellular transport
Decrease in transport of calcium and phosphorus
Parathyroid hormone
Increased secretion
Increased peripheral sensitivity of PTH
Gonads
Inhibition of synthesis of estrogen by ovary and testosterone by testes
Adrenal
Decrease in secretion (due to ACTH) of dehydroepiandrosterone and androstenedione

Effects on Pituitary Function

GROWTH HORMONE

The secretion of growth hormone is partially controlled by glucocorticoids. Prednisone inhibits pituitary secretion of growth hormone in response to GH-releasing hormone in normal men (Kaufmann *et al.,* 1988). Nevertheless, serum concentrations of growth hormone and insulin-like growth factor-1 (IGF-1) are normal in patients receiving glucocorticoids (Morris *et al.,* 1968; Gourmelen *et al.,* 1982; Kaufmann *et al.,* 1988). Despite normal levels, IGF-1 activity measured by bioassay is decreased in patients with glucocorticoid excess, perhaps because of an IGF-1 inhibitor that has been found in the serum of children receiving glucocorticoids (Unteman *et al.,* 1985). This inhibitory factor may be one of the IGF-binding proteins (IGFBP).

A clearer understanding of the role of IGF-binding proteins on IGF activity is emerging and shedding light on the mechanisms through which glucocorticoids may exert their effect. As discussed later, glucocorticoids may affect IGFBP, which inhibit or enhance IGF activity. Glucocorticoids increase circulating levels of IGFBP-1, which may limit the activity of IGF-1; this effect has been associated with glucocorticoid-induced fetal growth retardation (Prince *et al.,* 1992). The importance of serum levels of growth factors or their binding proteins is unknown, as growth factors are produced locally by bone cells. Growth hormone and PTH are trophic hormones (Ernst *et al.,* 1988; McCarthy *et al.,* 1989) for growth factors produced in bone, and the increase in bone density observed with the administration of PTH may be due to stimulation of the production of growth factors in bone (Lane *et al.,* 2000).

HYPOTHALAMIC–PITUITARY–GONADAL AXIS

Glucocorticoids blunt pituitary secretion of luteinizing hormone (Sakakura *et al.,* 1975). A subset of gonadotropin-releasing-hormone (GnRH)-containing neurons in the rat hypothalamus possesses glucocorticoid receptors that bind dexamethasone *in vitro* with high affinity. Glucocorticoids repress transcription in a hypothalamic cell line, and glucocorticoid receptors acting directly within GnRH neurons could be at least partly responsible for negative regulation of the hypothalamic–pituitary–gonadal axis. Glucocorticoids also have direct effects on gonads inhibiting follicle-stimulating hormone (FSH)-induced estrogen production by ovarian granulosa cells and testosterone production by the testes (Hsueh *et al.,* 1978). The adrenal secretion of androgens is also decreased due to suppression of ACTH secretion. Inhaled beclomethasone in doses of 1 mg/day or greater lower mean serum levels of dehydroepiandrosterone sulfate (DHEA) by 35% in postmenopausal women (Smith *et al.,* 1994). As a result of these combined effects, serum concentrations of estradiol, estrone, DHEA, androstenedione, and progesterone are decreased in women, whereas DHEA and testosterone are decreased in men receiving glucocorticoids (MacAdams *et al.,* 1986). It is very likely that deficiencies in these anabolic hormones accelerate bone loss. There is a direct correlation between bone mineral density and plasma estradiol levels in glucocorticoid-treated women (Montecucco *et al.,* 1992); furthermore, women receiving estrogen/progesterone replacement therapy and men given medroxyprogesterone acetate while taking glucocorticoids were protected against bone loss (Grecu *et al.,* 1990; Lukert *et al.,* 1992).

Calcium and Phosphorus Transport, Parathyroid Function, and Vitamin D Metabolism

Patients taking pharmacological doses of glucocorticoids have impaired gastrointestinal absorption of calcium, hypercalciuria, and phosphaturia and higher levels of serum PTH and $1,25(OH)_2D$ when compared to patients not taking steroid. (Favus *et al.,* 1973; Adams *et al.,* 1981; Bikle *et al.,* 1993; Shrivastava *et al.,* 2000). Even very small oral doses of beclomethasone, similar to doses that may be swallowed by patients using the drug in inhaled form, decrease calcium absorption for the intestine (Smith *et al.,* 1993). High PTH levels have traditionally been attributed to prolonged negative calcium balance, and the rise in $1,25(OH)_2D$ is due to the trophic effect of PTH. However, acute longitudinal studies have shown that serum $1,25(OH)_2D$ levels increase and serum phosphorus levels decrease within 2 hr after intravenous administration of methylprednisolone, before PTH had risen significantly. PTH levels increased progressively during the first 2 weeks of high-dose treatment. All of these parameters returned to baseline at 3 weeks when methylprednisolone was given orally but still at a high dose (Cosman *et al.,* 1994). These findings suggest that glucocorticoids alter transport across a number of biologic membranes. Whether these effects on transport are due to changes in calcium receptor or other proteins or enzymes involved in transport (a genomic effect) or to direct effects on membrane permeability remain unknown at this time.

Although acute studies showed that the changes in PTH and $1,25(OH)_2D$ were transient, it is important to note that others have found high PTH and $1,25(OH)_2D$ levels and higher levels of urinary cAMP and reduced tubular reabsorption of phosphate in patients taking glucocorticoids for more

than a year and in those with Cushing's disease (Lukert *et al.,* 1976; Findling *et al.,* 1982; Bikle *et al.,* 1993). This suggests that glucocorticoids have an acute effect on transport, which inhibits gastrointestinal absorption of calcium, decreases renal tubular reabsorption of calcium and phosphorus, and may decrease intracellular calcium and phosphorus. This in turn promotes synthesis of $1,25(OH)_2D$. Long-term glucocorticoid administration causes negative calcium balance, which perpetuates secondary hyperparathyroidism with its accompanying hypophosphatemia and elevated serum $1,25(OH)_2D$ levels. The high levels of cAMP and decreased TRP indicate that the increase in PTH (even though in the normal range) is of physiologic significance, as both of these changes are known effects of PTH on the kidney.

In addition to the effects of glucocorticoid-induced elevation of PTH levels on phosphate transport, glucocorticoids have direct effects on renal tubular reabsorption of phosphate acting through the Na^+-H^+ exchange activity in the proximal tubule, thus decreasing Na^+ gradient-dependent phosphate uptake (Frieberg *et al.,* 1982). Likewise, although glucocorticoids induce changes in vitamin D metabolism, which could affect calcium transport, non-vitamin D-dependent alterations in calcium transport have been observed in the gastrointestinal tract in the presence of glucocorticoids (Charney *et al.,* 1975; Adams *et al.,* 1980). The role of vitamin D metabolites and vitamin D-dependent mechanisms in the malabsorption of calcium in patients taking glucocorticoids is unclear. Active transport of calcium is inhibited by glucocorticoids in the presence of elevated levels of $1,25(OH)_2D$ and is only partially corrected by pharmacological levels of $1,25(OH)_2D$. Calbindin synthesis is stimulated by glucocorticoids (Corradino *et al.,* 1991), and the brush border uptake of calcium is not altered (Shultz *et al.,* 1982). These findings suggest that glucocorticoid-induced inhibition of calcium absorption is caused by alterations in posttranscriptional events, alterations in basolateral membrane transport, or other toxic mechanisms. Possible mechanisms include depletion of mitochondrial adenosine triphosphate (Krawitt, 1972) or pericellular back flux due to stimulation of the sodium–potassium–ATPase pump by glucocorticoids (Charney *et al.,* 1975; Adams *et al.,* 1980).

The sensitivity of osteoblasts to PTH is increased by glucocorticoids. Glucocorticoids probably act on or near the stimulatory guanine nucleotide-binding regulatory protein complex. The potentiation of PTH-induced increases in cAMP response appears to be due to increases in cAMP activity and inhibition of phosphodiesterase (Chen *et al.,* 1978). Whether renal tubules are more sensitive to PTH in the presence of glucocorticoids remains unclear.

Osteonecrosis

Osteonecrosis (avascular necrosis or aseptic necrosis) is a well-recognized complication of glucocorticoid excess. Glucocorticoid-induced osteonecrosis was first recognized in 1957. Previous administration of glucocorticoids can be implicated in 16–34% of patients presenting with "idiopathic" osteonecrosis (Fisher *et al.,* 1971). The femoral head is affected most frequently, followed by the head of the humerus and distal femur, but osteonecrosis may occur in other long bones and the bones of the feet. A similar lesion characterized by a transverse radiolucent cleft running under an end plate is seen in the vertebra and resembles subchondral fracture seen in long bones. The risk for osteonecrosis increases with both the dose of glucocorticoids and the duration of treatment (Zizic *et al.,* 1985). However, osteonecrosis may develop in patients who receive steroids in very high doses for a short period of time (Taylor, 1984), moderate doses over a long period of time (Metselaar *et al.,* 1985), or by intraarticular or epidural injection.

The mechanisms responsible for glucocorticoid-induced osteonecrosis remain obscure. Etiologic considerations invoke several theories. One is a mechanical theory that attributes ischemic collapse of the epiphysis to osteoporosis and the accumulation of unhealed trabecular microcracks resulting in fatigue fractures. Others include a vascular theory proposing that ischemia is caused by microscopic fat emboli and a theory that increased intraosseous pressure due to fat accumulation as part of the Cushing syndrome leads to mechanical impingement on the sinusoidal vascular bed and decreased blood flow (Mankin, 1992). The number of apoptotic osteoblasts and osteocytes is increased in femoral necks removed from patients developing avascular necrosis while taking steroids, whereas this phenomenon was not observed in patients with avascular necrosis due to other causes (Weinstein *et al.,* 1998). The induction of early cell death may play a pivotal role in the etiology of steroid-induced avascular necrosis.

Clinically, pain is the usual presenting symptom and may be mild or vague in chronic forms of the disease, but it is usually acute and severe. Osteonecrosis may remain silent as long as it is not associated with epiphyseal collapse, which appears to initiate symptoms (Maldague *et al.,* 1984). Early osteonecrosis of the hip may be managed by prolonged avoidance of weight bearing, but prosthetic replacement of the joint is frequently necessary. Surgical decompression may be attempted but the results are not encouraging (Mankin, 1992).

Summary of Cumulative Effects of Glucocorticoid-Induced Metabolic Changes on Bone

The overall effect of glucocorticoids is catabolic. Inhibition of pituitary secretion of growth hormone and alterations in IGF-binding proteins lead to a fall in the biologic activity of growth factors with loss of their anabolic effect on bone and other tissues. Gonadotrophin secretion is inhibited and, along with direct inhibitory effects of glucocorticoids on gonadal secretion of estrogen/testosterone, leads to a fall in circulating gonadal hormone concentrations. Deficiency in gonadal hormones causes an increase in bone resorption (Fig. 1).

Membrane transport systems are altered by glucocorticoids, resulting in inhibition of gastrointestinal absorption of calcium and decreased renal tubular absorption of calcium and phosphorus. Lowered intracellular phosphorus

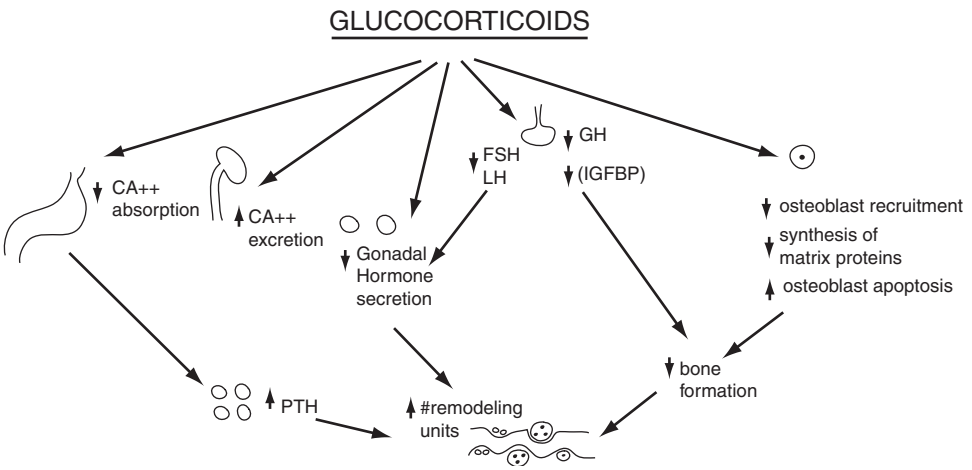

Figure 1 Effect of steroids on bone and calcium metabolism. Glucocorticoids (GC) inhibit gastrointestinal absorption and increase renal excretion of calcium. A negative calcium balance and perhaps failure to transport calcium into the parathyroid cell cause an increase in the secretion of parathyroid hormone (PTH). PTH increases the number of sites undergoing bone remodeling. Decreased levels of gonadal hormones caused by GC inhibition of their secretion further augment bone resorption. Glucocorticoids decrease recruitment of osteoblasts from osteoprogenitor cells, accelerate apoptosis of osteoblasts and osteocytes, and inhibit bone formation at each site. Combination of an increase in the number of sites undergoing remodeling and a decrease in bone formation at each site causes rapid bone loss.

causes an acute rise in $1,25(OH)_2D$ synthesis. PTH secretion is increased, despite elevated serum levels of calcium and $1,25(OH)_2D$. Chronically, increased PTH secretion and the resultant elevation in $1,25(OH)_2D$ production are perpetuated by a negative calcium balance. The elevated levels of PTH and $1,25(OH)_2D$, along with deficiency of gonadal hormones, increase the number of sites undergoing bone resorption. The direct inhibition of osteoblastic bone formation at each bone remodeling site further augments the rate of bone loss.

Glucocorticoid-induced bone loss can be prevented by bisphosphonates, hormone replacement, PTH, and perhaps calcitonin (Luengo *et al.,* 1990; Lukert *et al.,* 1992; Reid *et al.,* 1996; Lane *et al.,* 1998; Saag *et al.,* 1998; Cohen *et al.,* 1999). Fracture risk is reduced by bisphosphonates but fracture data are not available for the other modalities. In addition to inhibiting bone resorption, bisphosphonates and calcitonin appear to decrease the number of apoptotic osteocytes and osteoblasts observed in bone biopsies from patients treated with prednisolone (Plotkin *et al.,* 1999). Estrogen has a similar effect in estrogen-deficient states but it is not known whether estrogen prevents apoptosis in the presence of glucocorticoids. Protection from glucocorticoid-induced apoptosis may play a major role in the prevention of early bone loss in patients taking glucocorticoids.

Direct Actions of Glucocorticoids on Bone

Introduction

Glucocorticoids have a myriad of effects on osteoblastic cells, resulting in profound changes in bone remodeling (Delany *et al.,* 1994; Canalis, 1996; Ishida *et al.,* 1998; Cooper *et al.,* 1999; Manolagas *et al.,* 1999). A hallmark of glucocorticoid-induced osteoporosis in humans is decreased mean wall thickness of trabecular bone, reflecting a reduction in the amount of new bone replaced in each remodeling cycle (Dempster *et al.,* 1983). Cells of the osteoblast lineage contain glucocorticoid receptors (Chen *et al.,* 1977; Manolagas *et al.,* 1978; Haussler *et al.,* 1980; Abu *et al.,* 2000), and supraphysiological concentrations of glucocorticoids decrease protein, RNA, and DNA synthesis in primary bone cell cultures (Peck *et al.,* 1967; Chen *et al.,* 1977; Choe *et al.,* 1978; Wong, 1979). Teleologically, these studies are consistent with the catabolic effects of high levels of glucocorticoids on human bone. Likewise, glucocorticoid treatment also decreases bone formation in dogs, rats, and mice (Altman *et al.,* 1992; Quarles, 1992; Ortoft *et al.,* 1995; Turner *et al.,* 1995; Weinstein *et al.,* 1998). In some rat studies, glucocorticoid treatment decreases bone formation but does not induce osteopenia. This is due to an inhibition of turnover, as both bone resorption and formation are reduced, and bone mass does not decrease (Li *et al.,* 1996; Shen *et al.,* 1997). In mice, however, there is an increase in osteoclast surface shortly after glucocorticoid treatment, which is followed by a decrease in the rate of bone formation and a reduction in bone mass (Weinstein *et al.,* 1998). Thus, mice, compared to rats, may be more like humans in the response of bone to pharmacological doses of glucocorticoids (Manolagas *et al.,* 1999).

Bone Formation and Osteoblast Differentiation

In contrast to the marked inhibitory effect of pharmacological doses of glucocorticoids on bone formation *in vivo*, glucocorticoids cause both catabolic and anabolic effects

on bone formation and osteoblast differentiation *in vitro.* Although the relevance of the *in vitro* anabolic effect is not completely understood, it may reflect a permissive role in the maintenance of the osteoblast phenotype during bone remodeling. Analysis of data from *in vitro* studies is complicated by a plethora of experimental variables, including the concentration of hormone, the molecular form of the glucocorticoid used, the timing of hormone addition, the presence of serum, and the species, cellular heterogeneity, and developmental stage of the model system. However, some generalizations can be made. *In vitro,* physiological concentrations of glucocorticoids enhance the differentiation of early osteoprogenitors and stimulate the formation of bone in "developmental" models of bone formation. In contrast, pharmacological concentrations of glucocorticoids inhibit cell proliferation, impair the function of more mature osteoblasts, and increase osteoblast and osteocyte apoptosis. These effects ultimately lead to a decrease in bone mass.

Organ cultures reflect both anabolic and catabolic effects of glucocorticoids on bone formation. Organ explants of folded periostea from embryonic chick calvariae form new bone during culture (Tenenbaum *et al.,* 1985). When dexamethasone is added at the onset of culture, there is enhanced osteoid formation, alkaline phosphatase activity, and a transient increase in the replication of cells adjacent to the newly formed bone surface. However, when dexamethasone is added late in the culture period after bone has formed, there is a decrease in alkaline phosphatase activity (Tenenbaum *et al.,* 1985). Thus, it appears that glucocorticoids initially cause the proliferation and differentiation of a distinct population of osteoprogenitor cells that participate in bone formation, but then limit further cell proliferation in the cultures (McCulloch *et al.,* 1986).

Glucocorticoids both stimulate and inhibit type I collagen synthesis in serum-free organ cultures of fetal rat calvariae depending on the dose of hormone and duration of hormone treatment (Dietrich *et al.,* 1978; Canalis, 1983; Kream *et al.,* 1990b). In fetal rat calvariae, physiological concentrations of cortisol (30–100 n*M*) stimulate collagen synthesis after 24 hr, whereas pharmacological concentrations (1000 n*M*) are inhibitory at 48–96 hr (Dietrich *et al.,* 1978). Likewise, there is a rapid stimulatory effect of cortisol on collagen synthesis in newborn rat calvariae (Hahn, 1984). In fetal rat calvariae, the early stimulation of collagen synthesis is blocked by the addition of IGFBP-2, which binds and inactivates secreted IGFs (Kream *et al.,* 1997). These data suggest that the initial stimulation of collagen synthesis by glucocorticoids depends on the activity of endogenous IGF-1 and may be due to increased osteoblastic differentiation.

Many studies show that gluococorticoids enhance osteogenic differentiation in long-term primary calvarial cell cultures that form mineralized bone nodules in the presence of serum, ascorbic acid, and β-glycerolphosphate. These cultures are defined by the stages of cell proliferation, extracellular matrix maturation, and matrix mineralization, each characterized by the expression of cell growth and tissue-specific genes (Gerstenfeld *et al.,* 1987; Owen *et al.,* 1990; Stein *et al.,* 1990). Glucocorticoids increase the formation of bone nodules and the expression of genes associated with the osteoblast phenotype in primary rat osteoblastic cell cultures (Bellows *et al.,* 1987, 1989, 1990; Shalhoub *et al.,* 1992). The effect of glucocorticoids is biphasic: low concentrations of dexamethasone and hydrocortisone increase nodule formation, whereas pharmacological concentrations are less effective or not stimulatory (Bellows *et al.,* 1987). In this model, the anabolic effect of glucocorticoids has been attributed to the enhanced proliferation and differentiation of glucocorticoid-dependent osteoprogenitors (Bellows *et al.,* 1989, 1990). High concentrations of glucocorticoids inhibit osteogenic differentiation in MC3T3-E1 (Lian *et al.,* 1997) and primary murine osteoblast cultures (Bellows *et al.,* 1998).

Bone marrow stromal cell cultures grown in the presence of serum, ascorbic acid, and β-glycerolphosphate have been used extensively as a model system to study the stages of osteoblast differentiation. Osteoprogenitor cells within the bone marrow stromal network, when activated to differentiate, provide a renewable source of osteoblasts for endosteal and trabecular bone surfaces. Glucocorticoids enhance the expression of osteoblastic phenotypic traits such as alkaline phosphatase activity, osteocalcin, type I collagen, osteopontin, and bone sialoprotein and the formation of mineralized bone nodules in cultures of chick, rat, and human bone marrow stromal cells (Kasugai *et al.,* 1991; McCulloch *et al.,* 1991; Kamalia *et al.,* 1992; Cheng *et al.,* 1994, 1996; Malaval *et al.,* 1994; Rickard *et al.,* 1994; Herbertson *et al.,* 1995; Aubin, 1999). Bone stromal cell cultures contain glucocorticoid-dependent osteoprogenitor cells that gives rise to mineralized bone nodules (Aubin, 1999).

The pathway by which glucocorticoids enhance osteogenic differentiation *in vitro* is thought to involve members of the bone morphogenetic protein (BMP) family of proteins. BMP-2 and glucocorticoids exert a synergistic enhancement of the osteogenesis in rat bone marrow stromal cells (Rickard *et al.,* 1994) and fetal rat calvarial cells (Boden *et al.,* 1996). In the calvarial model, BMP-4 and BMP-6 are synergistic with glucocorticoids in promoting osteogenesis (Boden *et al.,* 1996). Moreover, glucocorticoid-dependent differentiation of fetal rat calvarial cells is blocked by antisense oligonucleotides to BMP-6 (Boden *et al.,* 1997).

High concentrations of glucocorticoids regulate preosteoblast replication and function. Glucocorticoids inhibit collagen synthesis in organ cultures of fetal rat (Dietrich *et al.,* 1978; Canalis, 1983) and mouse calvariae (Woitge *et al.,* 2000). In subclones of osteoblastic ROS 17/2 osteosarcoma cells, glucocorticoids either stimulate or inhibit collagen synthesis depending on the state of maturation of the cells; in less mature osteoblasts, glucocorticoids stimulate collagen synthesis, whereas in more mature osteoblasts they inhibit collagen synthesis (Hodge *et al.,* 1988). In confluent cultures of primary osteoblastic cells, glucocorticoids generally inhibit

collagen synthesis (Chen *et al.,* 1978; Kim *et al.,* 1989; Ng *et al.,* 1989). The inhibitory effect of glucocorticoids on collagen synthesis in fetal rat calvariae is accompanied by a decrease in periosteal cell content (Dietrich *et al.,* 1978; Canalis, 1984; Chyun *et al.,* 1984). Glucocorticoids also decrease the number of cells in the osteoblastic and periosteal layers of fetal rat parietal bone organ cultures (Gronowicz *et al.,* 1994). These effects in organ culture reflect at least in part the antiproliferative effect of glucocorticoids seen in osteoblastic cell cultures (Chen *et al.,* 1977; Hodge *et al.,* 1988; Hughes-Fulford *et al.,* 1992). Glucocorticoids decrease osteocyte formation in these cultures, which may reflect an inhibition of osteoblast renewal (Gohel *et al.,* 1995) and/or oseoblast apoptosis (Gohel *et al.,* 1999). High concentrations of glucocorticoids inhibit proliferation in primary human bone marrow stromal cell cultures (Silvestrini *et al.,* 2000; Walsh *et al.,* 2001). However, not all the effects of glucocorticoids on osteoblast function can be ascribed to an inhibition of cell replication. For example, the inhibitory effect of glucocorticoids on collagen synthesis, although blunted, still persists in the presence of DNA synthesis inhibitors, suggesting that glucocorticoids also inhibit the function of differentiated osteoblasts (Lukert *et al.,* 1991). In cultured bone marrow stromal cells, it was shown that a physiologically relevant concentration of dexamethasone (10 n*M*) promotes osteogenic differentiation, whereas a higher concentration (100 n*M*) was also osteogenic but decreased cell number. These data suggest that glucocorticoids enhance osteoblastic differentiation but that a decrease in the proliferation of osteogenic precursors ultimately limits the extent of bone formation (Walsh *et al.,* 2001).

Glucocorticoids increase the apoptosis of osteoblasts (Weinstein *et al.,* 1998; Gohel *et al.,* 1999; Silvestrini *et al.,* 2000) and osteocytes (Weinstein *et al.,* 1998; Plotkin *et al.,* 1999). In primary fetal rat calvariae cell cultures, the increase in osteoblast apoptosis is associated with a decrease in the Bcl-2/Bax protein ratio (Gohel *et al.,* 1999). Chronic treatment of adult mice with prednisolone increases apoptosis of osteoblasts in vertebrae and osteocytes in metaphyseal cortical bone and decreases bone mass (Weinstein *et al.,* 1998), which is reduced by bisphosphonate treatment (Plotkin *et al.,* 1999). Acute treatment of neonatal mice with dexamethasone increases apoptosis of osteoblasts in calvariae (Gohel *et al.,* 1999), and this effect was reversed by cotreatment with 17β-estradiol. Apoptotic osteocytes and cancellous-lining cells were seen in femoral heads from patients with glucocorticoid-induced osteonecrosis (Weinstein *et al.,* 2000).

Chronic glucocorticoid administration decreases osteoblast formation *in vivo.* When mice are treated with pharmacological levels of glucocorticoids *in vivo,* the generation of fibroblast colony forming units in *ex vivo* bone marrow cultures is decreased, suggesting that glucocorticoids deplete the bone marrow of osteogenic precursors (Simmons *et al.,* 1990). Likewise, glucocorticoids treatment of adult mice for 1 month suppresses osteogenic differentiation in *ex vivo* bone marrow cultures (Weinstein *et al.,* 1998).

Collectively, these data suggest that pharmacological doses of glucocorticoids induce osteoporosis due to a decrease in bone formation that results from an impairment of osteoblast function, inhibition of osteoblast renewal, and increased osteoblast apoptosis. However, physiological concentrations of glucocorticoids may be important for maintaining osteoblast differentiation.

Bone Resorption

Although the primary defect in glucocorticoid-induced osteoporosis is an inhibition of bone formation, glucocorticoids have direct effects on bone resorption. Glucocorticoids inhibit basal and agonist-stimulated resorption of fetal rat long bones (Raisz *et al.,* 1972) but increase resorption of fetal rat parietal bones (Gronowicz *et al.,* 1990) and mouse calvariae (Reid *et al.,* 1986b; Conaway *et al.,* 1996). Glucocorticoids decrease the activity and increase the apoptosis of rat osteoclasts (Tobias *et al.,* 1989; Dempster *et al.,* 1997). Dexamethasone increases osteoclastogenesis in mouse bone and spleen cell cocultures (Kaji *et al.,* 1997). In human stomal cell and osteoblastic cell cultures, glucocorticoids stimulate expression of the receptor activator of NF-κB (RANKL), the key stimulator of osteoclast formation, and decreases expression of osteoprotegerin (OPG), an inhibitor of osteoclast formation (Hofbauer *et al.,* 1999). This increase in the RANKL/OPG ratio is consistent with a stimulation of osteoclast formation and may explain the early stimulation of resorption in humans and mice.

Permissive Effects of Glucocorticoids on Osteoblasts

Some of the physiological effects of glucocorticoids on bone may be due in part to their ability to act as permissive hormones, thereby allowing other hormones to function optimally. Low doses of glucocorticoids enhance PTH-stimulated adenylate cyclase in rat, mouse, and human bone cells (Chen *et al.,* 1978; Wong, 1980; Rodan *et al.,* 1984; Wong *et al.,* 1990) and PTH-mediated bioactivities (Wong, 1979). The enhancement of the PTH-dependent cAMP response may be due to an increase in cAMP activity and a decrease in phosphodiesterase activity (Chen *et al.,* 1978). Glucocorticoids also increase the number of PTH receptors and levels of PTH/PTHrP receptor mRNA (Yamamoto *et al.,* 1988; Urena *et al.,* 1994).

The effect of glucocorticoids on 1,25$(OH)_2$D receptors and biological activity, however, is not as clear. In rat bone cytosol and primary rat osteoblastic cells, glucocorticoids maintain or increase 1,25$(OH)_2$D receptor number (Manolagas *et al.,* 1979; Chen *et al.,* 1983) and enhance the biological actions of 1,25$(OH)_2$D (Chen *et al.,* 1986). However, in one study using primary mouse osteoblastic cells, the effect of glucocorticoids on 1,25$(OH)_2$D receptor number was dependent on the stage of growth of the cells; receptor number was decreased at early log phase growth and at confluence and increased at late log phase growth (Chen *et al.,* 1982). In another study, glucocorticoids were shown to

increase 1,25$(OH)_2$D biological activities in primary mouse osteoblastic cells (Wong, 1980). In human MG-63 cells, glucocorticoids decrease the expression of 1,25$(OH)_2$D receptor mRNA (Godschalk *et al.,* 1992). Taken together, these findings indicate that glucocorticoids can increase or decrease 1,25$(OH)_2$D receptor levels depending on the experimental model.

Glucocorticoids alter the IGF-1 pathway. They inhibit IGF-1 mRNA and protein expression by osteoblasts (as discussed later) but increase IGF-1 receptor number (Bennett *et al.,* 1984). Physiological concentrations of cortisol enhance the stimulatory effects of IGF-1 on collagen synthesis, producing a larger anabolic effect than with IGF-1 alone (Kream *et al.,* 1990a). The ability of glucocorticoids to augment IGF-1 activity may represent a compensatory response that helps maintain bone mass and growth during periods of diminished IGF-1 supply, such as starvation. A similar enhancing effect of glucocorticoids on IGF-1 action occurs in fibroblast cultures (Conover *et al.,* 1986; Bird *et al.,* 1994). Cortisol enhances the anabolic effects of exogenous prostaglandins on collagen and DNA synthesis in organ cultures of rat calvariae, which may be dependent partly on the IGF-1 pathway (Raisz *et al.,* 1993). Physiological concentrations of glucocorticoids may amplify the stimulatory effect of PGE_2 on the IGF-1 promoter through the induction of C/EBP family transcription factors (McCarthy *et al.,* 2000b).

Target Cell Metabolism of Glucocorticoids

Target cell metabolism has emerged as an important mechanism for regulating the sensitivity of cells to glucocorticoids (Eyre *et al.,* 2001). Glucocorticoids can be modified by two 11β-hydroxysteroid dehydrogenases (Krozowski, 1999; Krozowski *et al.,* 1999; Stewart *et al.,* 1999). The NAD-dependent enzyme 11β-HSD type 2 (11β-HSD2) catalyzes the unidirectional conversion of biologically active glucocorticoids to inactive metabolites and the bidirectional interconversion of dexamethasone to 11-dehydrodexamethasone. The NADP-dependent 11β-HSD type 1 (11β-HSD1) has oxidoreductase activity and catalyzes the bidirectional conversion of inactive glucocorticoids to active metabolites. 11β-HSD2 is highly expressed in kidney where it protects the mineralocorticoid receptor from activation by glucocorticoids and is abundant in placenta where it protects the fetus from maternal glucocorticoids. Mice with a targeted deletion of 11β-HSD2 develop hypertension because glucocorticoids, which fail to be metabolized in kidney cells, evoke mineralocorticoid effects via the mineralocorticoid receptor (Kotelevtsev *et al.,* 1999). Both 11β-HSD1 and 11β-HSD2 are expressed in osteoblasts of rat and human osteoblasts (Cooper *et al.,* 2000). Rat and human osteoblastic osteosarcoma cell lines express 11β-HSD2 (Bland *et al.,* 1999; Eyre *et al.,* 2001). Both rat and mouse calvarial osteoblast cultures can convert inactive glucocorticoids to active metabolites (Bellows *et al.,* 1998). Primary human osteoblasts and adult human bone explants express both 11β-HSD1 and 11β-HSD2 (Bland *et al.,* 1999). The glucocorticoid sensitivity of osteosarcoma cell lines with equivalent numbers of glucocorticoid receptors is directly correlated with the level of 11β-HSD2 expression (Eyre *et al.,* 2001). Moreover, ROS 17/2.8 and MC3T3-E1 cells transfected with 11β-HSD2 show reduced responsiveness to natural glucocorticoids but maintain responsiveness to the synthetic glucocorticoid dexamethasone (Woitge *et al.,* 2001). Thus, 11β-HSD enzymes may regulate the sensitivity of osteoblasts to glucocorticoids.

Glucocorticoid-Regulated Gene Expression in Bone

Molecular Mechanisms of Glucocorticoid Action

At the molecular level, glucocorticoids alter the expression of a wide variety of genes in osteoblastic cells, including those for structural proteins, growth factors, receptors, and enzymes. Glucocorticoids elicit biological responses in their target cells by binding to and activating the intracellular glucocorticoid receptor. The structure and function of the glucocorticoid receptor, its intracellular trafficking, and glucocorticoid receptor-dependent transcription are discussed in detail in many excellent reviews (Beato *et al.,* 1995, 1996; McKay *et al.,* 1999; Webster *et al.,* 1999; Defranco, 2000). The glucocorticoid receptor is a modular protein containing an amino-terminal domain that encodes a transactivation function and a carboxyl-terminal domain that specifies ligand binding, dimerization, heat shock protein (HSP) binding, and transactivation functions. The most highly conserved region is the 66 amino acid DNA-binding domain consisting of two zinc finger motifs with cysteine residues that are coordinated with zinc atoms (Freedman *et al.,* 1988). Unligated glucocorticoid receptors are found in the cytoplasm in association with a variety of molecular chaperone proteins, including hsp90, an FK506-binding immunophilin protein, and p23 (Cheung *et al.,* 2000; Defranco, 2000). Upon hormone binding, a conformational change enables the receptor to translocate to the nucleus, dimerize, and bind to DNA. Transcriptional activation by a glucocorticoid receptor homodimer occurs when the DNA-binding domains interact with a glucocorticoid response element (GRE). The GRE consensus sequence is GGTACAnnnTGTTCT (Beato, 1989). Transcriptional activation involves protein–protein interactions between the receptor dimer and basal transcription factors and RNA polymerase II (Mitchell and Tijan, 1989). Glucocorticoid-dependent inhibition of gene expression has become a molecular paradigm for understanding mechanisms of transcriptional repression by steroid hormone receptors (Webster *et al.,* 1999). Transcriptional repression by glucocorticoid receptors occurs by direct interaction with DNA through negative GREs (Sakai *et al.,* 1988), by blocking the access of positive transcription factors to DNA sequences (Akerblom *et al.,* 1988), and by protein–protein interaction with transcription factors (Chatterjee *et al.,* 1991). An example of the latter mechanism is glucocorticoid

inhibition of collagenase expression, which is thought to occur by interaction of the glucocorticoid receptor with the AP-1 transcription factor complex (Jonat *et al.,* 1990; Schule *et al.,* 1990; Yang-Yen *et al.,* 1990).

Effects on Gene Expression

PROTOONCOGENES AND TRANSCRIPTION FACTORS

Glucocorticoids cause a rapid and transient increase in the mRNA levels of c-fos (Birek *et al.,* 1991; Shalhoub *et al.,* 1992; Subramaniam *et al.,* 1992) and c-myc (Subramaniam *et al.,* 1992) in human and rodent osteoblastic cells and chick periosteal cultures. The rat c-fos promoter contains a putative GRE that may mediate glucocorticoid-dependent induction (Wang *et al.,* 1994). Cell lines prepared from tumors of c-fos transgenic mice show changes in osteoblast phenotypic markers but have unaltered glucocorticoid responsiveness (Grigoriadis *et al.,* 1993). The role that protooncogene induction plays in glucocorticoid-mediated gene expression is unknown, although it may represent an example of cross-talk between signal transduction pathways. The induction of protooncogenes may be a primary event in the regulation of downstream genes such as those that encode growth factors and matrix proteins. Id (inhibitor of differentiation) is a member of the helix-loop-helix (HLH) family of transcription factors that binds to other HLH factors and suppresses differentiation (Benezea *et al.,* 1990). Id mRNA is detectable in early cultures of MC3T3-E1 cells and then decreases as the cells differentiate (Ogata *et al.,* 1993). Dexamethasone maintains the high levels of Id mRNA in confluent MC3T3-E1 cells (Ogata *et al.,* 1993).

TYPE I COLLAGEN

Type I collagen is the most abundant protein in the bone matrix and its expression is regulated by a wide variety of hormones, growth factors, and cytokines (Raisz, 1988). Glucocorticoids decrease α1(I) collagen (Col1a1) mRNA levels in osteoblastic cells and calvarial organ cultures (Kim *et al.,* 1989; Kream *et al.,* 1990a; Lukert *et al.,* 1991; Delany *et al.,* 1995a) and in calvariae of neonatal mice given *in vivo* dexa-methasone (Advani *et al.,* 1997). Glucocorticoid down-regulation of Col1a1 mRNA occurs by an inhibition of Col1a1 transcription and a decrease in the stability of Col1a1 mRNA (Delany *et al.,* 1995a). Dexamethasone decreases the activity of transfected Col1a1 mRNA promoter–reporter constructs in stably transfected osteoblastic cells, indicating a transcriptional effect (Petersen *et al.,* 1991). The precise molecular mechanisms by which glucocorticoids inhibit Col1a1 transcription in osteoblastic cells have not been elucidated. However, studies performed in fibroblasts provide mechanistic clues. Glucocorticoids decrease type I collagen mRNA levels in fibroblasts by decreasing the transcription of collagen genes and the stability of collagen mRNA (Hamalainen *et al.,* 1985; Cockayne *et al.,* 1986; Raghow *et al.,* 1986). Glucocorticoids also decrease Col1a1 mRNA stability by affecting protein binding to 3′ UTRs (Määttä *et al.,* 1993). Glucocorticoids decrease the activity of transfected mouse α2(I) collagen (Col1a2) promoter–reporter constructs in fibroblasts through sequences from −2048 to −981 bp and from −506 to −351 bp (Perez *et al.,* 1992). In stably transfected fetal skin fibroblasts, the inhibitory effect of dexamethasone on rat Col1a1 promoter activity is maintained when the promoter is deleted to −900 bp. This region contains a putative GRE half-site; however, a mutation of this site does not block glucocorticoid-dependent inhibition of Col1a1 promoter activity (Meisler *et al.,* 1995). In this study, it was suggested that glucocorticoids decrease Col1a1 transcription in fibroblasts by acting at a TGFβ responsive site (Meisler *et al.,* 1995).

NONCOLLAGEN PROTEINS OF BONE

Glucocorticoids alter the expression of a variety of noncollagenous structural proteins in bone. *In vivo* glucocorticoid treatment of rats and mice decreases osteocalcin mRNA levels in bone (Ikeda *et al.,* 1992; Advani *et al.,* 1997). Acute treatment of osteoblastic cell cultures with glucocorticoids inhibits basal and agonist-induced osteocalcin production and mRNA levels (Wong *et al.,* 1990; Schepmoes *et al.,* 1991). Glucocorticoids inhibit $1,25(OH)_2D$-mediated osteocalcin transcription (Morrison *et al.,* 1989). It has been proposed that this occurs by binding of the glucocorticoid receptor the TATA box in the proximal promoter region of the osteocalcin gene (Stromstedt *et al.,* 1991; Meyer *et al.,* 1997). Glucocorticoids may also modulate other cell-specific factors that control osteocalcin transcription (Morrison *et al.,* 1993).

Glucocorticoids increase alkaline phosphatase activity and mRNA levels in human osteoblastic cells (Subramaniam *et al.,* 1992), SaOS-2 osteosarcoma cells (Murray *et al.,* 1987), and ROS 17/2.8 cells (Majeska *et al.,* 1985). Glucocorticoids increase alkaline phosphatase mRNA levels in ROS 17/2.8 cells; the increase in mRNA occurred after a lag period of 12 hr and is blocked by cycloheximide, indicating the requirement for new protein synthesis (Green *et al.,* 1990). Actinomycin D does not block the stimulatory effect of glucocorticoids on alkaline phosphatase mRNA levels, indicating transcriptional regulation (Green *et al.,* 1990). Osteoblasts synthesize the bone/liver/kidney/placenta form of alkaline phosphatase; this gene contains two alternative promoters spaced 25 kb apart; baseline and glucocorticoid-stimulated alkaline phosphatase mRNA in calvariae and ROS 17/2.8 cells is transcribed from the upstream promoter (Zernick *et al.,* 1991).

Bone sialoprotein is a glycoprotein containing an arginine-glycine-aspartic acid (RGD) sequence that mediates the attachment of cells to extracellular matrix proteins. Glucocorticoids increase bone sialoprotein mRNA levels in fetal rat calvarial, bone marrow, ROS 17/2.8, and UMR-106–06 cells in part by a transcriptional mechanism (Ogata *et al.,* 1995). The bone sialoprotein promoter contains a GRE between −906 and −931 bp that may mediate this transcriptional effect of glucocorticoids (Ogata *et al.,* 1995). There have been few studies examining the direct effect of

glucocorticoids on the expression of osteonectin, an abundant noncollagenous glycoprotein that may have a role in mineralization. In one study, dexamethasone increased osteonectin mRNA levels and the activity of an osteonectin promoter–reporter construct in preosteoblastic UMR 201 cells (Ng *et al.,* 1989).

Gluococorticoids decrease fibronectin (Gronowicz *et al.,* 1991) and β1 integrin (Doherty *et al.,* 1995) mRNA levels in fetal rat parietal bone organ cultures. The inhibitory effect on β1 integrin expression is accompanied by a disruption of osteoblast organization on the bone surface and a decrease in calcification of the bone (DiPersio *et al.,* 1991). In primary rat osteoblastic cells and ROS 17/2.8 cells, glucocorticoids decrease plasma membrane β1-integrin staining, adhesion of the cells to bone matrix proteins, and β1 integrin mRNA levels (Gronowicz *et al.,* 1995). Glucocorticoids decrease the expression of cells containing the α2 and α4 integrin subunits in bone marrow stromal cultures (Walsh *et al.,* 2001).

Glucocorticoids decrease interstitial collagenase mRNA levels in human skin fibroblasts by reducing the half-life of collagenase mRNA (Delany *et al.,* 1992). In contrast, glucocorticoids increase the expression of collagenase mRNA in rat osteoblastic cells (Shalhoub *et al.,* 1992; Delany *et al.,* 1995b) by a mechanism that involves increased collagenase mRNA stability (Delany *et al.,* 1995b). In addition, cortisol antagonized the phorbol ester-mediated increase in activity of a transiently transfected rat collagenase promoter–reporter construct (Delany *et al.,* 1995b). Glucocorticoid induction of interstitial collagenase expression in osteoblasts may be related to biological activities, such as growth factor activation, or the activation of osteoclastic bone resorption (Delany *et al.,* 1995b).

Growth Factor Systems

IGF-1 is an important anabolic growth factor for bone (Rosen *et al.,* 1999). It has been suggested that the inhibitory effects of glucocorticoids on bone formation may be due in part to a decrease in the production of IGF-1 (McCarthy *et al.,* 1990). Glucocorticoids decrease IGF-1 mRNA expression in rat tibia, organ cultures of fetal rat calvariae, and primary osteoblastic cell cultures (Luo *et al.,* 1989; McCarthy *et al.,* 1990; Chen *et al.,* 1991). However, glucocorticoids do not regulate IGF-II mRNA levels in primary rat osteoblastic cells (McCarthy *et al.,* 1992), but they decrease IGF-II peptide production in fetal rat calvarial cultures (Canalis *et al.,* 1991). Inhibitory effects of glucocorticoids on bone formation persist when IGFBP-2 is added to cultures of fetal rat calvariae to inactivate IGFs (Kream *et al.,* 1997). Moreover, calvariae from mice with a complete ablation of the Igf1 gene maintain responsiveness to glucocorticoids (Woitge *et al.,* 2000). These studies suggest that inhibitory effects of glucocorticoids are partly independent of the IGF-1 pathway.

IGFBPs regulate the storage, transport, and bioactivities of IGFs (Clemmons *et al.,* 1993). Six IGFBPs, termed IGFBP-1 through -6, have been identified in a variety of tissues (Shimasaki *et al.,* 1991). The expression of IGFBPs in osteoblastic cells of different origins is cell line specific (Hassager *et al.,* 1992). IGFBPs generally inhibit IGF-1 action *in vitro* (Mohan *et al.,* 1989; Feyen *et al.,* 1991), except for IGFBP-5, which may act as an anabolic growth factor (Andress *et al.,* 1992; Miyakoshi *et al.,* 2001). Glucocorticoids decrease IGFBP-3, -4, and -5 production in normal human osteoblastic cells (Okazaki *et al.,* 1994) and decrease IGFBP-3 production in transformed osteoblastic cell lines (Nakao *et al.,* 1994). Glucocorticoids decrease IGFBP-5 transcription in rat osteoblasts (Gabbitas *et al.,* 1996b) and decrease IGFBP-2 production in rat calvarial osteoblastic cells (Chen *et al.,* 1991) and immortalized rat osteoblastic PyMS cells (Schmid *et al.,* 1988). However, glucocorticoids increase the expression of IGFBP-6 in fetal rat calvarial cell cultures (Gabbitas *et al.,* 1996a). Because IGFBP-6 has higher affinity for IGF-2 than IGF-1, glucocorticoid stimulation of IGFBP-6 may limit the availability of IGF-2 as an anabolic agent (Gabbitas *et al.,* 1996a). Because glucocorticoids decrease IGF-1 production in bone, the inhibitory effect of glucocorticoids on IGFBP expression may provide a mechanism by which osteoblastic cells are more responsive to the residual pool of IGF-1. Alternatively, downregulation of IGFBP-5 production could represent the removal of an anabolic factor and result in part in the inhibitory effects of glucocorticoids. Glucocorticoids alter the expression of other growth factor systems in cultured fetal rat calvarial osteoblasts, such as mac25 (IGFBP-related peptide), connective tissues growth factor, and hepatocyte growth factor and its receptor c-met (Pereira *et al.,* 1999, 2000; Blanquaert *et al.,* 2000). As of yet, the role of these factors in mediating glucocorticoid responses in bone is not known.

TGFβ is anabolic for bone formation and is either stimulatory or inhibitory for bone resorption depending on the experimental model and the culture conditions (Centrella *et al.,* 1994). TGFβ binds to three cell surface receptors, termed TGFβRI, II, and III, which are expressed in osteoblastic cells (Centrella *et al.,* 1991). Type I and II receptors are thought to mediate TGFβ signaling (Massague, 1992); the type III receptor, β-glycan, is a cell surface proteoglycan that is more abundant than type I and II receptors but has lower affinity for TGFβ1 (Lopez-Casillas *et al.,* 1993). Glucocorticoids modify the expression of molecules in the TGFβ pathway (McCarthy *et al.,* 2000a). Glucocorticoids decrease the stimulatory effects of TGFβ1 on DNA synthesis and collagen synthesis in fetal rat osteoblastic cells and increase the binding of TGFβ1 to β-glycan in primary cultures of fetal rat osteoblastic cells (Centrella *et al.,* 1991). Dexamethasone increases β-glycan mRNA levels in immortalized MC3T3-E1 and RCT1 osteoblastic cells (Nakayama *et al.,* 1994). If the function of β-glycan were to decrease the amount of TGFβ available for signaling, these effects of glucocorticoids would reduce the anabolic effects of TGFβ1 on osteoblastic cells. In fetal rat osteoblasts, glucocorticoids suppress Cbfa1 expression, which is associated with a decrease in the expression and activity of the TGFβRI (Chang *et al.,* 1998).

Plasminogen activator is a serine protease that activates plasminogen to the serine protease plasmin. The plasminogen activator–plasmin system may have a role in bone resorption by activating latent collagenase or TGFβ (Hamilton *et al.,* 1985). Glucocorticoids decrease plasminogen activator activity in normal rodent osteoblasts and UMR-106–01 cells (Hamilton *et al.,* 1985); this is due primarily to an increase in plasminogen activator inhibitor-1 mRNA and protein level (Fukumoto *et al.,* 1992). Glucocorticoid inhibition of plasminogen activator activity, therefore, might limit the activation of locally produced TGFβ, leading to a decrease in bone formation (Fukumoto *et al.,* 1992). However, glucocorticoids enhance the activation of latent TGFβ1 in normal human osteoblastic cells without an alteration of TGFβ1 mRNA levels (Oursler *et al.,* 1993). Such an effect of glucocorticoids might be expected to increase the availability of TGFβ as an anabolic bone growth factor; alternatively, enhanced TGFβ activation might lead to increased bone resorption. Taken together, the effect of glucocorticoids on TGFβ activity in bone may result from a combination of the actions described previously.

PROSTAGLANDINS

Prostaglandins are produced by bone cells and can affect both bone formation and resorption. Prostaglandins directly inhibit the activity of isolated osteoclasts (Fuller *et al.,* 1989) but increase bone resorption by increasing the formation of new osteoclasts (Dietrich *et al.,* 1975). Prostaglandins have both stimulatory and inhibitory effects on bone formation in organ cultures of rodent calvariae, depending on the dose and hormonal milieu that is used (Raisz *et al.,* 1990). Glucocorticoids decrease baseline and agonist-induced prostaglandin production in bone (Klein-Nulend *et al.,* 1991; Marusic *et al.,* 1991; Hughes-Fulford *et al.,* 1992). The mechanisms for this inhibition likely include both a decrease in arachidonic release from membranes and a decrease in the expression of the cyclooxygenases that convert arachidonic acid to prostaglandins. Osteoblasts express two cyclooxygenases, the constitutive prostaglandin synthase-1 (PGHS-1) and the inducible prostaglandin synthase-2 (PGHS-2) (Pilbeam *et al.,* 1993; Kawaguchi *et al.,* 1995). Endogenous glucocorticoids tonically suppress PGHS-2 in mice, and this suppression is relieved when the animals are adrenalectomized (Masferrer *et al.,* 1992). The induction of PGHS-2 by interleukin-1 and PTH in mouse calvariae and by serum in MC3T3-E1 cells is antagonized by glucocorticoids (Kawaguchi *et al.,* 1994). In summary, glucocorticoid inhibition of prostaglandin production in bone occurs primarily by a decrease in agonist-induced PGHS-2 expression.

Summary and Conclusions

A variety of *in vitro* models have been developed to examine the direct effects of glucocorticoids on bone formation and resorption. These experiments show that glucocorticoids have diverse and complex direct effects on bone and can modify the expression of a wide variety of genes in osteoblastic cells. It is likely that the experimental outcomes in different models are affected by the concentration of glucocorticoid used, the timing of glucocorticoid addition, the presence of serum and growth factors, the developmental stage of the model, and species differences. However, several general principles can be drawn from these studies. Glucocorticoids can either stimulate or inhibit bone formation *in vitro* and these effects depend on the developmental stage of the model. Low concentrations of glucocorticoids are permissive for hormone action in bone (allowing other hormones to have optimal activity) and are associated with increased osteoblastic differentiation and bone formation. The daily secretion of physiologic concentrations of cortisol may render osteoblasts and/or osteoprogenitor cells highly responsive to the effects of systemic and locally produced hormones. The ability of glucocorticoids to enhance the activity of some anabolic hormones may represent a compensatory response, which helps maintain bone mass during periods of diminished growth factor supply. A challenge will be to develop models that test the hypothesis that physiological glucocorticoids are required for bone formation and maintenance of the osteoblast phenotype *in vivo*. Pharmacological doses of glucocorticoids suppress bone formation by inhibiting osteoprogenitor proliferation, osteoblast renewal, and osteoblast function and by increasing osteoblast apoptosis. Understanding the molecular events that lead to the suppression of bone formation will enable the development of effective therapeutic modalities for glucocorticoid-induced osteoporosis.

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CHAPTER 42

Effects of Diabetes and Insulin on Bone Physiology

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Introduction

The liver, the skeletal muscles, and the adipose tissue are the main insulin-responsive tissues, yet insulin also influences the physiology of other tissues, including cartilage and bone. In conditions of hypoinsulinemia (e.g., type 1 diabetes) or hyperinsulinemia with or without glucose intolerance or fasting hyperglycemia (type 2 diabetes), endochondral bone growth and bone (re)modeling show significant alterations.

Type 1 diabetes is caused by pancreatic β-cell destruction (mostly immune mediated, or otherwise idiopathic), usually leading to absolute insulin deficiency. Type 2 diabetes is the most prevalent form of diabetes; its pathophysiology is heterogeneous, ranging from predominantly insulin resistance (i.e., at any of the main insulin-responsive tissues) with relative insulin deficiency to a predominantly insulin secretory defect with variable insulin resistance. Hence, insulin levels in type 2 diabetes vary widely, anywhere between hyper- and hypoinsulinemia. Most patients with type 2 diabetes are obese, and obesity itself causes some degree of insulin resistance. Other types of diabetes will not be considered herein.

Effect of Diabetes and Insulin on Endochondral Bone Growth

Type 1 Diabetes and Skeletal Growth and Maturation in Humans

At onset of type 1 diabetes, there is no difference in height compared with nondiabetic children; in fact, some, but not all, studies have documented that children are slightly taller at the onset of diabetes compared with reference values (Pond, 1970; Holl *et al.,* 1998).

However, growth is affected after the diagnosis of diabetes. In the preinsulin era, growth virtually stopped after the onset of diabetes in prepubertal or pubertal children, and stunted growth remained common in later decades of irregular insulin treatment (Pond, 1970). Even in the 1990s, the majority of studies document a slight reduction in growth velocity on the basis of height SDS or *Z* scores. This effect is more marked in type 1 diabetes with prepubertal than with pubertal onset (Holl *et al.,* 1998). The main predictor of reduction in growth velocity is the level of glycemic control, and glycohemoglobin levels—a reflection of glycemic control in the previous 2–3 months—correlate inversely with height velocity (Danne *et al.,* 1997; Holl *et al.,* 1998). In one recent cohort, final height was found to be reduced by a median of 0.5 SDS (2–3 cm) (Danne *et al.,* 1997).

At diagnosis, bone age—determined by radiographs of hand and wrist—is not different in diabetic and nondiabetic children (Holl *et al.,* 1994; Danne *et al.,* 1997). However, there is a small, but significant, retardation of bone age with increasing diabetes duration: the difference between chronological and bone age equals 1 year after a mean diabetes duration of 11 years (Holl *et al.,* 1994).

Skeletal Growth in Diabetic Experimental Animals

The effect of diabetes on bone growth has been examined in BB (Bio-Breeding) rats with spontaneous immune-mediated diabetes and in rats that received an injection of a diabetogenic drug such as alloxan or, more commonly,

Table I Histomorphometric Data from Proximal Tibial Metaphysis in Untreated and Insulin-Treated Male Spontaneously Diabetic BB Rats[a]

	Nondiabetic ($n = 14$)	Diabetic ($n = 11$)	Diabetic + insulin ($n = 15$)
Growth plate width (μm)	178 (8)	135 (8)***	230 (9)***,‡
Osteoblast surface (%)	1.5 (0.3)	0.04 (0.04)***	4.3 (0.8)**,‡
Osteoid surface (%)	1.5 (0.4)	0.04 (0.04)**	4.8 (1.0)**,‡
Osteoclast surface (%)	0.4 (0.1)	0*	0.5 (0.2)†

[a]From Verhaeghe *et al.* (1992), with permission of the Journal of Endocrinology Ltd. Measurements were performed about 4 weeks after onset of diabetes in diabetic rats and in nondiabetic littermates. Insulin-treated rats were treated with 3 U/day of insulin, infused sc with a miniosmotic pump for 14 days. Data are expressed as means (SEM). Statistical analysis:*, versus nondiabetic rats ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$);†, versus diabetic rats ($^{\dagger}P < 0.05$, $^{\ddagger}P < 0.001$).

streptozotocin (SZ). Spontaneously diabetic BB rats in our colony develop diabetes at a mean age of 13 weeks (Verhaeghe *et al.,* 2000), which is past the peak growth rate (week 7) in rats (Locatto *et al.,* 1993). Insulin levels in BB rats are very low or undetectable.

In rats in which diabetes is drug-induced at an early age, long bones such as the femur are shorter after 4 weeks of diabetes (Lucas, 1987). Growth plate width as well as endochondral bone growth—assessed by double fluorochrome labeling of the calcifying cartilage—of the proximal tibia are markedly lower in untreated diabetic rats, which is corrected by insulin treatment (Bain *et al.,* 1997; Epstein *et al.,* 1994; Scheiwiller *et al.,* 1986; Verhaeghe *et al.,* 1992) (Table I). Cartilage activity, assessed by the incorporation of [^{35}S]sulfate ($^{35}SO_4$) into proteoglycans, is reduced robustly in growth plate explants from diabetic rats as well as in demineralized bone particles implanted into diabetic rats, which again is reversed by insulin treatment (Axelsson *et al.,* 1983).

The effect of insulin on bone growth could be direct or indirect; the latter could be the result of normalizing hepatic production and circulating levels of insulin-like growth factor-I (IGF-I). Although one study reported that recombinant human IGF-I partly normalized growth plate width in diabetic rats (Scheiwiller *et al.,* 1986), this finding was not confirmed in other studies (Verhaeghe *et al.,* 1992). Kelley *et al.* (1993) confirmed that the low $^{35}SO_4$ uptake by rib cartilage explants from diabetic rats is unresponsive to recombinant bovine IGF-I administration; however, this unresponsiveness is restored by hypophysectomy, implying that a pituitary hormone-dependent factor induces IGF-I resistance in diabetic cartilage.

Effects of Insulin on Cartilage in Nondiabetic Animal Models and *in Vitro*

The classic experiments of Salter and Best (1953) demonstrated that insulin treatment increases growth plate width in hypophysectomized rats. Subsequent data by Heinze *et al.* (1989) confirmed that the administration of insulin to hypophysectomized rats increases body length, growth plate width of the proximal tibia, and $^{35}SO_4$ incorporation into rib cartilage. This is a local effect of insulin because insulin injection into the proximal tibia growth plate (Heinze *et al.,* 1989) or insulin infusion into one hindlimb (Alarid *et al.,* 1992) produces exclusive widening of the treated growth plates. The direct effect of insulin on the growth plate appears to be mediated by the *in situ* production of IGF-I: IGF-I is present on immunohistochemistry in hypertrophic chondrocytes of the insulin-treated growth plates only, and the effect of insulin on the growth plate is abolished by coinfusion of an IGF-I antibody (Alarid *et al.,* 1992).

In vitro studies have documented the presence of insulin receptors in a chondrosarcoma cell line (Foley *et al.,* 1982). Chondrocyte proliferation and $^{35}SO_4$ incorporation have been shown to be stimulated by insulin in a number of *in vitro* systems: organ and tissue cultures of neonatal mouse mandibular condyles (Maor *et al.,* 1993) and chondrocyte cultures from rat chondrosarcoma, rat rib cartilage, or fetal lamb growth plate cartilage (Foley *et al.,* 1982; Heinze *et al.,* 1989; Hill and De Sousa, 1990). These effects are obtained at physiological levels of insulin, as low as 1 n*M* (Hill and De Sousa, 1990). Proinsulin is only 3% as potent as insulin (Foley *et al.,* 1982), but equimolar IGF-I is more potent than insulin (Hill and De Sousa, 1990). Several data suggest that insulin stimulates chondrocyte proliferation and activity through its own receptor, not through the type 1 IGF receptor: (1) the effects of insulin and IGF-I on chondrocyte proliferation are additive (Maor *et al.,* 1993; Hill and De Sousa, 1990); (2) the stimulation of $^{35}SO_4$ incorporation by insulin is blocked by an insulin receptor antibody (Foley *et al.,* 1982); and (3) the stimulation of $^{35}SO_4$ is not affected by cotreatment with an antiserum against the type 1 IGF receptor (Maor *et al.,* 1993).

Conclusions on Effects of Diabetes and Insulin on Bone Growth

Insulin-deficient states are accompanied by stunted growth. Insulin stimulates the proliferation and metabolic activity of chondrocytes *in vitro,* as well as in insulin-deficient

and pituitary hormone-deficient conditions *in vivo.* This effect occurs at physiological insulin levels, presumably through interaction with the insulin receptor. The effect of insulin appears to be mediated by the local production of IGF-I in growth cartilage.

Effects of Diabetes and Insulin on Bone Remodeling, Bone Mass, and Bone Strength in Humans

Introductory Remarks

Published data on bone markers, bone mass, and bone strength in diabetic subjects are not infrequently difficult to interpret, for several reasons. The first problem pertains to the study subjects: (1) series often consist of a "mixed bag" of diabetic subjects (female and male, type 1 and type 2), with widely different diabetes duration and degree of long-term glycemic control; (2) the study population may include a variable proportion of subjects with diabetes complications such as retinopathy, neuropathy, or atherosclerotic disease, conditions that may limit physical activity and capabilities; and (3) diabetic subjects frequently take multiple medications, which may influence bone density and fracture risk, as has been shown for statins (Chung *et al.,* 2000). Second, diabetes may alter the reliability of bone and mineral measurements: (1) because diabetes affects bone size, areal bone mineral density (BMD) measurements may need to be adjusted; (2) the measurement of serum total alkaline phosphatase is meaningless in diabetic subjects because of overproduction of the intestinal and possibly the hepatic isoenzyme (Tibi *et al.,* 1988; Bouillon *et al.,* 1995); and (3) changes in collagen metabolism in tissues other than bone may interfere with the measurement of the excretion of collagen breakdown products, particularly hydroxyproline or pyridinoline cross-links, and reduce their usefulness as markers of bone resorption.

Type 1 Diabetes and Bone Remodeling

Formal bone histomorphometry data are lacking, but the measurement of biochemical markers of bone formation produces unequivocal evidence that bone formation is decreased. We found that serum osteocalcin concentrations are 24–28% lower in diabetic children, adolescents, and adults compared to age- and gender-matched nondiabetic subjects (Fig. 1) (Bouillon *et al.,* 1995). In the same study, serum levels of bone-specific alkaline phosphatase were decreased by 24% in diabetic adolescents, but there was no change in diabetic adults. Serum levels of PICP (procollagen carboxy-terminal extension peptide) were unchanged, however. Interestingly, serum osteocalcin levels were found to be below the control range at diagnosis of diabetes in 31 children aged 2–13 years and subsequently reverted to within the control range after 15 days of intensive insulin treatment; osteocalcin levels were correlated negatively with glycohemoglobin levels (Guarneri *et al.,* 1993). Again, PICP levels were unchanged at diagnosis of type 1 diabetes in prepubertal children (Bonfanti *et al.,* 1997).

Regarding bone resorption, plasma ICTP (collagen type I C-terminal telopeptide) levels were found to be normal at onset of diabetes (Bonfanti *et al.,* 1997). Urinary-free deoxypyridinoline cross-links (D-PYR) were measured by a commercial ELISA kit in 18 diabetic adolescents aged 12–17 years and compared with 69 nondiabetic adolescents of the same age group: D-PYR/creatinine levels were

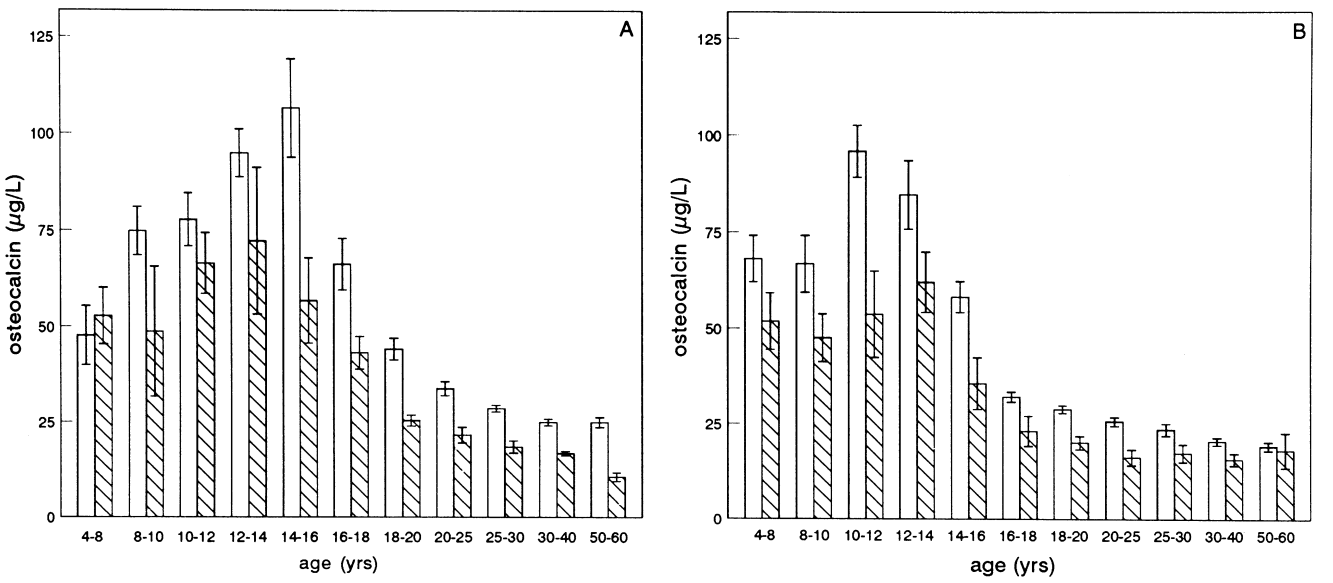

Figure 1 Serum osteocalcin concentrations in normal individuals (open bars) and individuals with type 1 diabetes (hatched bars): (A) male and (B) female. Data are presented as means ±SEM. Multiple regression analysis showed a positive correlation between age and serum osteocalcin levels below 16 and 12 years for boys and girls, respectively, and a negative correlation above those ages ($p < 0.001$). A significant ($p < 0.005$) decreasing effect of diabetes on serum osteocalcin was observed for both genders. From Bouillon *et al.* (1995), with permission. ©The Endocrine Society.

slightly ($p < 0.05$) higher in the diabetic group (Bjorgaas *et al.,* 1999). In contrast, total and free D-PYR excretion, measured by high-performance liquid chromatography (HPLC), were reported to be lower in a rather unspecified group of 84 adult type 1 diabetics compared with 99 adult controls, and the decrease was proportional to the level of glycosuria (Cloos *et al.,* 1998).

Type 2 Diabetes and Bone Remodeling

Bone formation is also dampened in type 2 diabetes. In a small series of 8 diabetic subjects (aged 37–67 years, diabetic for 2–36 years), 6 of whom had type 2 diabetes, a bone biopsy was carried out because of a low BMD *Z* score at the radius; histomorphometry showed a significant decrease in the osteoid thickness and in the dynamic bone formation rate (Krakauer *et al.,* 1995). Measurement of biochemical markers indicates lower serum osteocalcin levels, even in the presence of fasting hyperinsulinemia (Montecucco *et al.,* 1990). In a group of 37 Pima Indians, 22 of whom had impaired glucose tolerance or type 2 diabetes, osteocalcin levels correlated inversely with the 2-hr glucose value during an oral glucose tolerance test (OGTT) (Bouillon *et al.,* 1995). Poor glycemic control in subjects with type 2 diabetes impairs the response of osteocalcin to administration of $1,25(OH)_2D_3$ (Inaba *et al.,* 1999), whereas improvement in glycemic control augments serum osteocalcin levels (Okazaki *et al.,* 1997; Rosato *et al.,* 1998).

Measurement of D-PYR by HPLC showed that urinary D-PYR is decreased comparably in a group of 58 type 2 diabetics as in type 1 diabetics (Cloos *et al.,* 1998). Improvement in glycemic control increased D-PYR excretion measured by HPLC in one study (Rosato *et al.,* 1997), but not in another study in which urinary D-PYR and the type I collagen carboxy-terminal telopeptide (CTx) were measured with commercial kits (Okazaki *et al.,* 1997).

In conclusion, type 1 and type 2 diabetes are characterized by decreased bone formation and mineralization, as was shown in histomorphometrical and biochemical studies. Methodological issues obscure the interpretation of bone resorption markers, and more work is needed in this regard, perhaps focusing on other markers of bone resorption than collagen breakdown products, such as the measurement of the osteoclast-specific tartrate-resistant acid phosphatase (TRAP) isoform.

Type 1 Diabetes and Bone Mass

The effect of type 1 diabetes on axial bone density has been investigated in several relatively small studies. Ponder *et al.* (1992) studied 25 girls and 31 boys with diabetes, aged between 5 and 18 years, and compared their lumbar spine BMD (assessed by dual-photon absorptiometry) with those of 221 nondiabetic children/adolescents of the same age group. The BMD *Z* score was not significantly different from controls in diabetic subjects, regardless of diabetes duration. In the same age group, Roe *et al.* (1991) measured BMD by quantitative computed tomography (QCT) at the lumbar vertebrae in 23 girls and 25 boys with type 1 diabetes (aged 5–19 years) and in a similar number of age- and gender-matched controls: they found a small decrease ($p < 0.02$) in cortical bone density in the diabetic group, but no difference in trabecular bone density. Gallacher *et al.* (1993) reported on 20 type 1 diabetic women (aged 23–42 years, diabetic for 2–22 years): compared with an age-matched control group of 27 women, lumbar spine BMD (assessed by dual-energy X-ray absorptiometry, DXA) was higher ($p = 0.02$) in the diabetic group. Olmos *et al.* (1994) found no difference in lumbar spine BMD, measured by DXA, between 94 type 1 diabetic subjects (of whom 50 women; aged 18–62 years, diabetic for 1–42 years) and an age-matched control group of 34 women and 30 men. More recent studies present BMD data as *Z* scores compared with reference values provided by the manufacturer or validated in a local population. The conclusion from these studies would be that type 1 diabetic subjects have a mean *Z* score below, but generally within 1 SD, reference values at the lumbar spine (Lunt *et al.,* 1998; Miazgowski and Czekalski, 1998; Rix *et al.,* 1999). In addition, this BMD deficit does not appear to deteriorate, as the BMD did not change over 2 years in 54 subjects with long-term diabetes (Miazgowski and Czekalsi, 1998); in another study, BMD actually increased ($p = 0.002$) over 2 years in a group of 15 subjects with type 1 diabetes aged <30 years at the first evaluation (Kayath *et al.,* 1998), suggesting that type 1 diabetes does not impede the attainment of peak axial bone density.

At the femoral neck, results are comparable with those found at the lumbar spine. Some studies found no difference between diabetic subjects and controls (Gallacher *et al.,* 1993), whereas others reported a small decrease compared with controls or reference values, with a mean *Z* score between 0 and −1.0 SD (Lunt *et al.,* 1998; Rix *et al.,* 1999). Again, there is no reduction of femoral neck BMD 2 years after the initial evaluation (Kayath *et al.,* 1998).

Bone density at the appendicular skeleton was assessed in earlier studies. The control population is not well described or validated in many of these studies. With this caveat in mind, some studies conclude that BMC and BMC/width on single-photon absorptiometry (SPA) of the forearm (midshaft or distal one-third) are about 10% lower in diabetic children, adolescents, and adults than in control populations; the same is true for the cortical area measured by radiogrammetry of the metacarpal bones (McNair *et al.,* 1978; Wiske *et al.,* 1982). There is no correlation between diabetes duration and BMC deficit, and BMC deficit can be measured within a few years after diagnosis (McNair *et al.,* 1978).

Sixteen subjects with type 1 diabetes were studied by SPA at the radius at age 51 ± 13 years (mean ± SD) and again 12.5 ± 0.5 years later: BMC/width *Z* score had not changed significantly (Krakauer *et al.,* 1995). QCT of the ultradistal radius in 21 diabetic children/adolescents (of whom 8 girls; aged 6–19 years; diabetes duration 0.8–18 years) showed that cortical bone density was not different from age- and gender-matched controls, but trabecular bone density was 19% lower ($p < 0.01$) in the diabetic group.

The effect of diabetic neuropathy was investigated by Rix *et al.* (1999), who compared 21 male type 1 diabetics [aged 57 ± 6 (mean ± SEM) years; mean diabetes duration 28 (range 9–59) years] with severe neuropathy with 21 gender-, age-, and diabetes duration-matched diabetics with mild or no neuropathy and 21 age-matched controls. Subjects with neuropathy had a lower BMD than reference values (*Z* score around −1.0 SD) and a lower BMD at the proximal femur than controls, as well as lower broadband attenuation on quantitative ultrasound (QUS) of the calcaneus compared with diabetics without neuropathy and with controls. Whether this is caused by reduced physical activity is unknown.

In conclusion, a mild decrease in the BMD at the lumbar spine, proximal femur, and distal forearm has been found in some, but not all, studies in type 1 diabetics. The difference is maximally 10% or 1 SD compared with age-matched control groups. This effect can be seen within a few years after diagnosis and is not progressive. We speculate that this mild decrease is due to decreased appositional growth during the early hypoinsulinemic phase of the disease. Neuropathy appears to aggravate the BMD deficit.

Hyperinsulinemia and Bone Mass

Hyperinsulinemia is a marker of insulin resistance, the central mechanism in the pathogenesis of type 2 diabetes. Two population-based studies have addressed the relationship between insulin levels and BMD: the large Rotterdam study (5931 individuals, aged at least 55 years, including 578 diabetics) and the smaller Rancho Bernardo study (970 individuals, aged 50–89 years, diabetics excluded). It was shown that age-adjusted BMD at the lumbar spine, proximal femur, and radius correlated with fasting or post-OGTT insulin levels and with serum glucose levels. Excluding subjects with diabetes does not change this conclusion, but adjusting for body mass index (BMI) and other potentially confounding factors does reduce the correlation, and significance is lost in subgroups of individuals (Barrett-Connor and Kritz-Silverstein, 1996; Stolk *et al.,* 1996). Further studies should disentangle the respective effects of body weight and insulin levels on BMD in elderly individuals without type 2 diabetes.

Type 2 Diabetes and Bone Mass

Meema and Meema (1967) measured the cortical thickness at the radius in aged (65–101 years) Caucasian women and found that women with type 2 diabetes (average duration: 9 years) had higher cortical thickness compared with nondiabetics. In the Study of Osteoporotic Fractures, a population-based study of 7664–9704 nonblack women aged 65 years or older, type 2 diabetes (prevalence: 6%) was a significant predictor of BMD at the radius and the femoral neck, but not at the lumbar spine: in multivariate analyses, type 2 diabetes was associated with a 4.8% [95% confidence intervals (CI), 2.2–7.3] increase in BMD at the radius and a 3.4% (CI, 1.3–5.6) increase in BMD at the femoral neck (Bauer *et al.,* 1993; Orwoll *et al.,* 1996). In the Rotterdam study, the BMD of 578 subjects with type 2 diabetes was significantly (3–4%) higher at the lumbar spine and the femoral neck than in 5353 nondiabetic subjects, even after multivariate adjustment (van Daele *et al.,* 1995). The BMD of type 2 diabetics was found to be correlated with fasting insulin levels and urinary C-peptide levels (Rishaug *et al.,* 1995; Wakasugi *et al.,* 1993), again suggesting a link between hyperinsulinemia and an increase in BMD. In line with this contention, the BMD of insulin-treated type 2 diabetics (presumably with impaired β-cell function) was not different from controls (van Daele *et al.,* 1995; Tuominen *et al.,* 1999). While it has been reported that the BMD at the lumbar spine correlates inversely with the duration of type 2 diabetes (Wakasugi *et al.,* 1993), others found a significant increase in BMD (increase in *Z* score of 0.09 ± 0.01 per year, mean ± SD) at the radius in 19 type 2 diabetics (mean age: 63 years) 12.5 years after the initial evaluation (Krakauer *et al.,* 1995). Future studies should link changes in BMD in type 2 diabetics with circulating insulin levels.

The different effect of type 1 and type 2 diabetes on BMD was confirmed by Tuominen *et al.* (1999), who studied subjects (aged 52–72 years) who developed diabetes after 30 years of age (i.e., after achievement of peak bone mass) and were insulin treated, but differed in their insulin secretory response (C-peptide levels after glucagon). Subjects with deficient insulin secretion (classified as type 1 diabetes) had a lower BMD at the proximal femur than subjects with normal insulin secretion (type 2 diabetes) or controls. After adjusting for age, BMI, and other factors, the difference was less significant but still demonstrable.

In conclusion, type 2 diabetes is associated with a small but significant increase in areal BMD as measured by DXA, even after adjustment for BMI and other variables. Hence, low bone turnover in type 2 diabetes does not cause bone loss (Krakauer *et al.,* 1995). There is inconclusive evidence at this time that hyperinsulinemia is a causal factor.

Type 1 and Type 2 Diabetes and Fractures

Several cohort studies have shown that diabetes is a risk factor for fractures. In a study from Norway, 35 to 49-year-old women and men (24,000 each) were followed for at least 10 years: a self-reported diagnosis of diabetes (prevalence: 0.49% of women and 0.74% of men) was a strong predictor of hip fractures. Indeed, multivariate analysis showed a relative risk (RR) of 9.2 (CI, 3.4–24.9) in women with diabetes and 9.4 (2.9–30.5) in men with diabetes (Meyer *et al.,* 1993). In another Norwegian cohort study, 27,986 individuals aged 50–74 years were followed for 9 years. The age-, BMI-, and smoking-adjusted RR of hip fracture was 6.9 (CI, 2.2–21.6) in women with type 1 diabetes (prevalence: 0.17%) and 4.5 (CI, 0.6–31.9) in men (prevalence: 0.21%). However, the significantly increased RR in diabetic women was lost after adjusting for impaired vision, physical inactivity, impaired motor abilities, and a history of stroke. Type 2 diabetes for more than 5 years was associated with a slightly increased RR

of hip fracture in women only (1.8, CI, 1.1–2.9), which again was lost after adjusting for the factors mentioned earlier (Forsén *et al.,* 1999).

In the Study of Osteoporotic Fractures, insulin-treated diabetes (presumably type 2 diabetes in most women) was a strong predictor for fractures of the proximal humerus in multivariate analysis with a RR of 3.8 (CI, 1.2–12.4), during 2.2 years of follow-up (Kelsey *et al.,* 1992); there was no effect on distal forearm fractures, however. In the lower limbs, insulin-treated diabetes was an independent predictor of foot fractures, with a RR of 2.9 (CI, 1.2–7.2), during 5.9 years of follow-up; there was no significant effect on ankle fractures (Seeley *et al.,* 1996). In a subsequent analysis specifically studying the effect of type 2 diabetes on fractures, the risk of any nonspine fracture was found to be increased in diabetic women, particularly in diabetic women treated with insulin: the RR of nonspine fractures was 1.3 (CI, 1.1–1.6) in women not treated with insulin and 1.9 (CI, 1.3–2.8) in insulin-treated women (Schwartz *et al.,* 1999).

In the Rotterdam study, however, type 2 diabetes was not associated with an increased self-reported risk of nonspine fractures in the preceding 5 years; in fact, the RR was decreased (0.63, CI, 0.44–0.90) in women with type 2 diabetes (van Daele *et al.,* 1995).

Neuropathy is a predisposing factor for fractures: in a series of diabetic individuals (both type 1 and type 2, aged 50–73 years), foot fractures were detected radiographically in 12 of 54 (22%) subjects with a history of foot ulcers, but only in 3/83 subjects either without neuropathy or with neuropathy but no history of foot ulcers (Cavanagh *et al.,* 1994).

It is well known that diabetes can delay the healing of fractures (Loder, 1988). By analogy, it has been postulated that diabetes may impair the healing of fatigue microfractures (also called microdamage or microcracks) in load-bearing bones because of low bone formation and may thereby ultimately lead to overt fractures (Krakauer *et al.,* 1995). This interesting hypothesis has yet to be tested.

In conclusion, diabetes is an independent risk factor for the occurrence of nonspine fractures in the majority of studies. Type 1 diabetes appears to be associated with a stronger risk than type 2 diabetes, and neuropathy is an additional risk factor. Whether this is due to decreased intrinsic bone strength and/or an increased propensity to fall is uncertain at this time.

Diabetic Nephropathy and Bone

Several histomorphometric studies show that parameters of bone formation and resorption are lower in diabetic than in nondiabetic patients with chronic renal failure, and thus that mild or aplastic renal osteodystrophy is more prevalent in diabetics whereas high-turnover osteodystrophy is rare (Pei *et al.,* 1993, and references therein). Bone formation parameters were also found to be lower in 5/6 nephrectomized rats with SZ-induced diabetes compared with nondiabetic rats (Jara *et al.,* 1995).

Diabetic patients with chronic renal failure also have less Tc-99m methylenediphosphonate uptake on bone scintigraphy, confirming reduced osteoblastic activity; bone scans are, therefore, unreliable as a diagnostic method for renal osteodystrophy in diabetic subjects (So *et al.,* 1998). Importantly, diabetes was found to be the most significant predictor of fracture after renal transplantation; in a series of 193 transplant patients (of whom 35 had diabetes), followed for 6 months to 23 years, 40% of diabetics sustained at least one fracture, predominantly in ankles and feet, compared with 11% in nondiabetics (Nisbeth *et al.,* 1999). In another series of 35 kidney–pancreas transplant recipients, the 5-year fracture-free rate was only 48%; the cumulative steroid, but not cyclosporine, exposure was a significant predictor of fractures in this group (Chiu *et al.,* 1998). This confirms data in SZ diabetic rats that bone volume and bone remodeling parameters are unaffected by cyclosporine treatment (Epstein *et al.,* 1994).

The Effect of Diabetes and Insulin on Bone (Re)modeling, Bone Mass, and Bone Strength in Experimental Animals *in Vivo*

Effect of Insulin on Bone in Nondiabetic Animal Models

Significant *in vivo* insulin binding has been detected by autoradiography in osteoblasts, but not in osteocytes (Martineau-Doizé *et al.,* 1986). Cornish *et al.* (1996) injected human insulin over the periosteum of one hemicalvarium of normal adult mice for 5 days. In the insulin-injected hemicalvaria, histomorphometric parameters indicated a stimulation of bone formation (osteoblast number/perimeter, osteoid area), but no change in the osteoclast number/perimeter.

Bone (Re)modeling in Animal Models of Type 1 Diabetes

There is consensus that untreated severe diabetes is associated with low to virtually absent bone formation. First, this has been shown by biochemical markers, in particular plasma osteocalcin levels. Plasma osteocalcin levels drop exponentially after onset of diabetes in BB rats to about 25% of the levels in nondiabetic animals after 5 weeks (Verhaeghe *et al.,* 1997b) (Fig. 2); this was confirmed in SZ-induced diabetes (Epstein *et al.,* 1994). Interestingly, plasma osteocalcin levels are lower than in nondiabetic littermates on the first day of glycosuria (Verhaeghe *et al.,* 1997b), confirming data obtained in humans by Guarneri *et al.* (1993) that diabetes depresses bone formation parameters rapidly. Osteocalcin levels respond poorly, if at all, to exogenous $1,25(OH)_2D_3$ administration in diabetic rats (Verhaeghe *et al.,* 1989, 1993). The half-life of plasma osteocalcin is similar in diabetic and nondiabetic BB rats, indicating that circulating osteocalcin levels are not decreased because of faster clearance (Verhaeghe *et al.,* 1989). Osteocalcin levels gradually return to within the normal range with increasing insulin dose in

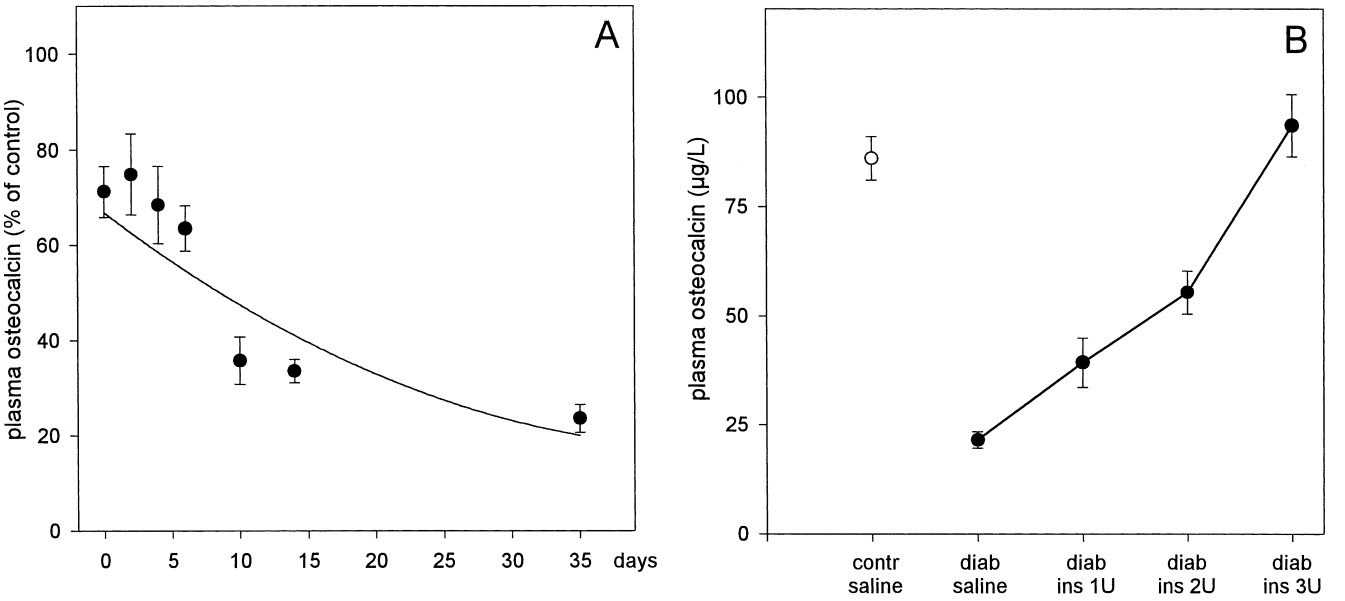

Figure 2 (A) Plasma osteocalcin concentrations after onset of diabetes in male spontaneously diabetic BB rats presented as the percentage (mean ±SEM) of values measured in nondiabetic (control) littermates. Osteocalcin levels were significantly different at the first day of glycosuria (71 ± 5% of paired controls) and decreased exponentially thereafter ($y = 67.e^{-0.0346}x$ (%); $R^2 = 0.62$; $p < 0.0001$). (B) Effect of sc-infused insulin, via miniosmotic pump, during 14 days on plasma osteocalcin concentrations in male spontaneously diabetic BB rats (diabetes duration about 5 weeks). Data are means ±SEM. Statistical analysis: significantly different compared with saline-infused control rats ($^{*}p < 0.001$); significantly different compared with saline-infused diabetic rats ($^{+}p < 0.001$). Modified from Verhaeghe *et al.* (1997b), with permission of Humana Press, Inc.

BB rats (Verhaeghe *et al.,* 1997b) (Fig. 2) and are also normalized by pancreas transplantation in SZ-diabetic rats (Ishida *et al.,* 1992).

Second, low to virtually absent bone formation has been shown by histomorphometry in many studies in SZ-diabetic and BB rats. This applies to all bone surfaces: trabecular-endosteal, endocortical, and periosteal (Bain *et al.,* 1997; Epstein *et al.,* 1994; Glajchen *et al.,* 1988; Shires *et al.,* 1981; Verhaeghe *et al.,* 1992). Static morphometry demonstrates a marked decline in osteoblast and osteoid surface/volume (Table I). Dynamic morphometry shows a decrease in both mineralizing surface (or labeled perimeter) and mineral apposition rate; however, the maturation and mineralization of osteoid remain normal when adjusted for the decrease in osteoid production rate (Goodman and Hori, 1984), indicating that the basic defect is in the number of active osteoblasts producing osteoid. Insulin treatment reverses these effects (Goodman and Hori, 1984; Verhaeghe *et al.,* 1992).

Electron microscopy of the endocortical surface in diabetic rats shows that active, cuboidal osteoblasts are virtually absent and are replaced by bone-lining cells with flattened nuclei, little or no rough endoplasmic reticulum, and no detectable alkaline phosphatase activity or uptake of [^{3}H]proline for collagen synthesis (Sasaki *et al.,* 1991).

Regarding bone resorption, the measurement of urinary D-PYR shows decreased total and creatinine-corrected D-PYR excretion in diabetic BB rats (Verhaeghe *et al.,* 2000). Most histomorphometric data confirm that the osteoclast surface/number is decreased moderately to severely in diabetic rats, which is reversed by insulin treatment (Glajchen *et al.,* 1988; Shires *et al.,* 1981; Verhaeghe *et al.,* 1992). Electron microscopy shows that most osteoclasts in diabetic rats lack a ruffled border–clear zone complex and that acid phosphatase activity is rarely detected (Kaneko *et al.,* 1990).

In conclusion, there is strong evidence from *in vivo* studies in animal models that diabetes is associated with a marked dampening of both resorption and formation. The bone surface engaged in remodeling is scant, whereas most of the bone surface is in a quiescent state.

Bone Mass in Animal Models of Type 1 Diabetes

Physicochemical measurements in rats in which diabetes was drug-induced at an early age show a gradual decline in both body weight and skeletal weight (Locatto *et al.,* 1993). Thus, femoral (wet and dry) weight, length, and diaphyseal width are lower in diabetic than in nondiabetic rats, and this decrease in bone size is more apparent with longer diabetes duration and with higher average glycemia (Dixit and Ekstrom, 1980; Einhorn *et al.,* 1988; Locatto *et al.,* 1993; Lucas, 1987). This corresponds with a decrease in bone blood flow, as measured using radiolabeled microspheres (Lucas, 1987). In diabetic BB rats, which develop diabetes at an average age of 13 weeks, femur weight and diaphyseal width, but not femur length, are decreased after 8–12 weeks of diabetes (Verhaeghe *et al.,* 1994, 2000). However, the ash percentage and calcium/phosphate concentration of long bones remain within the normal range in SZ-diabetic and BB-diabetic rats (Bain *et al.,* 1997; Dixit and Ekstrom, 1980; Shires *et al.,* 1981; Verhaeghe *et al.,* 1989, 1990, 1994). After 1 year of SZ diabetes, Einhorn *et al.* (1988) found a small decrease in bone ash percentage at the femoral metaphyseal level, but an increase at the diaphyseal

level; Bain *et al.* (1997) also reported an increase in tibial cortical bone density in SZ-diabetic rats.

Using DXA technology, we confirmed that diabetes (for 6–12 weeks) affects bone size, as reflected by the measurement of bone area, in BB rats; consequently, BMC is decreased, particularly at the diaphysis (Verhaeghe *et al.,* 1997a, 2000). However, the BMD (BMC/area) at the distal metaphysis and the BMD or the BMAD (bone mineral apparent density, BMC/area^2) at the diaphysis are not significantly different from values in nondiabetic rats (Verhaeghe *et al.,* 1994, 1997a, 2000).

Histomorphometry, again, shows a decrease in total and cortical area at the tibial diaphysis, whereas the cortical area as a percentage of total area remains unchanged (Epstein *et al.,* 1994). Thus, physiochemical, DXA, and histomorphometry consistently show a decrease in bone size, and consequently in the total amount of bone (mineral), whereas bone density (mineral per unit bone) is normal.

There is some inconsistency in the reported effect of diabetes on trabecular (cancellous) bone volume at the proximal tibial metaphysis, as measured by histomorphometry. In SZ- or alloxan-diabetic rats, trabecular bone volume was found to be decreased in most studies, and this reduction correlates with the average glycemia (Bain *et al.,* 1997; Epstein *et al.,* 1994; Glajchen *et al.,* 1988; Locatto *et al.,* 1993); further analysis showed a decrease in trabecular thickness, but not in the number of trabeculae (Epstein *et al.,* 1994). In diabetic BB rats, we found a normal trabecular bone volume at the secondary spongiosa in most (Verhaeghe *et al.,* 1989, 1992, 1993, 1994, 2000), but not all (Verhaeghe *et al.,* 1990), experiments. The discrepancy in results may be explained by the differences in age at onset or induction of diabetes and the method of measuring trabecular volume (i.e., the distance from the distal growth plate). Ovariectomy (6–12 weeks) results in trabecular bone loss at the proximal tibial metaphysis in adult diabetic BB rats, but not in diabetic ovariectomized rats treated with 17β-estradiol (Verhaeghe *et al.,* 1994, 1997a). In contrast, we found no trabecular bone loss in diabetic BB rats after 8 weeks of skeletal disuse induced by unilateral sciatic neurectomy, in contrast to what was observed in paralyzed limbs of nondiabetic rats (Verhaeghe *et al.,* 2000); urinary D-PYR excretion was increased in nondiabetic rats only, suggesting that bone resorption is not stimulated by immobilization in diabetic rats.

Bone Remodeling and Bone Mass in Animal Models of Type 2 Diabetes

Data are scarce. Takeshita *et al.* (1993) examined the effects on bone in genetic Wistar fatty rats and in rats treated neonatally with SZ, a frequently used animal model for type 2 diabetes. Wistar fatty rats are markedly obese, hyperinsulinemic, and hyperglycemic. Despite this, femur size (length and weight) is decreased and femur calcium content is decreased, as well as plasma osteocalcin levels. Rats with neonatal SZ treatment have a normal weight and are hypoinsulinemic and hyperglycemic. Their bone size and calcium content are normal, but plasma osteocalcin levels are again lower than in controls. These effects are reminiscent of those found in animal models of type 1 diabetes and indicate that the effect of diabetes on bone size and bone formation parameters is, at least to some extent, independent of plasma insulin levels and body weight.

Bone Strength in Animal Models of Type 1 and Type 2 Diabetes

The breaking strength of the femur—assessed by torsion or by pressure with a knife edge—has been shown to be lower after 8 weeks of diabetes or more in SZ-diabetic and diabetic BB rats (Dixit and Ekstrom, 1980; Einhorn *et al.,* 1988; Verhaeghe *et al.,* 1990, 1994), but not after 4 weeks (Funk *et al.,* 2000). Decreased torsional strength in rats diabetic for 1 year persisted after correcting for smaller bone size (Einhorn *et al.,* 1988). Similarly, decreased breaking strength was found at the femoral neck of SZ-diabetic rats, which was partly restored by insulin treatment (Hou *et al.,* 1993). Femoral breaking strength was also found to be decreased in models of type 2 diabetes (Takeshita *et al.,* 1993).

We carried out a detailed analysis of bone strength in BB rats at the fifth lumbar vertebra and at the femoral neck, diaphysis, and distal metaphysis; diabetic rats were poorly controlled, and the rats had received either no intervention or had received running exercise for 8 weeks (starting within 4 days of diagnosis in diabetic rats). Using two-factor ANOVA, diabetes had no effect on biomechanical competence at the lumbar vertebra, femoral neck, and diaphysis. At the femoral metaphysis, however, load/density was reduced in diabetic rats; moreover, biomechanical competence at the femoral metaphysis improved after a running exercise program in nondiabetic rats only so that the biomechanical differences between diabetic and nondiabetic rats were more marked in the exercise group (Verhaeghe *et al.,* 2000).

As in human diabetics, fracture repair is delayed in diabetic rats: callus volume and BMC after fracture of the fibula were found to be lower in SZ-diabetic than in nondiabetic rats (Kawaguchi *et al.,* 1994), as well as biomechanical properties after fracture of the femur (Funk *et al.,* 2000). The postfracture expression of basic fibroblast growth factor (FGF-2) in the soft callus and periosteum is impaired in diabetic rats and is restored by insulin treatment. Administration of FGF-2 dose dependently facilitates callus volume in both nondiabetic and diabetic rats (Kawaguchi *et al.,* 1994).

Insulin and Bone Cells *in Vitro*

High-affinity insulin receptors have been documented in several mature osteoblastic cell lines: in UMR-106, a clonal rat osteogenic osteosarcoma cell line (De Luise and Harker, 1988; Hickman and McElduff, 1989; Ituarte *et al.,* 1989; Pun *et al.,* 1989; Thomas *et al.,* 1996), and in ROS-17/2.8, a

rat osteogenic osteosarcoma cell line (Levy *et al.,* 1986). However, there is no insulin binding in UMR-201, a rat calvaria-derived preosteoblastic clonal cell line (Thomas *et al.,* 1996). Half-maximal displacement of ^{125}I-labeled insulin is attained at physiological concentrations of unlabeled insulin (between 0.5 and 1.0 n*M*) (Fig. 3) (Ituarte *et al.,* 1989; Pun *et al.,* 1989). In cultures from neonatal rat calvaria, positive immunostaining was observed within the cytoplasm of mature cuboidal osteoblasts, and insulin binding was found to be higher in osteoblast-enriched populations of cells with high alkaline phosphatase activity than in less mature cell populations (Thomas *et al.,* 1996). In UMR-106, the number of high-affinity receptors is estimated to be around 80,000 per cell (Ituarte *et al.,* 1989; Pun *et al.,* 1989); insulin binding and the number of insulin receptors are downregulated by supraphysiological levels of insulin (10^{-7} *M*) but are stimulated by dexamethasone (from 10^{-8} *M*) (De Luise and Harker, 1988; Ituarte *et al.,* 1989; Pun *et al.,* 1989).

Osteoblast proliferation, assessed by [^{3}H]thymidine incorporation, is stimulated by physiological concentrations of insulin (0.5–1.0 n*M*) in UMR-106 cells (Hickman and McElduff, 1989). In other cell culture systems, osteoblast proliferation can be achieved only at supraphysiological or pharmacological doses of insulin (10^{-7}–10^{-6} *M*), e.g., in osteoblastic cells obtained from fetal rat calvariae or from human femoral trabecular bone (Hock *et al.,* 1988; Wergedal *et al.,* 1990). Physiological concentrations of insulin (0.5–1.0 n*M*) stimulate glucose uptake in UMR-106 osteoblastic cells (Ituarte *et al.,* 1989; Thomas *et al.,* 1996). Glucose uptake is generally examined using radiolabeled 2-deoxyglucose (2-DG), which cannot be metabolized beyond 2-DG-6-phosphate. Ituarte *et al.* (1989) found that the half-maximal concentration of insulin needed for displacement of radiolabeled insulin corresponds with the half-maximal concentration needed to stimulate 2-DG (Fig. 3). Insulin at 10^{-8} *M* also increases the mRNA level of the glucose transporter GLUT1 by threefold in UMR-106 cells (Thomas *et al.,* 1996). Insulin, even at 10^{-6} *M,* does not stimulate glucose uptake in preosteoblastic UMR-201 cells, as expected in the absence of insulin binding (Thomas *et al.,* 1996); surprisingly, insulin is also ineffective in stimulating glucose uptake in ROS 17/2.8 osteoblastic cells, which contain insulin receptors (Levy *et al.,* 1986). Insulin also affects ion fluxes across the osteoblast membrane in UMR-106: there is stimulation of the Na^+-K^+ pump-mediated K^+ uptake with half-maximal stimulation at 0.25 n*M* (De Luise and Harker, 1988) and a stimulation of the Na^+-dependent PO_4^{2-} uptake by 10^{-9}–10^{-7} *M* insulin (Kunkler *et al.,* 1991).

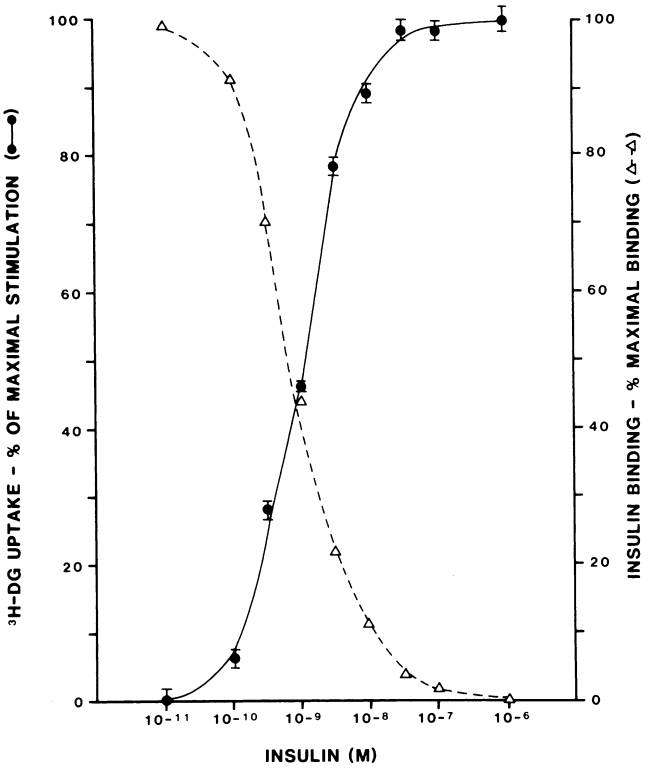

Figure 3 Relationship between insulin receptor occupancy and stimulation of 2-deoxyglucose (2-DG) uptake in UMR-106 (rat osteosarcoma) cells. Insulin displacement of [^{125}I]insulin binding and stimulation of 2-DG were performed in parallel cultures. The half-maximal insulin concentration for both processes was 0.8 n*M*. Each point represents the mean ±SEM of 12 determinations. From Ituarte *et al.* (1989b), with permission.

Furthermore, insulin at 1 n*M* stimulates the incorporation of collagen synthesis—measured by the uptake of [^{3}H]proline into collagen—in UMR-106 cells (Pun *et al.,* 1989), but also in fetal rat calvariae, especially in its osteoblast-rich central bone area (Kream *et al.,* 1985); at the latter site, insulin at 3 n*M* stimulates the level of α-1(I)-procollagen mRNA, possibly by modulating the stability of the procollagen mRNA (Craig *et al.,* 1989). Thus, in fetal rat calvariae, there is a discrepancy between the effect of insulin on collagen synthesis and matrix apposition, assessed by histomorphometry (effective from 10^{-9} *M*) versus its effect on osteoblast replication (from 10^{-7}–10^{-6} *M*), indicating that the stimulating effect of insulin on collagenous matrix production is not explained by its effect on osteoblast replication (Hock *et al.,* 1988). Proinsulin is only 0.1–1% as effective as insulin in stimulating collagen synthesis in UMR-106 and fetal rat calvariae (Kream *et al.,* 1985; Pun *et al.,* 1989).

Thomas *et al.* (1998) produced evidence that there is insulin binding in primary mouse and rat osteoclasts and in cultured osteoclast-like cells; the receptor density was higher in purified osteoclast-like cells than in primary osteoblast cultures. Insulin dose dependently inhibited pit formation in a dentine slice assay. These results would indicate that insulin inhibits bone resorption, but further studies are needed to corroborate this conclusion.

In conclusion, high-affinity insulin receptors are present in mature osteoblastic cells but not in preosteoblastic cell lines. Insulin at physiological concentrations (1 n*M* or lower) stimulates osteoblastic cell function, including glucose and phosphate uptake, and collagenous matrix synthesis. In mature osteoblastic cell lines, insulin at the same concentration also stimulates osteoblast replication; however, experiments in fetal rat calvariae indicate that the effect of insulin on osteoblastic function is not fully explained by its effect on cell proliferation. Osteoclasts also appear to contain insulin receptors.

Causative Factors and Mechanisms of Diabetic Bone Disease

The *in vivo* repression of bone growth and remodeling in diabetic subjects and animal models can be replicated *in vitro* using diabetic serum. Indeed, diabetic rat serum inhibits collagen production in rib cartilage from hypophysectomized rats (Spanheimer, 1992, and references therein), and sera from diabetic individuals with poor glycemic control inhibit the proliferation of human osteoblastic cells and osteoblastic collagen production (Brenner *et al.,* 1992). Hence, diabetic bone disease appears to be induced by one or several circulating factors. These factors almost certainly include insulin, but may also include glucose, IGF-I, the IGF-binding proteins (IGFBPs), and glucocorticoids. Indeed, insulin-deficiency *per se* does not appear to explain all clinical and experimental data: (1) low osteocalcin levels have been measured in subjects and animal models with type 2 diabetes and hyperinsulinemia (Montecucco *et al.,* 1990; Takeshita *et al.,* 1993) and (2) the addition of insulin to diabetic sera *in vitro* does not reverse defective collagen synthesis, whereas prior *in vivo* treatment of the animals does (Spanheimer, 1992).

A very high glucose environment has been shown to inhibit basal and IGF-I-induced osteoblastic cell proliferation and $1,25(OH)_2D_3$-induced osteocalcin secretion in human MG-63 cells *in vitro;* this was not replicated in mannitol cultures (Terada *et al.,* 1998). However, in another study, the effect of high extracellular glucose concentrations on osteocalcin gene expression in mouse osteoblasts was mimicked by mannitol so that the effects of hyperglycemia versus osmotic stress must be further delineated (Zayzafoon *et al.,* 2000). In addition, we found no *in vivo* effect of hyperglycemia on plasma osteocalcin levels in chronically catheterized nondiabetic BB rats (Verhaeghe *et al.,* 1997b).

IGF-I is well known to promote osteoblast proliferation and bone matrix formation (Hock *et al.,* 1988). Numerous reports have shown that circulating IGF-I levels are decreased in subjects with type 1 diabetes; in addition, we found a correlation between IGF-I levels and biochemical markers of bone formation in subjects with type 1 diabetes (Bouillon *et al.,* 1995). We confirmed this correlation between plasma IGF-I and osteocalcin levels in several studies in diabetic BB rats (Verhaeghe *et al.,* 1997b). Insulin-deficient diabetic subjects also have increased serum IGFBP1 concentrations (Bereket *et al.,* 1995) due to increased hepatic IGFBP1 gene expression; IGFBP1 has been shown to inhibit IGF-I-induced DNA synthesis in human osteosarcoma cells (Campbell and Novak, 1991). Moreover, the osteoblastic expression of IGFBP1 is potently inhibited by insulin in human osteoblasts *in vitro* (Conover *et al.,* 1996) and would thus be expected to be increased in type 1 diabetes.

Glucocorticoid excess also appears to play a role in the development of diabetic bone disease. Children with newly onset type 1 diabetes have increased cortisol levels (Bereket *et al.,* 1995), and higher corticosterone levels were found in some, but not all, studies in animal models of severe type 1 diabetes (Verhaeghe *et al.,* 1997b). We showed that osteocalcin levels consistently rose to within the control range 4 days after adrenalectomy in severely diabetic BB rats, but not in corticosterone-treated adrenalectomized diabetic rats (Verhaeghe *et al.,* 1997b) (Fig. 4). The effect of glucocorticoid excess on bone is mediated, in part, by altered IGF and IGFBP gene expression in hepatocytes (affecting circulating levels) and in osteoblasts, i.e., decreased IGF-I but increased IGFBP1 gene

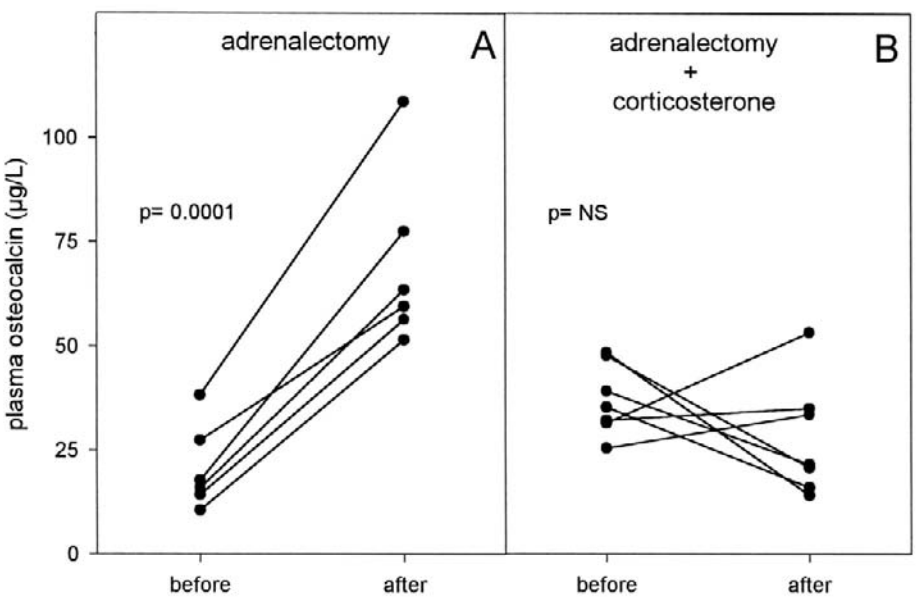

Figure 4 Effect of adrenalectomy (A) and adrenalectomy with corticosterone treatment (15 mg/day, sc) (B) on plasma osteocalcin concentrations in male spontaneously diabetic BB rats. Individual data are shown before adrenalectomy and 96 hr after adrenalectomy. Modified from Verhaeghe *et al.* (1997b), with permission of Humana Press, Inc.

expression (reviewed by Verhaeghe *et al.,* 1997b). Glucocorticoid excess is well known to decrease osteoblastogenesis and stimulate osteoblast apoptosis. However, in contrast to diabetic bone disease, corticosteroid excess is associated with an early increase in bone resorption (reviewed by Manolagas, 2000).

Diabetes is associated with changes in the circulating levels of the calciotropic hormones PTH and (total) $1,25(OH)_2D_3$, which were found to be decreased in some, but not all, studies in type 1 diabetic subjects and in animal models of type 1 diabetes (reviewed by Verhaeghe *et al.,* 1999). However, we found that $1,25(OH)_2D_3$ injections, even at doses that cause hypercalcemia, do not increase bone formation in diabetic BB rats (Verhaeghe *et al.,* 1993). Intermittent administration of PTH_{1-34} was found to normalize trabecular bone formation rate in SZ-injected diabetic rats (Tsuchida *et al.,* 2000); however, intermittent PTH is effective in increasing trabecular bone formation and bone volume in rats with various osteopenic conditions not necessarily associated with hypoparathyroidism (e.g., after ovariectomy, glucocorticoid administration).

In diabetic rats treated with tetracycline or a nonmicrobial tetracycline analog, there is normalization of both growth plate width and bone formation rate at the trabecular-endosteal bone surface, as well as an unequivocal increase in bone formation rate at the periosteal surface (Bain *et al.,* 1997). On electron microscopy, inactive bone-lining cells are reverted into active osteoblasts, and osteoclasts are structurally normal (i.e., containing a ruffled border) in tetracycline-treated diabetic rats (Kaneko *et al.,* 1990; Sasaki *et al.,* 1991). Tetracycline is known to inhibit collagenase (matrix metalloproteinase) activity in osteoblastic cells. Collagenase activity is increased in the skin and periodontium of diabetic rats and presumably in bone as well (Bain *et al.,* 1997, and references therein). Low IGF-I levels and glucocorticoid excess in diabetic serum may be involved, as collagenase expression in osteoblastic cells is known to be repressed by IGF-I but stimulated by glucocorticoids. An interesting preliminary report also showed that femoral neck BMD is determined by type 1 collagen gene polymorphism in women with (type 1 or type 2) diabetes (Hampson *et al.,* 1998).

Although there is evidence from electron microscopic studies that (some) osteoblasts revert to an inactive state as bone-lining cells in severe diabetes (Sasaki *et al.,* 1991), the possibility that low bone formation would also result from reduced osteoblastogenesis from marrow stromal cells and/or increased osteoblast apoptosis has not been studied to date. Osteoblast survival *in vitro* has been found to be promoted by insulin and IGF-I (Hill *et al.,* 1997), and glucocorticoid excess stimulates osteoblast apoptosis *in vivo* (Manolagas, 2000). Further studies are needed in this regard. Insulin and IGF-I bind to insulin and type 1 IGF receptors, which are both tyrosine kinase receptors. The subsequent intracellular signaling involves the phosphorylation of insulin receptor substrate (IRS)-1 and -2. Mice deficient for the IRS-1 gene have been generated: interestingly, the bone changes in these animals are virtually identical to those observed in severely diabetic rats, with a dramatic decline in bone-remodeling parameters. Osteoblastic cells from IRS −/− mice show decreased proliferation and differentiation *in vitro,* but increased apoptosis, which is unaffected by insulin or IGF-I administration (Ogata *et al.,* 2000). These experiments underscore the potent *in vivo* effects of insulin and/or IGF-I on bone.

Postfracture periosteal FGF-2 expression has been shown to be decreased in insulin-deficient rats, which is restored by insulin administration (Kawaguchi *et al.,* 1994). Mice with a disruption of the FGF-2 gene show decreased trabecular bone formation and bone volume, although to a lesser extent than is observed in severely diabetic rats or IRS-1-gene null mice (Montero *et al.,* 2000). The effect of diabetes on the expression of growth factors in bone needs to be studied further.

The collagenous matrix is not only scant in diabetic rats, but type 1 collagen is glycated to a larger extent as well, which increases with longer diabetes duration. *In vitro,* osteoblastic cells cultured on glycated collagen show decreased proliferation and differentiation (Katayama *et al.,* 1996). Thus, glycosylation of collagen may constitute yet another mechanism for low bone formation in diabetic rats, although it is unlikely to be the initiating pathogenetic mechanism of diabetic bone disease.

In conclusion, the pathogenesis of diabetic bone disease is complex: a serum factor(s) is believed to inititate low bone formation, but the role of insulin and IGF-I deficiency, hypercortisolism, and changes in the IGFBP concentrations must be delineated further. At the bone level, there is evidence of a reversible swap of active osteoblasts into inactive bone-lining cells and increased collagenase (metalloproteinase) activity. Clearly, more research is needed as understanding of the mechanisms involved in osteoblast generation might be important for all metabolic bone diseases.

General Conclusions

Insulin stimulates endochondral bone growth and osteoblast proliferation and function *in vitro* and *in vivo* at physiological concentrations. Severe diabetes in animal models typically induces a "freeze" effect on bones, with robust reductions in bone blood flow, bone growth, periosteal bone apposition, and bone remodeling (both resorption and formation). Consequently, when diabetes is present in fast-growing animals or/and is long-standing, bone size and bone mass (BMC) are reduced. However, when adjusted for bone size, there is no effect of diabetes on bone mineral density. These changes are less apparent in (insulin-treated) human type 1 diabetes, although many studies report (1) a mild reduction in growth velocity in prepubertal children with type 1 diabetes, (2) a mild deficit in areal BMD (maximum 10% or *Z* score between 0 and −1.0 SD), which does not deteriorate with longer diabetes duration, and (3) significantly reduced bone remodeling parameters. Individuals with hyperinsulinemia and/or type 2 diabetes, however, have a mild increase (3–5%) in areal BMD.

Whereas the effect of diabetes in humans on bone density is mild, diabetes increases the risk of nonspine fractures, particularly of the lower extremities. Peripheral neuropathy and steroid exposure after kidney (pancreas) transplantation aggravate the risk of nonspine fractures. However, the effect of diabetes on intrinsic bone strength in animal models remains controversial, and diabetes-related factors that predispose to falls may be important to explain increased fracture risk.

Apart from insulin deficiency, there are likely to be other causative factors in the development of diabetic bone disease, including alterations in the IGF-IGFBP system and hypercortisolism. The cellular and molecular mechanisms by which diabetes affects chondrocyte, (pre)osteoblast and (pre)osteoclast proliferation, and function still need to be elucidated.

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CHAPTER 43

Androgens

Receptor Expression and Steroid Action in Bone

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Introduction

The obvious impact of menopause on skeletal health has focused much of the research on the general action of gonadal steroids on the specific effects of estrogen. However, androgens, independently, have important beneficial effects on skeletal development and on the maintenance of bone mass in both men and women. Thus, androgens (1) influence growth plate maturation and closure, helping to determine longitudinal bone growth during development, (2) participate in the dichotomous regulation of bone mass that leads to a sexually dimorphic skeleton, (3) modulate peak bone mass acquisition, and (4) inhibit bone loss (Vanderschueren and Bouillon, 1995, 1996; Orwoll, 1996; 1999). In castrate animals, replacement with nonaromatizable androgens (e.g., dihydrotestosterone) yields beneficial effects that are clearly distinct from those observed with estrogen replacement (Turner *et al.,* 1990, 1994). In intact females, blockade of the androgen receptor with the specific androgen receptor antagonist hydroxyflutamide results in osteopenia (Goulding and Gold, 1993). Data suggests that combination therapy with both estrogen and androgenic steroids is more effective than estrogen replacement alone (Watts *et al.,* 1995; Raisz *et al.,* 1996; Rosenberg *et al.,* 1997; Barrett-Connor, 1998). At the same time, nonaromatizable androgen alone and in combination with estrogen also results in distinct changes in bone mineral density in females (Coxam *et al.,* 1996). These reports illustrate the independent actions of androgens and estrogens on the skeleton. Thus, in both men and women it is probable that androgens and estrogens each have important, yet distinct, functions during bone development and in the subsequent maintenance of skeletal homeostasis. With the awakening awareness of the importance of the effects of androgen on skeletal homeostasis, and the potential to make use of this information for the treatment of bone disorders, much is to be learned.

The mechanisms by which androgens affect skeletal homeostasis are thus the focus of intensified research. Androgen receptors have been identified in a variety of cells found in bone tissue (Abu *et al.,* 1997). Characterization of androgen receptor expression in these cells thus clearly identifies bone as a target tissue for androgen. Direct actions of androgen that influence the complex processes of proliferation, differentiation, mineralization, and gene expression in the osteoblast have also been documented (Hofbauer and Khosla, 1999). Androgen effects on bone may also be indirectly modulated and/or mediated by other autocrine and paracrine factors in the bone microenvironment. This chapter reviews progress on the characterization of androgen action in bone cells.

Molecular Mechanisms of Androgen Action in Bone Cells: The Androgen Receptor

A steroid hormone target tissue can be defined as one that possesses both functional levels of the steroid receptor and a measurable biological response in the presence of hormone. As described in this chapter, bone tissue clearly meets this standard with respect to androgen. Direct characterization of androgen receptor expression in a variety of tissues, including bone, was made possible by the cloning of the androgen receptor cDNA (Chang *et al.,* 1988; Lubahn *et al.,* 1988). Colvard *et al.,* (1989) first described the presence of androgen

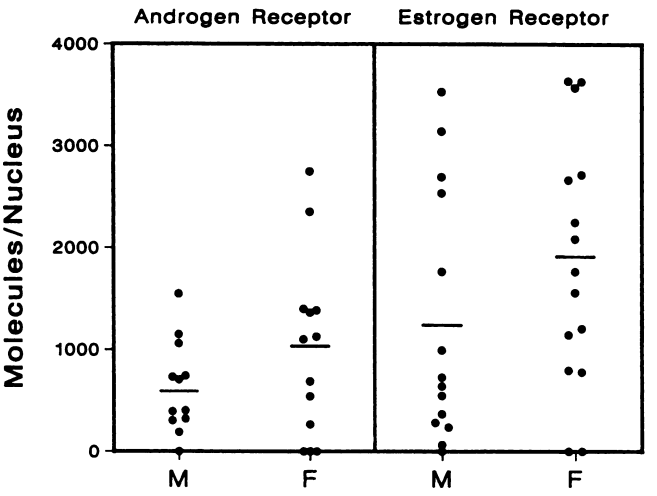

Figure 1 Nuclear androgen and estrogen receptor binding in normal human osteoblast-like cells. Dots represent the mean calculated number of molecules per cell nucleus for each cell strain. (Left) Specific nuclear binding of [^{3}H]R1881 (methyltrienolone, an androgen analog) in 12 strains from normal men and 13 strains from normal women. (Right) Specific nuclear [^{3}H]estradiol binding in 15 strains from men and 15 strains from women. Horizontal lines indicate the mean receptor concentrations (Colvard *et al.,* 1989).

receptor mRNA and specific androgen-binding sites in normal human osteoblastic cells. This report characterized the abundance of both androgen and estrogen receptor proteins as similar in osteoblasts (Fig. 1), suggesting that androgens and estrogens each play important roles in skeletal physiology. Subsequent reports have confirmed androgen receptor mRNA expression and/or the presence of androgen-binding sites in both normal and clonal, immortalized or transformed osteoblastic cells, derived from a variety of species (Benz *et al.,* 1991; Orwoll *et al.,* 1991; Zhuang *et al.,* 1992; Liesegang *et al.,* 1994; Nakano *et al.,* 1994; Takeuchi *et al.,* 1994). The size of the androgen receptor mRNA transcript in osteoblasts (about 10 kb) is similar to that described in prostate and other tissues (Chang *et al.,* 1988), as is the size of the androgen receptor protein analyzed by Western blotting (~110 kDa) (Nakano *et al.,* 1994). There is a report of two isoforms of androgen receptor protein in human osteoblast-like cells (~110 and ~97 kDa) (Kasperk *et al.,* 1997) similar to that observed in human prostate tissue (genital skin) (Wilson and McPhaul, 1994). Whether these isoforms possess similar functional activities in bone, when expressed at similar levels as described in other tissue (Gao and McPhaul, 1998), has yet to be determined.

The number of specific androgen-binding sites in osteoblasts varies, depending on methodology and the cell source, from 1000 to 14000 sites/cell (Masuyama *et al.,* 1992; Liesegang *et al.,* 1994; Nakano *et al.,* 1994; Kasperk *et al.,* 1997a), but is in a range seen in other androgen target tissues. Furthermore, the binding affinity of the androgen receptor found in osteoblastic cells ($K_d = 0.5–2 \times 10^{-9}$) is typical of that found in other tissues. Androgen binding is specific, without significant competition by estrogen, progesterone, or dexamethasone (Colvard *et al.,* 1989; Orwoll *et al.,* 1991; Nakano *et al.,* 1994; Kasperk *et al.,* 1997a). Finally, testosterone and dihydrotestosterone (DHT) appear to have similar binding affinities (Benz *et al.,* 1991; Nakano *et al.,* 1994). All these data are consistent with the notion that the direct biologic effects of androgenic steroids in osteoblasts are mediated, at least in part, via classic mechanisms associated with the androgen receptor.

The Androgen Receptor Signaling Pathway

The androgen receptor is a member of the class I (so-called classical or steroid) nuclear receptor superfamily, as are the estrogen receptor, the progesterone receptor, and the mineralocorticoid and glucocorticoid receptor (Mangelsdorf *et al.,* 1995). These steroid receptors are ligand-inducible transcription factors with a highly conserved modular design consisting of transactivation, DNA binding, and ligand-binding domains. Cellular localization of the androgen receptor in the absence of ligand is somewhat controversial. The unliganded androgen receptor has been found both predominantly in the cytoplasmic compartment (Georget *et al.,* 1997; Noble *et al.,* 1998; Tyag *et al.,* 2000) or, predominantly in the nucleus in a large complex of molecular chaperonins consisting of loosely bound heat-shock and other accessory proteins (Zhuang *et al.,* 1992). As lipids, androgenic steroids can diffuse freely through the plasma membrane to bind the androgen receptor. Once bound by ligand, the androgen receptor is activated and released from this protein complex, allowing the formation of homodimers (or potentially heterodimers) that bind to DNA at palindromic androgen response elements (AREs) in androgen responsive gene promoters (Fig. 2). ARE sequences are found characteristically as a motif represented by an inverted repeat separated by 3 bp (Whitfield *et al.,* 1999), but similar to glucocorticoid response elements (Denison *et al.,* 1989); androgen-specific regulation at nonconventional direct repeat AREs has also been shown (Verrijdt *et al.,* 1999). DNA binding of the activated androgen receptor organizes a cascade of events in the nucleus, leading to transcription and translation of a specific network of genes that is responsible for the cellular response to the steroid (Chang *et al.,* 1995). In the classic model of steroid action, the latent receptor is converted into a transcriptionally active form by simple ligand binding. This model is now considered an oversimplification, with the understanding that signaling pathways and additional proteins (e.g., coactivators or corepressors as described later) within the cell can influence steroid receptor transduction activity. For example, steroid receptor phosphorylation can result from signal transduction cascades initiated at the cell membrane, e.g., with cyclin-dependent kinases (Rogatsky *et al.,* 1999). It has been shown that steroid receptor phosphorylation can lead to alteration of the responsiveness of steroid receptors to cognate ligands or, in some cases, even result in ligand-independent activation (Fig. 2).

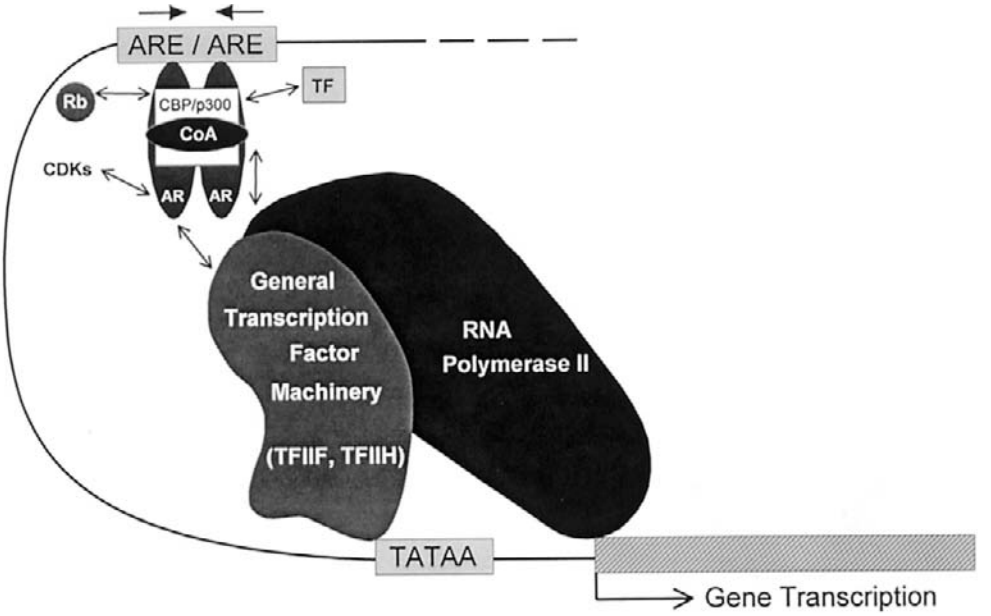

Figure 2 Model of androgen receptor regulation of gene expression. Binding of androgen promotes high-affinity dimerization, followed by DNA binding at the androgen response element (ARE) in an androgen-responsive gene promoter. Coactivators may remodel chromatin through histone acetylase activity to open chromatin structure (Spencer *et al.,* 1997) or act as a bridge to attract TFs that target binding of TATA-binding protein to the TATAA sequence (Beato and Sanchez-Pacheco, 1996). The retinoblastoma tumor suppressor product also activates androgen receptor transactivation (Yeh *et al.,* 1998). Phosphorylation of receptor may result from activation of phosphorylation cascades, such as by cyclin-dependent kinases (CDKs). Androgen receptor can also directly contact TFIIH (Lee *et al.,* 2000) and TFIIF (McEwan and Gustafsson, 1997) in the general transcription machinery. Such interactions between the androgen receptor and the general transcription machinery, leading to stable assembly, result in recruitment of RNA polymerase II and subsequent increased gene transcription.

Such a potential modification(s) of androgen receptor action in bone cells is poorly characterized. Whether the androgen receptor in osteoblasts undergoes posttranslational processing that might thus influence receptor signaling (stabilization, phosphorylation, etc.), as described for androgen receptor in other tissues (Kemppainen *et al.,* 1992; Ikonen *et al.,* 1994), the potential functional implications of such modifications (Blok *et al.,* 1996), are unknown. Ligand-independent activation of AR by cellular phosphorylation cascades has been described in other tissues (Culig *et al.,* 1994; Nazareth and Weigel, 1996), but has not been explored in bone. Androgen receptor activity may also be influenced by receptor modulators, such as the nuclear receptor coactivators or corepressors (Horwitz *et al.,* 1996; McKenna *et al.,* 1999). As outlined in Fig. 2, these coactivators/corepressors can influence the downstream signaling of nuclear receptors through multiple mechanisms, including histone acetylation/deacetylation to remodel chromatin. These activities reflect both the cellular context and the particular promoter. In addition, direct acetylation of the androgen receptor by p300/CBP has been documented (Fu *et al.,* 2000). Androgen receptor-specific coactivators have been identified (MacLean *et al.,* 1997), many of which interact with the ligand-binding domain of the receptor (Yeh and Chang, 1996). Expression and regulation of these modulators may thus influence the ability of steroid receptors to regulate gene expression in bone (Haussler *et al.,* 1997), but this has been underexplored with respect to androgen action. A preliminary report has suggested the presence of androgen-specific coactivators in osteoblastic cells (Wiren *et al.,* 1997).

Another means by which androgen receptor action in bone may be affected is via polymorphisms in the androgen receptor that affect function. Loss of function in the androgen receptor is well known to be associated with reduced bone mass, but less dramatic sequence variations may also be important. For instance, in the first exon of the androgen receptor is a CAG repeat of variable length. Shorter repeat lengths have been associated with increased transcriptional effects, and preliminary data suggest that men with longer repeat lengths have lower bone mineral density.

In addition to the classical androgen receptor present in bone cells, several other androgen-dependent signaling pathways have been described. Specific binding sites for weaker adrenal androgens (dehydroepiandrosterone, DHEA) have been described (Meikle *et al.,* 1992; Kasperk *et al.,* 1997a), raising the possibility that DHEA or similar androgenic compounds may also have direct effects in bone. In fact, Bodine *et al.,* (1995) showed that DHEA caused a rapid inhibition of c-*fos* expression in human osteoblastic cells that was more robust than that seen with the classical androgens (DHT, testosterone, androstenedione). Nevertheless, all androgenic compounds significantly increased Transforming growth factor-β (TGF-β) activity in osteoblastic cells. Androgens may also be specifically bound in osteoblastic cells by a

63-kDa cytosolic protein (Wrogemann *et al.,* 1991). There are reports of distinct androgen receptor polymorphisms identified in different races that may have a biological impact on androgen responses (Pettaway, 1999), but this has not been explored with respect to bone tissue. These different isoforms have the potential to interact in distinct fashions with other signaling molecules, such as c-Jun (Grierson *et al.,* 1999). Androgens may also regulate osteoblast activity via rapid nongenomic mechanisms through elevations in intracellular calcium levels (Benten *et al.,* 1999; Peterziel *et al.,* 1999) mediated by receptors at the bone cell surface (Lieberherr and Grosse, 1994), as has also been shown for estrogen (Lieberherr *et al.,* 1993). Finally, the androgen receptor may also interact with other transcription factors, such as NF-κB, CREB-binding protein, and different forms of AP-1, to generally repress transcription without DNA binding (Aarnisalo *et al.,* 1998, 1999). The role and biologic significance of these nonclassical signaling pathways in androgen-mediated responses in bone are still relatively uncharacterized.

Localization of Androgen Receptor Expression

Clues about the potential sequela of androgen receptor signaling might be derived from a better understanding of the cell types in which expression is documented. In the bone microenvironment, the localization of androgen receptor expression in osteoblasts has been described in intact human bone using immunocytochemical techniques (Abu *et al.,* 1997; Noble *et al.,* 1998). In developing bone from young adults, Abu *et al.* (1997) showed that androgen receptors were expressed predominantly in active osteoblasts at sites of bone formation (Fig. 3). Androgen receptors were also observed in osteocytes embedded in the bone matrix. Importantly, the pattern of both androgen receptor distribution and the level of expression was similar in males and in females. Furthermore, the androgen receptor was also observed within the bone marrow in mononuclear cells and endothelial cells of blood vessels. Expression of the androgen receptor has also been characterized in cultured osteoblastic cell populations isolated from bone biopsy specimens, determined at both the mRNA level and by binding analysis (Kasperk *et al.,* 1997a). Expression varied according to the skeletal site of origin and age of the donor of the cultured osteoblastic cells: AR expression was higher at cortical and intramembranous bone sites and lower in cancellous bone. This distribution pattern correlates with androgen responsiveness. Androgen receptor expression was highest in osteoblastic cultures generated from young adults and somewhat lower in samples from either prepubertal or senescent bone. Again, no differences were found between male and female samples, suggesting that differences in receptor number per se do not underlie development of a sexually dimorphic skeleton. Androgen and estrogen receptors have also been shown in bone marrow-derived stromal cells (Bellido *et al.,* 1995), which are responsive to sex steroids during the regulation of osteoclastogenesis. Because androgens are so important in bone development at the time of puberty, it is not surprising that androgen receptors are also present in epiphyseal chondrocytes (Carrascosa *et al.,* 1990; Abu *et al.,* 1997). Noble *et al.* (1998) described androgen receptor expression mainly in the narrow zone of proliferating chondrocytes in the growth plate, with reduced expression in hypertrophied cells. The expression of androgen receptors in such a wide variety of cell types known to be important for bone modeling during development, and remodeling in the adult, provides evidence for direct actions of androgens in bone and cartilage tissue. These results illustrate the complexity of androgen effects on bone.

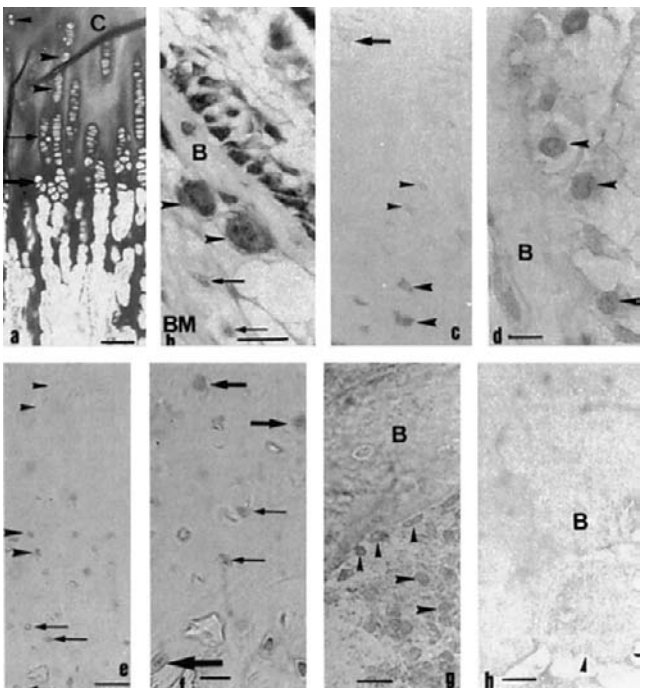

Figure 3 Localization of AR in normal tibial growth plate and adult osteophytic human bone. (a) Morphologically, sections of the growth plate consist of areas of endochondral ossification with undifferentiated (small arrowhead), proliferating (large arrowheads), mature (small arrow), and hypertrophic (large arrow) chondrocytes. Bar: 80 μm. An inset of an area of the primary spongiosa is shown in b. (b) Numerous osteoblasts (small arrowheads) and multinucleated osteoclasts (large arrowheads) on the bone surface. Mononuclear cells within the bone marrow are also present (arrows). Bar: 60 μm. (c) In the growth plate, AR is expressed predominantly by hypertrophic chondrocytes (large arrowheads). Minimal expression is observed in mature chondrocytes (small arrowheads). The receptors are rarely observed in the proliferating chondrocytes (arrow). (d) In the primary spongiosa, AR is predominantly and highly expressed by osteoblasts at modeling sites (arrowheads). Bar: 20 μm. (e) In osteophytes, AR is also observed at sites of endochondral ossification in undifferentiated (small arrowheads), proliferating (large arrowheads), mature (small arrows), and hypertrophic-like (large arrow) chondrocytes. Bar: 80 μm. (f) A higher magnification of e showing proliferating, mature, and hypertrophic-like chondrocytes (large arrows, small arrows, and very large arrows, respectively). Bar: 40 μm. (g) At sites of bone remodeling, the receptors are highly expressed in the osteoblasts (small arrowheads) and also in mononuclear cells in the bone marrow (large arrowheads). Bar: 40 μm. (h) AR is not detected in osteoclasts (small arrowheads). Bar: 40 μm. B, bone: C, cartilage; BM, bone marrow Abu *et al.* (1997).

Osteoclasts may be a target for sex steroid regulation, as estrogen receptors have been reported to be present in osteoclastic cells (Oursler *et al.,* 1991), but a direct effect of androgens on osteoclast function has not been demonstrated. Mizuno *et al.* (1994) described the presence of androgen receptor immunoreactivity in mouse osteoclast-like multinuclear cells, but expression was not detected in *bona fide* osteoclasts in human bone slices (Abu *et al.,* 1997). Because the major effects of androgens on skeletal remodeling and maintenance of bone mineral density seem to be mediated by cells of the osteoblast lineage (Weinstein *et al.,* 1997), the biologic relevance of potential androgen receptor expression osteoclasts is unclear.

Regulation of Androgen Receptor Expression

The regulation of androgen receptor expression in osteoblasts is incompletely characterized. Homologous regulation of the androgen receptor by androgen has been described that is tissue specific; upregulation by androgen exposure is seen in a variety of osteoblastic cells (Zhuang *et al.,* 1992; Takeuchi *et al.,* 1994; Wiren *et al.,* 1997, 1999), whereas in prostatic tissue, downregulation of the androgen receptor after androgen exposure is observed. The androgen-mediated upregulation of the androgen receptor observed in osteoblasts, at least in part, occurs through changes in androgen receptor gene transcription (Fig. 4). As in other tissues, increased androgen receptor protein stability may also play a part. No effect, or even inhibition, of androgen receptor mRNA by androgen exposure in other osteoblastic models has also been described (Hofbauer *et al.,* 1997; Kasperk *et al.,* 1997a). The mechanism(s) that underlies tissue specificity in autologous androgen receptor regulation and the possible biological significance of distinct autologous regulation of androgen receptor are not yet understood. It is possible that receptor upregulation by androgen in bone may result in an enhancement of androgen responsiveness at times when androgen levels are rising or elevated. In addition, androgen receptor expression in

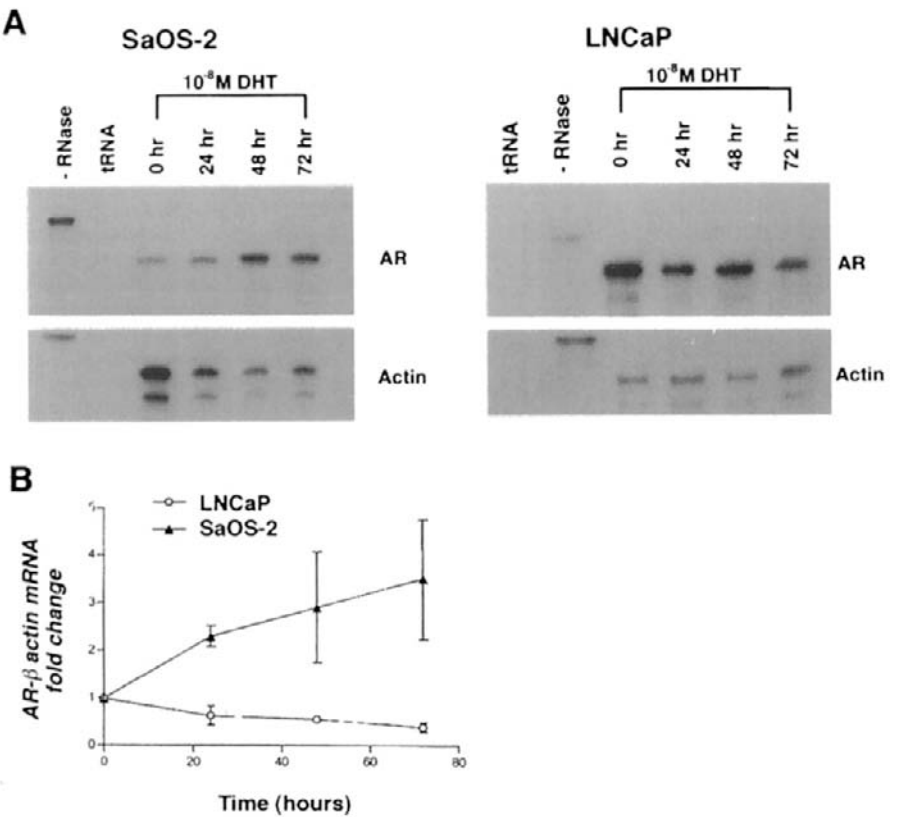

Figure 4 Dichotomous regulation of AR mRNA levels in osteoblast-like and prostatic carcinoma cell lines after exposure to androgen. (A) Time course of changes in AR mRNA abundance after DHT exposure in human SaOS-2 osteoblastic cells and human LNCaP prostatic carcinoma cells. To determine the effect of androgen exposure on hAR mRNA abundance, confluent cultures of either osteoblast-like cells (SaOS-2) or prostatic carcinoma cells (LNCaP) were treated with 10^{-8} *M* DHT for 0, 24, 48, or 72 hr. Total RNA was then isolated and subjected to RNase protection analysis with 50 μg total cellular RNA from SaOS-2 osteoblastic cells and 10 μg total RNA from LNCaP cultures. (B) Densitometric analysis of AR mRNA steady-state levels. The AR mRNA to β-actin ratio is expressed as the mean $\pm$SE compared to the control value from three to five independent assessments (Wiren *et al.,* 1997).

osteoblasts may be upregulated by exposure to glucocorticoids, estrogen, or 1,25-dihydroxyvitamin D_3 (Kasperk *et al.,* 1997a). Except for the immunocytochemical detection of androgen receptor expression in bone slices described earlier, regulation during osteoblast differentiation has not been well characterized. A preliminary report describes changes in mRNA expression of the three sex steroid receptors: androgen receptor and estrogen receptor α and β during osteoblast *in vitro* differentiation (Wiren *et al.,* 2000). Whether any other hormones, growth factors, or agents influence androgen receptor expression in bone is unknown.

Effects of Androgens on Proliferation and Apoptosis of Osteoblastic Cells

Androgens have direct effects on osteoblast proliferation and expression *in vitro.* The effect of androgen exposure on osteoblast proliferation remains controversial; both stimulation and inhibition of osteoblast proliferation have been reported as summarized in Table I. Benz *et al.* (1991) have shown that prolonged androgen exposure in the presence of serum inhibited proliferation (cell counts) by 15–25% in a transformed human osteoblastic line (TE-85). Testosterone and DHT were nearly equally effective regulators. Hofbauer *et al.* (1998) examined the effect of DHT exposure on proliferation in hFOB/AR-6, an immortalized human osteoblastic cell line stably transfected with an androgen receptor expression construct (with ~4,000 receptors/cell). In this line, DHT treatment inhibited cell proliferation by 20–35%. Finally, Kasperk *et al.* (1997b) reported that prolonged DHT pretreatment inhibited normal human osteoblastic cell proliferation (cell counts) in cultures pretreated with DHT.

In contrast, the same group (Kasperk *et al.,* 1989, 1990) also demonstrated that a variety of androgens in serum-free medium increase DNA synthesis ([^{3}H]thymidine incorporation) up to nearly 300% in osteoblast-like cells in primary culture (murine, passaged human). Again, testosterone and nonaromatizable androgens (DHT and fluoxymesterone) were nearly equally effective regulators. Consistent with increased proliferation, testosterone and DHT have also been reported to cause an increase in creatine kinase activity and [^{3}H]thymidine incorporation into DNA in rat diaphyseal bone (Somjen *et al.,* 1989). Variable results have also been reported with the adrenal androgen DHEA on osteoblast proliferation: DHEA was shown to stimulate osteoblast proliferation, but with less potency than DHT (Kasperk *et al.,* 1997b); however, no effect of DHEA alone has been described (Scheven and Milne, 1998). The differences observed with androgen-mediated changes in osteoblastic cell proliferation may be due to the variety of model systems employed (transformed osteoblastic cells vs passaged normal cells) and/or may reflect differences in the culture conditions (e.g., state of differentiation, receptor number, times of treatment, phenol red containing vs phenol red free, or serum containing, charcoal stripped vs serum free). These differences suggest

Table I Complex Effects of Androgens on Proliferation of Osteoblastic Cells

Cells[a]	Steroid[b]	Conditions[c]	% change	Reference
h TE-85 (osteosarcoma)	DHT (10 n*M*), 72 hr	2% FBS	↓ 25	Benz *et al.* (1991)
	T (10 n*M*), 72 hr	2% FBS	↓ 20	Benz *et al.* (1991)
h FOB/AR6 (immortalized)	DHT (10 n*M*), 6 days	1% csFBS	↓ 30	Hofbauer *et al.* (1998)
m MC3T3–E1 (immortalized)	DHT (10 n*M*), 24 hr	1.5% csFBS	↑ ~ 32	Nakano *et al.* (1994)
	T (10 n*M*), 24 hr	1.5% csFBS	↑ ~ 28	Nakano *et al.* (1994)
m MC3T3–E1 (immortalized)	DHT (10 n*M*), 72 hr	SF	↑ ~ 15	Masuyama *et al.* (1992)
r normal calvarial OBs	DHT (10 n*M*), 5 days	First psg	↑ ~ 48	Gray *et al.* (1992)
r normal long bone OBs	T (50 n*M*), 5 days	First psg	↑ ~ 60	Gray *et al.* (1992)
r normal explanted OBs	DHT (10 n*M*), 8 days	First psg	↑ ~105	Gray *et al.* (1992)
h normal OBs	DHT (1 n*M*), 48 hr; (24-hr pretreatment 10 n*M* DHT)	First/Second psg, SF	↓ ~ 40	Kasperk *et al.* (1997b)
h mandibular OBs	DHT (1 n*M*), 3 days	First/Second psg, SF	↑ ~ 46	Kasperk *et al.* (1997b)
h iliac crest OBs	DHT (1 n*M*), 3 days	First/Second psg, SF	No effect	Kasperk *et al.* (1997b)
h cortical OBs	DHT (10 n*M*) 48 hr	First/Second psg, 1% csFBS	↑ ~ 230	Kasperk *et al.* (1997b)
h cortical OBs	DHEA (10 n*M*) 48 hr	First/Second psg, 1% csFBS	↑ ~ 170	Kasperk *et al.* (1997b)
r diaphysis	DHT (50 μg), 24 hr	20 days rats; *in vivo*	↑ 98	Somjen *et al.* (1989)
r epiphysis	DHT (50 μg), 24 hr	20-days rats; *in vivo*	↑ 83	Somjen *et al.* (1989)

[a] h, human; m, mouse; r, rat; OBs, osteoblasts.
[b] T, testosterone.
[c] cs, charcoal stripped; SF, serum free; psg, passage.

an underlying biologic complexity for the androgen regulation of osteoblast proliferation.

As a component of the control of osteoblast survival, it is also important to consider programmed cell death, or apoptosis (Wyllie *et al.,* 1980). A variety of skeletal cell types have been shown to undergo apoptosis (Hughes and Boyce, 1997; Manolagas and Weinstein, 1999). In particular, as the osteoblast population differentiates *in vitro,* the mature bone cell phenotype undergoes apoptosis (Lynch *et al.,* 1998). Modulation of bone cell apoptosis by steroid hormones has been shown: glucocorticoids enhance apoptosis of osteoclasts (Dempster *et al.,* 1997) and osteoblasts/osteocytes (Manolagas, 1998; Weinstein *et al.,* 1999), which estrogen treatment prevents (Gohel *et al.,* 1999). Furthermore, evidence shows that the osteocytic population is particularly sensitive to the effects of estrogen withdrawal, which induces apoptosis (Tomkinson *et al.,* 1997; 1998). Androgen exposure has been shown to influence apoptosis in other tissues (Lim *et al.,* 1997; Abreu-Martin *et al.,* 1999), but the effects of either androgen exposure or androgen withdrawal in bone have not been described.

In an interesting series of experiments, Manolagas (2000) showed that the effects of androgens (and estrogens) on bone cell proliferation are dependent on classical transcriptional mechanisms, whereas the antiapoptotic effects in osteoblastic cells, and the proapoptotic effects in osteoclastic cells, may be related to a nonclassical effect mediated by the activation of extracellular signal-related kinases (ERKs). The ERK-mediated actions were mechanistically dissociable from transcriptional effects, but were nevertheless receptor dependent. Of potential importance, the ERK-mediated actions were transmitted by either androgens or estrogens via both androgen and estrogen receptors. Obviously, this added complexity offers some intriguing explanations for the observation that androgens and estrogens have similar effects on remodeling in both genders.

Effects of Androgens on Differentiation of Osteoblastic Cells

Osteoblast differentiation can be characterized by changes in alkaline phosphatase activity and/or alterations in the expression of important extracellular matrix proteins, such as type I collagen, osteocalcin, and osteonectin. Enhanced osteoblast differentiation, as measured by increased matrix production, has been shown to result from androgen exposure. Androgen treatment in both normal osteoblasts and transformed clonal human osteoblastic cells (TE-89) appears to increase the proportion of cells expressing alkaline phosphatase activity, thus representing a shift toward a more differentiated phenotype (Fig. 5) (Kasperk *et al.,* 1989). Kasperk and colleagues subsequently reported dose-dependent increases in alkaline phosphatase activity in both high and low alkaline phosphatase subclones of SaOS2 cells (Kasperk *et al.,* 1996) and human osteoblastic cells (Kasperk *et al.,* 1997b). However, there are also reports, in a variety of model systems, of androgens either inhibiting (Hofbauer *et al.,* 1998) or having no effect on alkaline phosphatase activity (Gray *et al.,* 1992; Takeuchi *et al.,* 1994), which may reflect both the complexity and the dynamics of osteoblastic differentiation. There are also reports of androgen-mediated increases in type I α-1 collagen protein and mRNA levels (Benz *et al.,* 1991; Gray *et al.,* 1992; Kasperk *et al.,* 1996; Davey *et al.,* 2000) and increased osteocalcin mRNA or protein secretion (Kasperk *et al.,* 1997b; Davey *et al.,* 2000). Consistent with increased collagen production, androgen treatment has also been shown to stimulate mineral accumulation in a time-and dose-dependent manner (Kapur and Reddi,

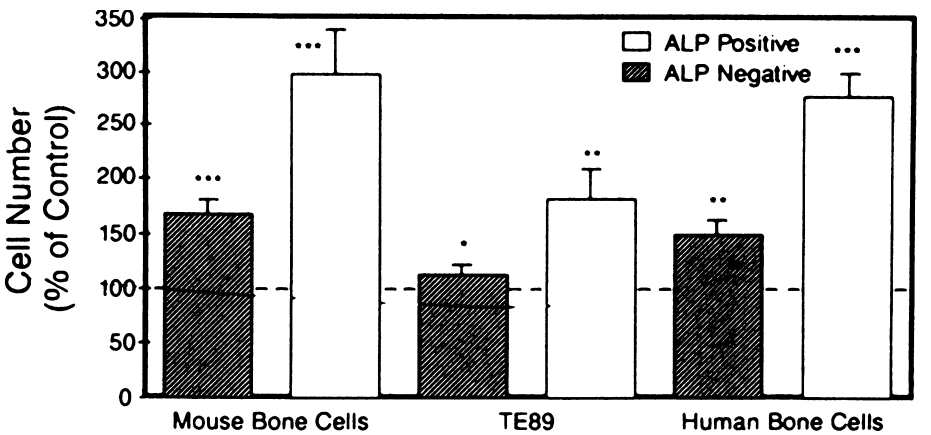

Figure 5 Effect of DHT on ALP-positive (ALP^+) and ALP-negative (ALP^-) cells in a normal mouse, a normal human osteoblast line, and a human osteosarcoma (TE89) monolayer cell culture. ($^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.1$). Control values in cells per mm^2 for mouse bone cells, TE89 cells, and human bone cells were 90 ± 5, 75 ± 7, and 83 ± 14, respectively (Kasperk *et al.,* 1989).

1989; Takeuchi *et al.,* 1994; Kasperk *et al.,* 1997b). These results suggest that androgens can enhance osteoblast differentiation under certain conditions and may thus play an important role in the regulation of bone matrix production and/or organization. This effect is also consistent with an overall stimulation of bone formation, as is observed clinically after androgen treatment.

Interaction with Other Factors to Modulate Bone Formation and Resorption

The effects of androgens on osteoblast activity must certainly also be considered in the context of the very complex endocrine, paracrine, and autocrine milieu in the bone microenvironment. Systemic and/or local factors can act in concert, or can antagonize, to influence bone cell function. This has been well described with regard to modulation of the effects of estrogen on bone (see e.g., Horowitz, 1993; Kawaguchi *et al.,* 1995; Kassem *et al.,* 1996). Androgens have also been shown to regulate well-known modulators of osteoblast proliferation or function. The most extensively characterized growth factor influenced by androgen exposure is TGF-β. TGF-β is stored in bone (the largest reservoir for TGF-β) in a latent form and has been show under certain conditions to be either a mitogen for osteoblasts (Centrella *et al.,* 1994; Harris *et al.,* 1994) or to inhibit proliferation (Noda and Rodan, 1986; Pfeilschifter *et al.,* 1987; Datta *et al.,* 1989). Androgen treatment has been shown to increase TGF-β activity in human osteoblast primary cultures (Fig. 6). The expression of some TGF-β mRNA transcripts (apparently TGF-β2) was increased, but no effect on TGF-β1 mRNA abundance was observed (Kasperk *et al.,* 1990; Bodine *et al.,* 1995). At the protein level, specific immunoprecipitation analysis reveals DHT-mediated increases in TGF-β activity to be predominantly TGF-β2 (Kasperk *et al.,* 1990; Bodine *et al.,* 1995). DHT has also been shown to inhibit both TGF-β gene expression and TGF-β-induced early gene expression that correlates with growth inhibition in this cell line (Hofbauer *et al.,* 1998). The TGF-β-induced early gene has been shown to be a transcription factor that may mediate some TGF-β effects (Subramaniam *et al.,* 1995). However, TGF-β1 mRNA levels are increased by androgen treatment in human clonal osteoblastic cells (TE-89), under conditions where osteoblast proliferation is slowed (Benz *et al.,* 1991). These results are consistent with the notion that TGF-β may mediate the complex androgen effects on osteoblast proliferation. Furthermore, the specific TGF-β isoform may determine osteoblast responses. It is interesting to note that at the level of bone, orchiectomy drastically reduces bone content of TGF-β levels, and testosterone replacement prevents this change, (Gill *et al.,* 1998) (Fig. 7). These data support the findings that androgens influence cellular expression of TGF-β and suggest that the bone loss associated with castration could be related to a reduction in growth factor abundance, induced by androgen deficiency.

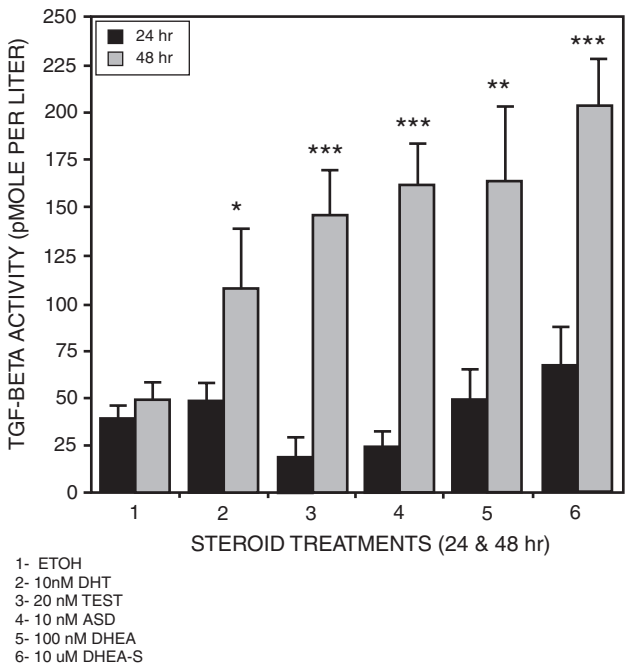

Figure 6 Induction of total TGF-β activity by gonadal and adrenal androgens in human osteoblast (hOB) cell-conditioned media. Cells were treated for 24 or 48 hr with vehicle or steroids. After treatment, conditioned media were saved and processed for the TGF-β bioassay. Results are presented as the mean ±SEM of three to four experiments. $^{*}p < 0.05$; $^{**}p < 0.02$, $^{***}p < 0.0005$ (Behren's Fisher *t* test) compared to the 48-hr ethanol control. ETOH, ethanol; TEST, testosterone; DHT, dihydrotestosterone; ASD, androstenedione; DHEA, dehydroepiandrosterone; DHEA-S, DHEA-sulfate (Bodine *et al.,* 1995).

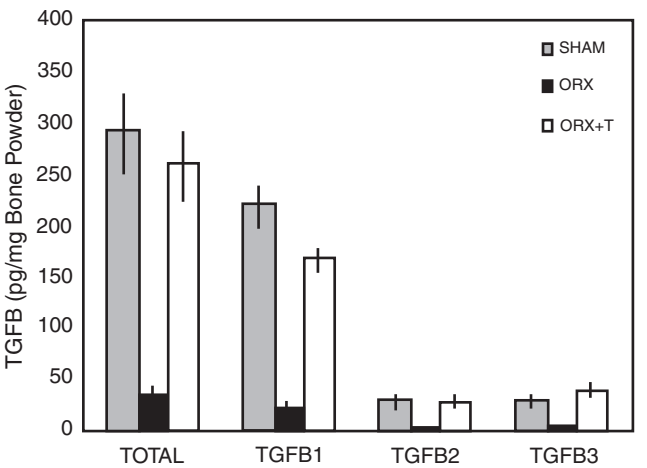

Figure 7 Effects of orchiectomy and T replacement on isoforms of TGF-β in long bones. Results are mean ±SE of four to six animals. Rats underwent sham operation or orchiectomy and 1 week later were given either placebo or 100 mg of testosterone in 60–day slow-release pellets. Specimens were obtained 6 weeks after surgery. All forms of TGF-β were reduced by orchiectomy (at least $p < 0.0002$), while there was no change in those with testosterone replacement (Gill *et al.,* 1998).

Other growth factor systems may also be influenced by androgens. Conditioned media from DHT-treated normal osteoblast cultures are mitogenic, and DHT pretreatment increases the mitogenic response of osteoblastic cells to fibroblast growth factor and to insulin-like growth factor II (IGF-II) (Kasperk *et al.,* 1990). In part, this may be due to slight increases in IGF-II binding in DHT-treated cells (Kasperk *et al.,* 1990), as IGF-I and IGF-II levels in osteoblast-conditioned media are not affected by androgen (Kasperk *et al.,* 1990; Canalis *et al.,* 1991). Although most studies have not found regulation of IGF-I or IGF-II abundance by androgen exposure (Kasperk *et al.,* 1990; Canalis *et al.,* 1991; Nakano *et al.,* 1994), there is a report that IGF-I mRNA levels are significantly upregulated by DHT (Gori *et al.,* 1999). Androgens may also modulate expression of components of the AP-1 transcription factor, as has been shown with inhibition of c-*fos* expression in proliferating normal osteoblast cultures (Bodine *et al.,* 1995). Thus, androgens may accelerate osteoblast differentiation via a mechanism whereby growth factors or other mediators of differentiation are regulated by androgen exposure.

Finally, androgens may modulate responses to other important osteotropic hormones/regulators. Testosterone and DHT specifically inhibit the cAMP response elicited by parathyroid hormone or parathyroid hormone-related protein in the human clonal osteoblast-like cell line SaOS-2, whereas the inactive or weakly active androgen 17α-epitestosterone had no effect (Fig. 8). This inhibition may be mediated via an effect on the parathyroid hormone receptor-G_s-adenylyl cyclase (Fukayama and Tashjian, 1989; Gray *et al.,* 1991; Vermeulen, 1991). The production of prostaglandin E_2 (PGE_2), another important regulator of bone metabolism, is also affected by androgens. Pilbeam and Raisz (1990) showed that androgens (both DHT and testosterone) were potent inhibitors of both parathyroid hormone (Fig. 9) and interleukin 1-stimulated prostaglandin E_2 production in cultured neonatal mouse calvaria. The effects of androgens on parathyroid hormone action and PGE_2 production suggest that androgens could act to modulate (reduce) bone turnover in response to these agents.

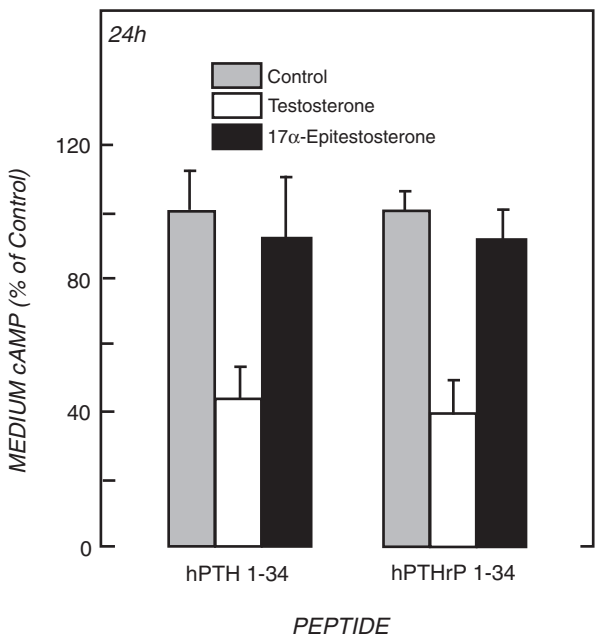

Figure 8 Actions of testosterone and 17α-epitestosterone on cAMP accumulation stimulated by $hPTH^{1-34}$ (5.0 n*M*) or $hPTHrP^{1-34}$ (5.0 n*M*) in human SaOS-2 cells. Cells were pretreated without or with the steroid hormones (10^{-9} *M*) for 24 hr. Each bar gives the mean value, and brackets give the SE for four to five dishes (Fukayama and Tashjian, 1989).

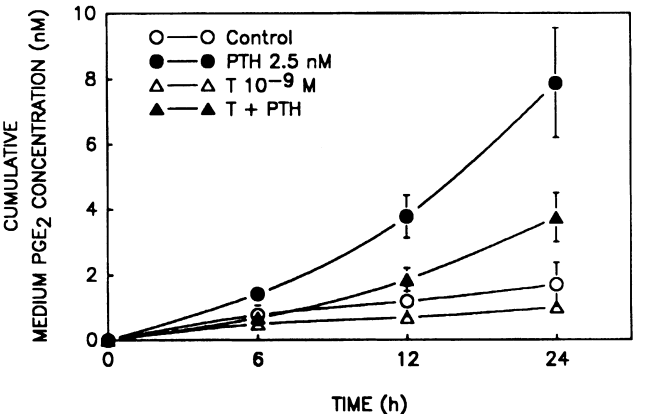

Figure 9 Effect of T on PTH-stimulated PGE_2 production in cultured neonatal calvariae as a function of time. Each bone was precultured for 24 hr in 1 ml medium with or without 10^{-9} *M*T and then transferred to similar medium with 2.5 n*M* PTH. Media were sampled (0.1 ml) at the indicated times. Data were corrected for the media removed. Each point represents the mean ±SEM for six bones in one experiment. The effect of T on PTH-stimulated PGE_2 production was significant ($p < 0.05$) at 6, 12, and 24 hr (Pilbeam and Raisz, 1990).

Finally, both androgen (Hofbauer and Khosla, 1999) and estrogen (Passeri *et al.,* 1993; Kassem *et al.,* 1996) inhibit production of interleukin-6 by osteoblastic cells (but see Rifas *et al.,* 1995). In stromal cells of the bone marrow, androgens have been shown to have potent inhibitory effects on the production of interleukin-6 (Table II) and the subsequent stimulation of osteoclastogenesis by marrow osteoclast precursors (Bellido *et al.,* 1995). Interestingly, adrenal androgens (androstenediol, androstenedione, dehydroepiandrosterone) have similar inhibitory activities on interleukin-6 gene expression and protein production by stroma (Bellido *et al.,* 1995). The loss of inhibition of interleukin-6 production by androgen may contribute to the marked increase in bone remodeling and resorption that follows orchidectomy. Moreover, androgens inhibit the expression of the genes encoding the two subunits of the IL-6 receptor (gp80 and gp130) in the murine bone marrow, another mechanism which may blunt the effects of this osteoclastogenic cytokine in intact animals (Lin *et al.,* 1997). In these aspects, the effects of androgens seem to be very similar to those of estrogen, which may also inhibit osteoclastogenesis via mechanisms that involve interleukin-6 inhibition.

Table II Effect of Androgens on Cytokine-Induced IL-6 Production by Murine Bone Marrow Stromal Cells[a]

Treatment	IL-6
IL-1 + TNF	4.27 ± 1.43
IL-1 + TNF+ testosterone (10^{-12} *M*)	3.87 ± 0.33
IL-1 + TNF+ testosterone (10^{-11} *M*)	2.90 ± 0.42
IL-1 + TNF+ testosterone (10^{-10} *M*)	2.09 ± 0.33
IL-1 + TNF+ testosterone (10^{-9} *M*)	1.12 ± 0.49
IL-1 + TNF+ testosterone (10^{-8} *M*)	1.03 ± 0.04
IL-1 + TNF+ testosterone (10^{-7} *M*)	1.01 ± 0.48
IL-1 + TNF+ dihydrotestosterone (10^{-12} *M*)	4.05 ± 0.19
IL-1 + TNF+ dihydrotestosterone (10^{-11} *M*)	2.97 ± 0.48
IL-1 + TNF+ dihydrotestosterone (10^{-10} *M*)	2.31 ± 0.86
IL-1 + TNF+ dihydrotestosterone (10^{-9} *M*)	1.72 ± 0.43
IL-1 + TNF+ dihydrotestosterone (10^{-8} *M*)	0.65 ± 0.21
IL-1 + TNF+ dihydrotestosterone (10^{-7} *M*)	1.41 ± 0.82

[a] Murine stromal cells (+/+ LDA11 cells) were cultured for 20 hr in the absence or the presence of different concentrations of either testosterone or dihydrotestosterone. Then IL-1 (500 U/ml) and TNF (500 U/ml) were added and cells were maintained for another 24 hr in culture. Values indicate means (±SD) of triplicate cultures from one experiment. Data were analyzed by one-way ANOVA. $^{*}p < 0.05$, versus cells not treated with steroids as determined by Dunnet's test. Neither testosterone nor dihydrotestosterone had an affect on cell number (Bellido *et al.,* 1995).

Metabolism of Androgens in Bone: Aromatase and 5α-Reductase Activities

There is abundant evidence in a variety of tissues that the eventual cellular effects of androgens may be the result not only of direct action of androgen, but also of the effects of sex steroid metabolites formed as the result of local enzyme activities. The most important of these androgen metabolites are estradiol (formed by the aromatization of testosterone) and 5α-DHT (the result of 5α reduction of testosterone). Evidence shows that both aromatase and 5α-reductase activities are present in bone tissue, at least to some measurable extent, but the biologic relevance of androgen metabolism is still controversial.

5α-reductase activity was first described in crushed rat mandibular bone by Vittek *et al.* (1974), and Schweikert *et al.* (1980) reported similar findings in crushed human spongiosa. Two different 5α-reductase genes encode type 1 and type 2 isozymes in many mammalian species (Russell and Wilson, 1994), but the isozyme present in human bone has not been characterized. In osteoblast-like cultures derived from orthopedic surgical waste, androstenedione (the major circulating androgen in women) can be reversibly converted to testosterone via 17β-hydroxysteroid dehydrogenase activity and to 5α-androstanedione via 5α-reductase activity, whereas testosterone is converted to DHT via 5α-reductase activity (Bruch *et al.,* 1992). The principal metabolite of androstenedione is α-androstanedione in the 5α-reductase pathway and testosterone in the 17β-hydroxysteroid dehydrogenase pathway. Essentially the same results were reported in experiments with human epiphyseal cartilage and chondrocytes (Audi *et al.,* 1984). In general, K_m values for bone 5α-reductase activity are similar to those in other androgen responsive tissues (Schweikert *et al.,* 1980; Nakano *et al.,* 1994).

The cellular populations in these studies were mixed and hence the specific cell type responsible for the activity is unknown. Interestingly, Turner *et al.* (1990a) found that periosteal cells do not have detectable 5α-reductase activity, raising the possibilities that the enzyme may be functional in only selected skeletal compartments and that testosterone may be the active metabolite at this clinically important site.

From a clinical perspective, the general importance of this enzymatic pathway is suggested by the presence of skeletal abnormalities in patients with 5α-reductase type 2 deficiency (Fisher *et al.,* 1978). However, Bruch *et al.* (1992) found no significant correlation between enzyme activities and bone volume. In mutant null mice lacking 5α-reductase type 1 (mice express very little type 2 isozyme), the effect on the skeleton cannot be analyzed due to midgestational fetal death (Mahendroo *et al.,* 1997). Treatment of male animals with finasteride (an inhibitor of type 2 5α-reductase activity) does not recapitulate the effects of castration (Rosen *et al.,* 1995), indicating that the reduction of testosterone to DHT by the type 2 isozyme is not a major determinant in the effects of gonadal hormones on bone. Whereas available data point to a possible role for 5α-reduction in the mechanism of action for androgen in bone, the clinical impact of this enzyme, which isozyme may be involved, whether it is present uniformly in all cells participating (involved) in bone modeling/remodeling, or whether local activity is important at all remains uncertain.

The biosynthesis of estrogens from androgen precursors is catalyzed by the microsomal enzyme aromatase cytochrome P450 ($P450_{arom}$, the product of the CYP19 gene). It is an enzyme known to be both expressed and regulated in a very pronounced tissue-specific manner (Simpson *et al.,* 1994). Aromatase activity has been reported in bone from mixed cell populations derived from both sexes (Nawata *et al.,* 1995; Schweikert *et al.,* 1995; Sasano *et al.,* 1997) and from osteoblastic cell lines (Purohit *et al.,* 1992; Tanaka *et al.,* 1993; Nakano *et al.,* 1994). Aromatase expression in intact bone has also been documented by *in situ* hybridization and immunohistochemical analysis (Sasano *et al.,* 1997) (Table III). Aromatase mRNA is expressed predominantly in lining cells, chondrocytes, and some adipocytes, but there is no detectable expression in osteoclasts. At least in vertebral bone, the aromatase fibroblast (1b type) promoter is utilized predominantly (Sasano *et al.,* 1997). The enzyme kinetics in bone cells seem to be similar to those in other tissues, although the V_{max} may be increased by glucocorticoids (Tanaka *et al.,* 1993).

Aromatase can produce the potent estrogen estradiol, but can also result in the weaker estrogen estrone from its adrenal precursors androstenedione and dehydroepiandrosterone (Nawata *et al.,* 1995). In addition to aromatase itself,

Table III Steroid Metabolism in Human Bone[a]

		Dihydrotestosterone			Androstenedione		
Crystallization	Solvent	^{3}H (cpm/mg)	^{14}C (cpm/mg)	$^{3}H/^{14}C$	^{3}H (cpm/mg)	^{14}C (cpm/mg)	$^{3}H/^{14}C$
1	Acetone	409	26	16	811	14	56
2	Benzene-heptane	408	25	16	811	14	57
3	Ethylacetate-cyclohexane	411	26	16	786	13	60
4	Ethylether-hexane	411	28	15	775	13	59
5	Methanol	393	26	15	791	16	48
Mother liquor		405	24	17	848	16	53

[a] From Schweikert *et al.,* (1980). Confirmation by recrystallization of the identities of [^{3}H]dihydrotestosterone and [^{3}H]androstenedione recovered following the incubation of normal and osteoporotic human bone (ground spongiosa) with [1,2,76,7^{-3}H]testosterone. Pooled samples from 18 separate incubations of bone from various anatomical origins were chromatographed by preparative thin-layer chromatography. Material tentatively identified as [^{3}H]dihydrotestosterone and [^{3}H]androstenedione, respectively, was mixed with 200 mg of the appropriate carrier steroid and with ^{14}C-labeled steroid for recrystallization as described in the text.

osteoblasts contain enzymes that are able to interconvert estradiol and estrone (estradiol-17β hydroxysteroid dehydrogenase) and to hydrolyze estrone sulfate to estrone (estrone sulfatase) (Purohit *et al.,* 1992). Nawata *et al.* (1995) have reported that dexamethasone and 1α,25$(OH)_2D_3$ synergistically enhance aromatase activity and aromatase mRNA ($P450_{arom}$) expression in human osteoblast-like cells. There is no other information concerning the regulation of aromatase in bone, although this is an area of obvious interest given the potential importance of the enzyme and its regulation by a variety of mechanisms (including androgens and estrogens) in other tissues (Abdelgadir *et al.,* 1994; Simpson *et al.,* 1994).

The clinical impact of aromatase activity has been suggested by the reports of skeletal changes in women (Conte *et al.,* 1994) and men (Morishima *et al.,* 1995; Carani *et al.,* 1997) with aromatase deficiencies. The presentation of men with aromatase deficiency is very similar to that of a man with estrogen receptor α deficiency, namely an obvious delay in bone age, lack of epiphyseal closure, and tall stature (Smith *et al.,* 1994), suggesting that aromatase (and thus estrogen action) has a substantial role to play during skeletal development in the male. In one case, estrogen therapy of a man with aromatase deficiency was associated with an increase in bone mass (Bilezikian *et al.,* 1998). Inhibition of aromatization in young growing orchidectomized males, with the nonsteroidal inhibitor vorozole, results in decreases in bone mineral density and changes in skeletal modeling, as does castration, which reduces in both androgens and estrogens. However, vorozole therapy induces less dramatic effects on bone turnover (Vanderschueren *et al.,* 1997). Inhibition of aromatization in older orchidectomized males resembles castration with similar increases in bone resorption and bone loss, suggesting that aromatase activity may also play a role in skeletal maintenance in males (Vanderschueren *et al.,* 1996). Aromatase inhibition results in cancellous and cortical bone loss on older animals (Vanderschueren *et al.,* 2000). Interestingly, the development of aromatase knockout mice revealed the skeletal phenotype to be remarkably mild (Oz *et al.,* 2000), although the data suggested that there were gender differences in effects on bone remodeling (increased remodeling in the females, reduced in the males). These studies herald the importance of aromatase activity (and estrogen) in the mediation of androgen action in bone. The finding of these enzymes in bone clearly raises the difficult issue of the origin of androgenic effects. Do they arise from direct androgen effects (as is suggested by the actions of nonaromatizable androgens), to some extent from the local production of estrogenic intermediates, or both?

Nevertheless, there is substantial evidence that some, if in fact not most, of the biologic actions of androgens in the skeleton are direct. As noted previously, both *in vivo* and *in vitro* systems reveal the effects of the nonaromatizable androgen DHT to be essentially the same as those of testosterone (*vida infra*). In addition, blockade of the androgen receptor with the receptor antagonist flutamide results in osteopenia as a result of reduced bone formation (Goulding and Gold, 1993). These reports clearly indicate that androgens, independent of estrogenic metabolites, have primary effects on osteoblast function. However, the clinical reports of subjects with aromatase deficiency also highlight the relevance of androgen metabolism to biopotent estrogens in bone. Elucidation of the regulation steroid metabolism, and the potential mechanisms by which androgenic and estrogenic effects are coordinated, may have physiological, pathophysiological, and therapeutic implications.

Direct Effects of Androgens on Other Cell Types in Bone *in Vitro*

Similar to the effects noted in osteoblastic populations, androgens regulate chondrocyte proliferation and expression. Androgen exposure promotes chondrogenesis,

as shown with increased creatine kinase and DNA synthesis after androgen exposure in cultured epiphyseal chondrocytes (Carrascosa *et al.,* 1990; Somjen *et al.,* 1991). Increased [^{35}S] sulfate incorporation into newly synthesized (Corvol *et al.,* 1987) and increased alkaline phosphatase activity (Schwartz *et al.,* 1994) are androgen mediated. Regulation of these effects is obviously complex, as they were dependent on the age of the animals and the site from which chondrocytes were derived. Thus, in addition to effects on osteoblasts, multiple cell types in the skeletal milieu are regulated by androgen exposure.

Gender Specificity in Actions of Sex Steroids

Although controversial, there may be gender-specific responses in osteoblastic cells to sex steroids. Somjen and colleagues have shown that the increase in creatine kinase that occurs from bone cells *in vivo* and *in vitro* is gender specific (i.e., male animals, or cells derived from male bones, respond only to androgens, whereas females or female-derived cells respond only to estrogens) (Weisman *et al.,* 1993; Somjen *et al.,* 1994). This gender specificity appears to depend on the previous history of exposure of animals to androgens (or estrogens). How much gender-specific effects might affect bone metabolism in the intact animal is completely unknown. In addition, in most mammals, there is a marked gender difference in morphology that results in a sexually dimorphic skeleton. The mechanisms responsible for these differences are obviously complex and presumably involve both androgenic and estrogenic actions on the skeleton. It is becoming increasingly clear that estrogens are particularly important for the regulation of epiphyseal function and act to reduce the rate of longitudinal growth via influences on chondrocyte proliferation and function, as well as on the timing of epiphyseal closure (Turner *et al.,* 1994). Androgens, however, appear to have opposite effects on the skeleton. Androgens tend to promote long bone growth, chondrocyte maturation, and metaphyseal ossification. Furthermore, the most dramatic effect of androgens is on bone size, particularly cortical thickness (Turner *et al.,* 1994; Kasra and Grynpas, 1995), as androgens appear to have gender-specific effects on periosteal bone formation (Turner *et al.,* 1990b). This difference of course has biomechanical implications, with thicker bones being stronger bones. At the same time, the response of the adult skeleton to the same intervention results is distinct responses in males and females. For example, in a model of disuse osteopenia, in mice antiorthostatic suspension results in significant reduction in bone formation rate at the endocortical perimeter in males. In females, however, a decrease in bone formation rate occurred along the periosteal perimeter (Bateman *et al.,* 1997). Gender-specific responses *in vivo* and *in vitro,* and the mechanism(s) that underlies such responses in bone cells, may thus have significant implications in treatment options for metabolic bone disease.

Summary

Thus, the effects of androgens on bone health are both complex and pervasive. The effects of androgens are particularly dramatic during growth in boys, but almost certainly play an important role during this period in girls as well. Throughout the rest of life, androgens affect skeletal function and maintenance in both sexes. Nevertheless, relatively little has been done to unravel the mechanisms by which androgens influence the physiology and pathophysiology of bone, and there is still much to be learned about the roles of androgens at all levels. The interaction of androgens and estrogens and how their respective actions can be utilized for specific diagnostic and therapeutic benefit are important but unanswered issues. With an increase in the understanding of the nature of androgen effects will come greater opportunities to use their positive actions in the prevention and treatment of a wide variety of bone disorders.

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CHAPTER 44

Kinins and Neuro-osteogenic Factors

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Introduction

Inflammatory processes within soft tissues are characterized by intense vasodilatation, increased blood vessel permeability, and exudation of plasma to the perivascular adjacent tissue, followed by the migration of different leukocytes from the blood into the surrounding tissues. These vascular and cellular reactions are associated with clinical symptoms of inflammation and with altered metabolism in the surrounding milieu. Bone cells can react to a nearby inflammatory process with both anabolic and catabolic reactions. In most cases, this results in osteolytic loss of bone tissue, but in some cases, sclerotic reactions can be seen. These reactions are induced by inflammatory mediators capable of interacting not only with the inflammatory process, but also with bone cells. In recent years, much experimental work has been performed to study the possible role of cytokines in bone metabolism (Rodan and Martin, 2000). This research area has been directed not only because of the possible role of cytokines in inflammatory bone disease, but also due to their possible roles in physiological bone remodeling and osteoclast development (reviewed in Martin *et al.,* 1998; Suda *et al.,* 1999; Hofbauer *et al.,* 2000; Lerner, 2000a), as well as their possible pathophysiological role in osteoporosis (reviewed in Manologas and Jilka, 1995; Spelsberg *et al.,* 1999). The discovery of the pleiotropic cellular effects of cytokines, and their important roles in the communication between different cells in inflammatory processes, has led to that the interest in inflammation-induced bone loss has been focused on the role of these peptides. However, it has been known for many years that other peptides and nonpeptides are also involved in nonimmune, or classical, inflammatory reactions, e.g., the kallikrein–kinin system, the coagulation cascade, the fibrinolytic pathway, and prostaglandins are activated in inflammatory processes and play important roles in the tissue inflammatory response. Although these systems are most well known for their effects on vessel permeability and dilatation, pain, extravascular coagulation, and fibrinolysis, it has been demonstrated that they are also involved in cell activation, proliferation, migration, and control of proteolysis. As regarding bone, we and others have shown that kinins and thrombin stimulate bone resorption *in vitro* (reviewed in Lerner, 1994, 1997). Osteoblasts synthesize plasminogen activators and inhibitors of these activators in a manner controlled by stimulators of bone resorption (Leloup *et al.,* 1991); *in vitro* data indicate that this system may be involved in the degradation of noncollageonous bone matrix proteins without having any effect on osteoclast formation (Daci *et al.,* 1999). Interestingly, *in vivo* data show that the lack of plasminogen activator inhibitor 1 protects ovariectomized mice from trabecular bone loss without affecting cortical bone loss (Daci *et al.,* 2000).

The activities of bone cells can be regulated at a local level not only by cytokines and kinins. The immunohistochemical demonstration of nerve fibers containing different neuropeptides in the vicinity of bone tissue (reviewed in Bjurholm, 1989; Kontinnen *et al.,* 1996; Lundberg, 2000) raises the possibility that neuropeptides, via neuro-osteogenic interactions, may directly or indirectly modulate the activity of bone cells in physiological and pathological conditions (Lerner, 2000b), in line with the view of neuroendocrine and neuroimmune interactions (van Hagen *et al.,* 1999). The presence of receptors for several neuropeptides on osteoblasts (Bjurholm *et al.,* 1992) and the finding that vasoactive intestinal peptide (VIP)

can stimulate bone resorption in organ culture (Hohmann *et al.,* 1983) provide evidence for a possible direct effect of neuropeptides on bone. The reports that neuropeptides enhance the production of cytokines from a variety of cell types, including monocytes and bone marrow cells, indicate that signaling molecules from skeletal nerve fibers may indirectly affect the skeleton via a neuroimmune control of bone cells, in line with the view that neuroimmunoendocrine interactions are important for the regulation of a variety of cells and tissues.

This chapter summarizes the knowledge of the effects of kinins on bone and the neuronal influence on bone tissue, as well as the interactions among kinins, neuro-osteogenic factors, and cytokines, on bone metabolism.

Activation of the Kallikrein–Kinin System

Kinins are blood-derived short peptides released from kininogens due to the enzymatic action of kallikreins, proteolytic enzymes present in most tissues and body fluids (Fig. 1). The biological effects of the kallikrein–kinin system are mainly exerted by bradykinin (BK) and kallidin (Lys-BK) acting on a variety of cells via cell surface receptors of the B2 subtype. In addition, BK and kallidin, without the carboxy-terminal arginine residue (des-Arg^9-BK and des-Arg^{10}-Lys-BK), can exert effects via BK B1 receptors. The kallikrein–kinin system is briefly summarized here, without giving any references to original reports. Readers are referred to extensive reviews in which relevant references can be found (Hall, 1997; Kaplan *et al.,* 1997; Marceau and Bachvarov, 1998; Marceau *et al.,* 1998; Pesquero and Bader, 1998; Raidoo and Bhoola, 1998; Regoli *et al.,* 1998; Stewart *et al.,* 1999).

Hageman Factor

Activation of the plasma kallikrein system is initiated by the Hageman factor (coagulation factor XII), a single chain globulin (molecular weight 80,000), which can be activated by exposure to an activating macromolecular anionic surface and by endotoxin, as well as by an autocatalytic mechanism. The kallikrein system is activated by the Hageman factor by the action of this enzyme on plasma prekallikrein (Fig. 1).

Kallikreins

Plasma prekallikrein is a single chain globulin encoded by a single gene and is synthesized and secreted by hepatocytes as an inactive proenzyme. Activated plasma kallikrein acts on high molecular weight (HMW) kininogen at two sites, Lys-Arg and Arg-Ser, to release BK, a peptide consisting of nine amino acids with arginine at both the amino- and the carboxy-terminal ends (Fig. 1).

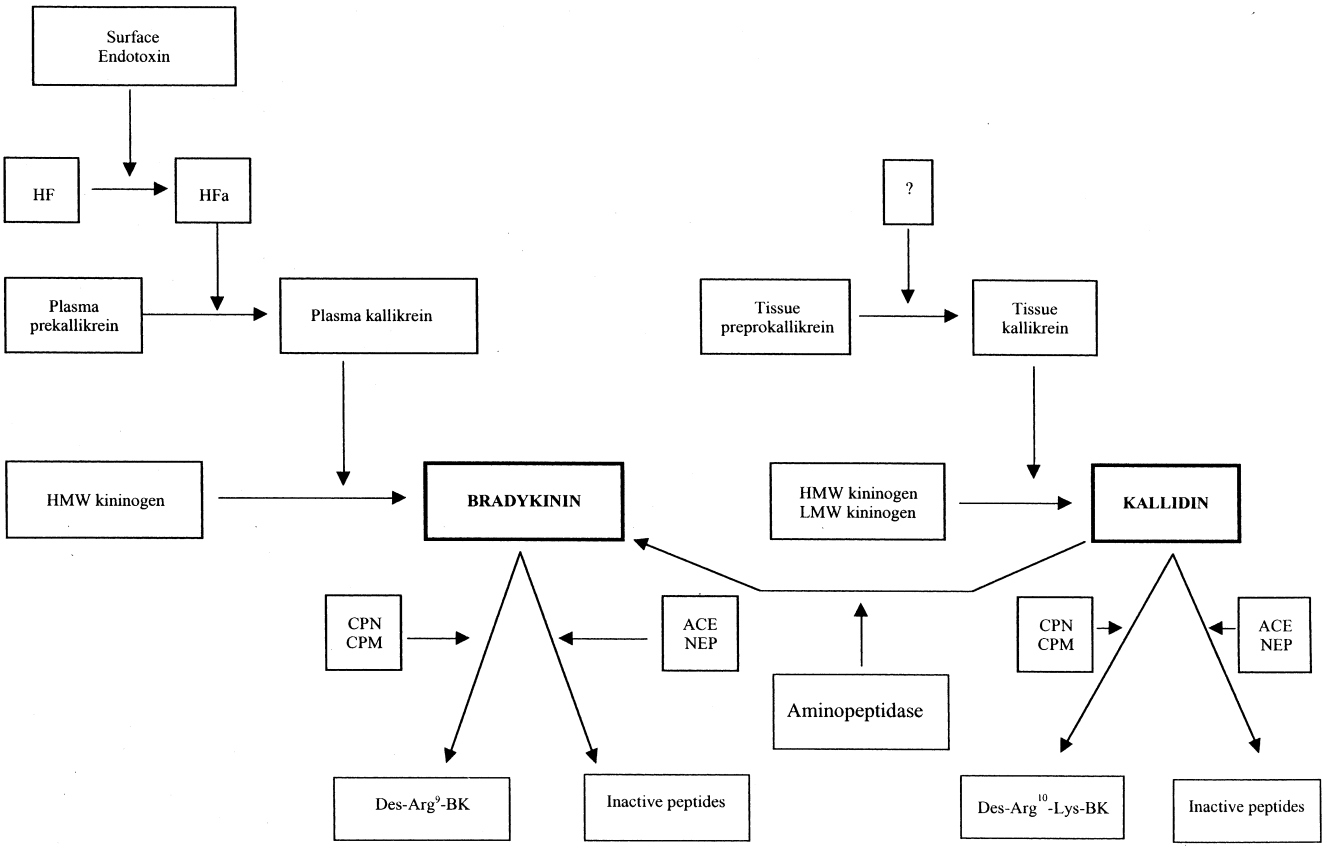

Figure 1 Schematic representation of activation of the kallikrein–kinin system and the formation of kinins.

Plasma kallikrein is inactivated rapidly by the C1 inhibitor and may also be inhibited by α_2-macroglobulin and antithrombin III.

Tissue kallikrein is a member of a multigene family with tissue-specific expression. Several of the serine proteases belonging to this family have important roles in the activation of peptide prohormones and growth factors. Tissue kallikrein liberates kallidin (Lys-BK) from both HMW and (LMW) kininogens, but because LMW kininogen is the most abundant kininogen, the enzyme preferentially uses LMW kininogen as substrate (Fig. 1).

As compared to plasma kallikrein, tissue kallikrein is less susceptible to inhibition. In humans, mainly α_1-antiprotease has some inhibitory capacity. However, a new inhibitor belonging to the serpine superfamily has been identified. In inflammatory processes, tissue kallikrein may be more important for kinin generation, because it seems less susceptible to degradation and because it can use both HMW and LMW kininogen as substrate.

Kininogens

HMW (88–120 kDa) and LMW (50–68 kDa) kininogens are synthesized by hepatocytes as single chain glycoproteins with one amino-terminal heavy chain and one carboxy-terminal light chain. The HMW and LMW kininogens are coded for by a single gene, and the different forms are a consequence of alternate splicing of the gene transcript. The heavy chain of both kininogens contains a domain with cysteine proteinase inhibitory capacity, suggesting the possibility that kininogens may possess both pro- and anti-inflammatory activities.

Rats have a unique kininogen, T-kininogen (68 kDa), from which T-kinin (Ile-Ser-BK) is released by T-kininogenase(s). The levels of T-kininogen in plasma seem to be influenced by estrogen, with concentrations being increased in females at puberty and decreased in mature females by ovariectomy.

Kinins

The term kinin is derived from the Greek word *kineo* (= to move) and was originally used for substances acting on smooth muscles. Today the term kinin is mainly restricted to peptides related to the nonapeptide BK. In this chapter, the word kinin is used for endogenous mammalian peptides with sequence homology to BK, and the kinin analogs refer to synthetic peptides whose amino acid sequence is modified from that of BK.

The kinins are not synthesized and released by cells, but are bioactive, short, and potent peptides that constitute a small part of large proteins (kininogens) from which they are released extracellularly by kininogenases. Four different, but closely related, primary kinins have been described: BK, kallidin, Hyp^3-BK, and T-kinin. The amino acid sequences of these peptides are shown in Table I.

Kinins released have a very short half-life *in vivo,* being estimated to approximately 30 sec, due to the action of different kininases. An interesting aspect is that one of these kininases, cleaving off the carboxy-terminal arginine, gives rise to peptides (des-Arg^9-BK and des-Arg^{10}-Lys-BK) that are biologically active in several cell types and therefore des-Arg^9-BK and des-Arg^{10}-Lys-BK are also included in the group of naturally produced kinins with biological activities.

Kininases

Kinins formed have a short half-life because they are destroyed rapidly by the enzymatic action of different proteases, collectively called kininases (Fig. 2). These enzymes are present both as circulating and as cell-bound enzymes. Several kininases have been described, including carboxypeptidase N (CPN) and carboxypeptidase M (CPM), together called kininase-I. Other kininases are two different kininase-II enzymes called angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP). A third type of kininases are prolidase and aminopeptidase.

Table I Amino Acid Sequences of Natural Kinins and Bradykinin Analogs with Receptor Antagonistic Properties

			1	2	3	4	5	6	7	8	9
B2 receptor agonists											
Bradykinin			Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
Kallidin		Lys-	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
Met-Lys-Bradykinin	Met-	Lys-	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
Hyp^3-bradykinin			Arg-	Pro-	Hyp-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
T-kinin	Ile-	Ser-	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe-	Arg
B1 receptor agonists											
Des-Arg^9-bradykinin			Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe	
Des-Arg^9-Lys-bradykinin		Lys-	Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Phe	
B1 receptor antagonist											
Des-Arg^9-[Leu^8]-bradykinin			Arg-	Pro-	Pro-	Gly-	Phe-	Ser-	Pro-	Leu	
B2 receptor antagonists											
D-Arg-[Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin		Arg-	Arg-	Pro-	Hyp-	Gly-	Thi-	Ser-	Phe-	Thi-	Arg
Hoe 140		Arg-	Arg-	Pro-	Hyp-	Gly-	Thi-	Ser-	Tic-	Oic-	Arg

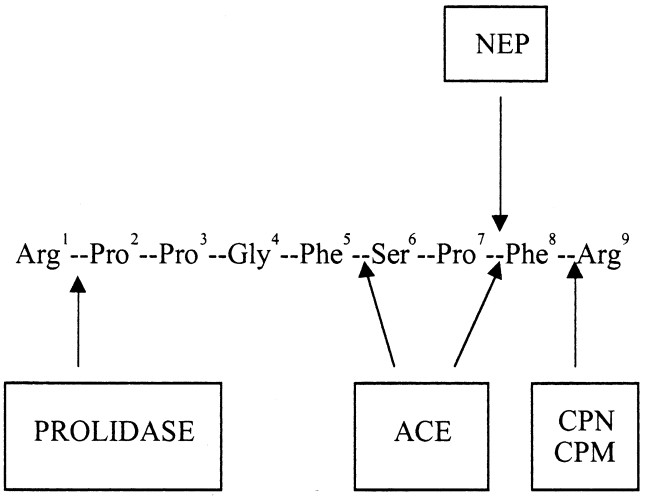

Figure 2 Cleavage sites for different kininases in the bradykinin molecule.

Kininases have been described in biological fluids and in a variety of cells, but no information on the presence of these enzymes in bone is available. However, the fact that some (but not all) kininase II inhibitors potentiate the bone-resorbing effect of BK (Lerner *et al.,* 1987) provides indirect evidence for the presence of BK-inactivating enzymes in bone.

Bradykinin Receptors

Two different BK receptors, termed B1 and B2, have been demonstrated using pharmacological methods such as rank order potencies for different agonists, sensitivity to receptor antagonists, and radioligand-binding studies.

Des-Arg^9-BK and des-Arg^{10}-Lys-BK are the natural agonists for B1 receptors with the latter being the more potent ligand. Substitution of phenylalanine at position 8 in des-Arg^9-BK by an amino acid with an aliphatic side chain gives rise to B1 receptor antagonists, the classical one being des-Arg^9-[Leu^8]-BK. Hoe-140-des-Arg^{10} has also been found to be a potent and selective antagonist for B1 receptors.

The natural kinins with affinity for the B2 receptors are BK and Lys-BK. These agonists are equipotent, or sometimes Lys-BK are slightly more potent than BK. Since the discovery that substitution of proline at position 7 of BK with phenylalanine converts BK agonists to antagonists, several B2 receptor antagonists have been developed, with D-Arg-[Hyp^3, Thi^{5,8}, D-Phe^7]-BK being a very potent one. Hoe 140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK has been shown to be the first totally selective B2 receptor antagonist. The first nonpeptide BK receptor antagonists with affinity for B2 receptors (the phosphonium WIN 64338 and the quinoline FR 173657) have also been developed.

The distribution of BK receptors in different cells and tissues and the relative expression of B1 and B2 receptors have been studied extensively, mostly by the use of pharmacological methods. B2 receptors seem to be constitutively expressed in a variety of tissues. B1 receptor expression has, so far, been demonstrated in certain cell types. In most cells, however, B1 receptor expresssion can be induced by tissue injury and cytokines/growth factors, including interleukin-1 (IL-1), IL-2, oncostatin M, interferon-γ, and epidermal growth factor, by a mechanism likely to be mediated by mitogen-activated protein (MAP) kinases.

B1 and B2 receptors have been cloned molecularly from human, rabbit, rat, and mice species and found to be highly conserved (70–80% homology). In humans, the predicted sequences of the B1 and B2 receptors show proteins of 357 and 364 amino acids, respectively. However, the homology between the two receptor types is only 36%. Both receptors contain seven transmembrane-spanning domains, typical of G protein-coupled receptors, and are located on chromosome 14q32, in very close proximity to each other. Targeted disruption of the B1 receptor gene by homologous recombination results in mice that develop normally with normotension, although failing to respond to B1 receptor agonists. However, lipopolysaccharide-induced hypotension is blunted and the number of polymorphonuclear leukocytes is reduced in inflamed tissues. Moreover, B1 receptor knockout mice are analgesic in behavioral tests of nociception (Pesquero *et al.,* 2000). Targeted deletion of the B2 receptor gene results in mice with severe hypertension, with end-organ damage, when challenged to excess dietary sodium chloride (Alfie *et al.,* 1996). No data are available concerning the skeletal phenotype of B1 and B2 receptor knockout mice. Both B1 and B2 receptor genes have been found to express several allelic polymorphisms, although any association to physiological or pathophysiological bone metabolism has not been studied.

Effects of Kinins on Bone Metabolism

Studies on the effects of kinins on bone metabolism have been performed *in vitro,* mainly in bone organ cultures. Most of these investigations have used a mouse

calvarial bone culture system, in which bones, prelabeled with ^{45}Ca or [^{3}H]proline *in vivo,* are precultured for 18–24 hr in serum-free medium with added indomethacin. The reason for the preculture period is not to "wash out" loosely bound isotope (not necessary because the skeletons of the young mice are prelabeled for 4 days), but to inhibit the initial rise of endogenously produced prostaglandins often causing a high control (or basal) rate of resorption. After the preculture period, the bones are washed extensively for 24 hr to get rid of indomethacin present in the tissues and then cultured in the absence or presence of different test substances (Lerner, 1987; Ljunggren *et al.,* 1991b).

Treatment of mouse calvarial bones, precultured as described earlier, with BK for 72–96 hr results in increased bone resorption, as assessed either by the release of ^{45}Ca or by the mobilization of stable calcium and inorganic phosphate (Gustafson and Lerner, 1984; Lerner *et al.,* 1987). Also, bone matrix degradation, as assessed by the release of ^{3}H from [^{3}H]proline-labeled bones, is increased by BK (Lerner *et al.,* 1987). BK can stimulate the release of ^{45}Ca also from fetal rat long bones, although the response is less than that seen in mouse calvarial bones (Ljunggren and Lerner, unpublished results). The threshold for action of BK in the mouse calvariae is 3 n*M* and half-maximal stimulation (EC_{50}) is obtained at 100 n*M* (Lerner *et al.,* 1987).

Calcitonin, added simultaneously with BK, inhibits the bone resorptive effect of BK (Lerner *et al.,* 1987). Because calcitonin can inhibit the activity of multinucleated osteoclasts, as well as the recruitment of new osteoclasts, data do not reveal if BK stimulates bone resorption by enhancement of the activity of preformed osteoclasts or by the formation of new osteoclasts. However, morphological studies using both light and electron microscopy have shown that osteoclasts present in the calvariae, when bones are dissected from the mice, disappear after the preculture period (Reinholt and Lerner, unpublished results). This implies that the stimulation of bone resorption in this system is dependent on proliferation(?)/differentiation/fusion of osteoclast progenitor cells to multinucleated active osteoclasts and thus suggests that BK stimulates bone resorption by enhancing osteoclast recruitment, a hypothesis supported by the fact that the action of BK on bone resorption is delayed, with no effect observed until after 24 hr (Lerner *et al.,* 1987). Because no hematopoetic cells are present in the mouse calvarial explants, the osteoclast precursor cells in these bones are probably late precursor cells in the osteoclastic cell lineage. Some stimulators, including PTH and $1,25(OH)_2$ vitamin D_3, enhance bone resorption and osteoclast formation by mechanisms insensitive to inhibition of cell proliferation, probably by stimulating differentiation and/or fusion of a postmitotic pool of mononuclear precursor cells (Lorenzo *et al.,* 1983; Lerner and Hänström, 1989). In contrast, stimulation of bone resorption in the mouse calvariae by glucocorticoids and transforming growth factor β (TGF-β) is decreased by mitotic inhibition (Conaway *et al.,* 1996; Lerner, 1996), indicating that these substances act at a level proximal to the level at which the calcium-regulating hormones do. At which level BK acts is not yet known. In ongoing studies, we have not found any effect on osteoclast formation in mouse bone marrow cultures by BK itself. The stimulatory effect of IL-1 β, however, is clearly potentiated by BK (Lerner *et al.,* in preparation). One possibility may be that BK does not share the stimulatory effects of PTH and $1,25(OH)_2$ vitamin D_3 on early precursor cells, but that it may act at later stages downstream of the effect of IL-1. Another possibility may be that BK potentiates the mechanism by which IL-1 stimulate osteoclastogenesis. Whether stromal cells or osteoclast precursor cells are the target cells for BK is not known at present.

The fact that BPP5a, which is a kininase-II inhibitor, potentiates the bone resorptive effect of BK, without affecting those of PTH and PGE_2 (Lerner *et al.,* 1987), supports the hypothesis that the capacity of BK to stimulate bone resorption is decreased by the action of kininase-II enzymes present in mouse calvarial bones. For reasons not known, the ACE inhibitor captopril does not potentiate BK-induced release of ^{45}Ca.

Inflammatory bone loss may not only be due to enhanced bone resorption, but also to decreased bone formation. As regarding osteoblast cell proliferation, biosynthesis of bone matrix proteins, and the activity of alkaline phosphatase, very little is known about the possible effects of BK. In the human osteoblastic osteosarcoma cell line MG-63, BK does not stimulate cell proliferation or the biosynthesis of type I collagen and osteocalcin (Rosenquist *et al.,* 1996), although these cells express BK B2 receptors coupled to a burst of prostanoid formation (Bernhold and Lerner, in preparation). BK B1 receptors have been suggested to play a role in fibrinogenesis in fibrotic disorders, and B1 receptor agonists have been shown to stimulate type I collagen biosynthesis in human fibroblasts due to the stabilization of connective tissue growth factor mRNA (Ricupero *et al.,* 2000). In MG-63 cells, however, we have not been able to find any effect on type I collagen biosynthesis by B1 agonists, although these cells express B1 receptors (Rosenquist *et al.,* 1996; Bernhold and Lerner, in preparation). In agreement with the findings in MG-63 cells, BK has no effect on the proliferation of osteoblast-like cells isolated from human bone (Frost *et al.,* 1999).

Interestingly, whey protein obtained from milk and known to contain a variety of growth factors has been found to increase bone strength in ovariectomized rats (Takada *et al.,* 1997) and to stimulate proliferation and collagen synthesis in MC3T3-E1 cells (Takada *et al.,* 1996). The growth-promoting activity of milk has been purified and found to be a 17-kDa protein with an amino-terminal amino acid sequence very similar to an internal sequence of HMW kininogen (Yamamura *et al.,* 2000). This observation suggests that kininogens may not only be important for BK formation and cysteine protease inhibition, but also for bone growth.

Prostaglandins as Mediators of Bone Resorption Induced by BK

It was noted early that bone resorption induced by BK was inhibited by indomethacin, a potent inhibitor of prostaglandin biosynthesis (Gustafson and Lerner, 1984). It was later shown that several inhibitors of the cyclooxygenase pathway of arachidonic acid metabolism, including indomethacin, naproxen, meclofenamic acid, and flurbiprofen, abolish BK-induced mineral mobilization and bone matrix degradation (Lerner *et al.,* 1987). Similarly, all these nonsteroidal anti-inflammatory drugs also completely inhibit the bone resorptive effect of kallidin and Met-Lys-BK (Gustafson *et al.,* 1986; Ljunggren and Lerner, 1988). The glucocorticoids hydrocortisone and dexamethasone, which are potent inhibitors of prostaglandin biosynthesis, also inhibit BK-induced bone resorption (Lerner *et al.,* 1987). These observations indicate that the bone resorptive effect of BK is totally dependent on the capacity of this peptide to activate prostaglandin formation. Interestingly, most stimulators of bone resorption *in vitro* also stimulate prostanoid formation in bone tissue and bone cells, although the magnitude of the prostaglandin response varies considerably between different stimulators. However, the bone resorptive effect and the biosynthesis of prostaglandins are not necessarily linked to each others. There are stimulators of bone resorption, including PTH, $1,25(OH)_2$ vitamin D_3, TNF-α/TNF-β, and TGF-β, that are totally independent of prostaglandin formation (Ljunggren and Lerner, 1989; Lerner and Ohlin, 1993; Lerner, 1996). Other stimulators, *e.g.,* IL-1, have a larger capacity to stimulate bone resorption in the presence of endogenous prostaglandin production, although a bone resorptive effect of IL-1 still can be seen in the absence of prostaglandins (Lerner *et al.,* 1991). To a third group of stimulators, being unable to stimulate bone resorption in the absence of prostaglandin production, belong BK, kallidin, and Met-Lys-BK.

In primary cultures of mouse calvarial osteoblasts, BK causes a rapid burst of PGE_2 and 6-keto-$PGF_{1\alpha}$ (the stable breakdown product of PGI_2) that is maximal after 5–10 min (Lerner *et al.,* 1989). The half-maximal effect for the prostaglandin response (10 n*M*) is less than that for the bone resorptive effect (100 n*M*), again probably due to differences in the degradation of BK in short-term cell incubations compared to long-term organ cultures. The nontransformed mouse calvarial osteoblastic cell line MC3T3-E1, which both enzyme-histochemically and biochemically express a significantly lower activity of alkaline phosphatase compared to primary mouse calvarial osteoblasts (indicating that the MC3T3-E1 cells may represent a preosteoblastic phenotype; Lundberg and Lerner, unpublished results), also responds to BK with a burst of prostanoid formation (Lerner *et al.,* 1989). The time course, threshold for action, and EC_{50} value are similar to those found in primary mouse calvarial osteoblasts. A very similar prostanoid response to BK is also obtained in nonenzymatically isolated human bone cells (Ljunggren *et al.,* 1990; Rahman *et al.,* 1992) and in the human osteoblastic osteosarcoma cell line MG-63 (Bernhold and Lerner, in preparation).

BK-induced prostanoid biosynthesis can be abolished by a variety of structurally different nonsteroidal anti-inflammatory drugs and glucocorticoids (Lerner *et al.,* 1989). The inhibitory effect of glucocorticoids is only seen in cells pretreated with the steroids for several hours, in contrast to the effect of the nonsteroidal anti-inflammatory drugs, which require a very short preincubation period to be fully active. This is not an unexpected finding considering the different mechanisms by which these compounds are regarded to inhibit prostaglandin biosynthesis; nonsteroidal drugs inhibiting cyclooxygenase activity and steroids exerting their effects via a genomic action. However, bone resorption induced by BK can be inhibited by the simultaneous addition of glucocorticoids (Lerner *et al.,* 1987). This could be explained if the rapid burst of prostaglandins produced is not sufficient to stimulate bone resorption, but that prostaglandins synthesized later during the bone organ cultures are rate limiting in the bone resorption process. Such a hypothesis raises the possibility that the kininase-I metabolite of BK, des-Arg^9-BK, may contribute to the effect of BK. This octapeptide has also been found to stimulate bone resorption and prostaglandin production, but the effects are very much delayed as compared to those by BK (Lerner *et al.,* 1987; Ljunggren and Lerner, 1990), thereby making it possible for glucocorticoids to exert an inhibitory effect. The fact that glucocorticoids, added together with BK, inhibit the bone resorptive effect could also indicate that the effect of these compounds is unrelated to the inhibition of prostaglandin biosynthesis, but exerted at a step distal to the burst of prostaglandin biosynthesis. However, the observation that glucocorticoids potentiate the bone resorptive effect of exogenous PGE_2 (Conaway *et al.,* 1997) does not support such a view. Whatever the mechanism in glucocorticoid-induced inhibition of BK-induced bone resorption is, the findings that several different cyclooxygenase inhibitiors, as well as 5,8,11,14-eicosatetraynoic acid, a competitive inhibitor of arachidonic acid metabolism, abolish the bone resorptive effect of BK convincingly demonstrate the importance of prostanoids as mediators of the bone resorptive action of BK.

Kinin Receptors in Bone Cells

BK, Lys-BK, and Met-Lys-BK have been demonstrated to stimulate bone resorption in mouse calvariae, indicating the presence of B2 receptors (Gustafson *et al.,* 1986; Lerner *et al.,* 1987; Ljunggren and Lerner, 1988). This view is further supported by the fact that the B1 receptor antagonist des-Arg^9-[Leu^8]-BK does not affect the bone resorptive effect of BK (Lerner *et al.,* 1987), an observation that also suggests that the effect of BK is not due to the conversion of BK by kininase-I to the the B1 receptor agonist des-Arg^9-BK.

Pretreatment of BK with kininase-I does not affect BK-induced bone resorption, whereas the effect of PTH is reduced significantly (Lerner *et al.,* 1987). This observation

suggests that des-Arg^9-BK, a B1 receptor agonist, may be able stimulate bone resorption. In agreement with this view, it has been shown that the addition of des-Arg^9-BK to mouse calvarial bones results in enhanced release of ^{45}Ca (Lerner *et al.,* 1987; Ljunggren and Lerner, 1990), an effect that is inhibited by the B1 receptor antagonist des-Arg^9-[Leu^8]-BK (Ljunggren and Lerner, 1990), indicating that bone cells are also equipped with B1 receptors. The effect of des-Arg^9-BK is abolished by indomethacin, flurbiprofen, and hydrocortisone. In addition, prostanoid biosynthesis in mouse calvarial bones is stimulated by des-Arg^9-BK in 72-hr cultures, but, in contrast to BK, no prostaglandin response is seen in bones incubated for 30 min with des-Arg^9-BK.

Data obtained with mouse calvarial bones demonstate the presence of both B1 and B2 receptors linked to bone resorption by a process requiring the stimulation of prostaglandin biosynthesis. Differences in the kinetics for the prostaglandin response indicate different molecular mechanisms of action in B1 and B2 receptor stimulation of bone resorption.

Using the burst of PGE_2 and 6-keto-$PGF_{1\alpha}$ biosynthesis in primary mouse calvarial osteoblasts and in the osteoblastic cell line MC3T3-E1 as parameters, the following rank order potency for different agonist has been shown: BK = Lys-BK>Met-Lys-BK>>>>des-Arg^9-BK, demonstrating the presence of B2 receptors on these osteoblasts (Ljunggren *et al.,* 1991c). The fact that D-Arg^0-[Hyp^3, Thi^{5,8}, D-Phe^7]-BK, but not des-Arg^9-[Leu^8]-BK, inhibits the inital rise of prostaglandins induced by BK further supports the presence of B2, and not B1, receptors on mouse osteoblasts. It has been shown that the human osteosarcoma cell line MG-63 shows a prostanoid burst in response to a wide variety of natural kinins and kinin analog with affinity to BK B2 receptors. The effect of BK in these cells is inhibited by B2 receptor antagonists, but not by B1 receptor antagonists (Bernhold and Lerner, in preparation). These observations and the finding that [Hyp^3]-BK is a weak agonist and T-kinin is a potent agonist further indicate that osteoblasts are equipped with B2 receptors linked to a burst of prostaglandin biosynthesis. This view is also compatible with the observations that BK and D-Arg^0-[Hyp^3, Thi^{5,8}, D-Phe^7]-BK, but not des-Arg^9-BK, compete with the binding of [^{3}H]BK to osteoblasts (Ljunggren *et al.,* 1991c). Leis *et al.* (1997) have demonstrated that responsiveness to BK, specific binding of [^{3}H]BK, and mRNA expression of BK B2 receptors in subclones of the murine osteoblastic cell line MC3T3-E1 are highest in clones with low alkaline phosphatase activity, indicating that it is mainly osteoblasts at early stages of differentiation that are responsive to BK. This observation is in agreement with findings by Lerner *et al.* (1989) demonstrating that the more confluent mouse calvarial osteoblasts are in cell cultures, the less is the BK responsiveness. Using BK-sensitive MC3T3-E1 cells, it has been shown that these cells express a single category of binding sites for [^{3}H]BK (Windischhofer and Leis, 1997). Radioligand-binding assays in MG-63 cells, using [^{3}H]BK as ligand, have demonstrated specific binding sites that can be competed for by B2 receptor agonists and antagonists. The rank order potency for kinin-induced stimulation of prostaglandin formation and radioligand-binding studies strongly indicate the constitutive expression of B2 receptors in MG-63 cells, a conclusion further supported by RT-PCR analysis showing mRNA expression of BK B2 receptors (Bernhold and Lerner, in preparation).

The acute rise of prostaglandin production in osteoblasts in response to BK is preceded by an accumulation of inositol phosphates, a transient increase of intracellular calcium, and an activation of protein kinase C (Ljunggren *et al.,* 1991a, 1993; Leis *et al.,* 1997). The initial, transient rise of intracellular calcium and the sustained influx of extracellular calcium seem to be regulated by different protein kinase C isoenzymes (Sakai *et al.,* 1992). By studying BK-induced release of arachidonic acid from MC3T3-E1 cells, evidence shows that BK receptors are linked to G proteins (Yanaga *et al.,* 1991), well in agreement with cloning data. These findings suggest that activation of BK receptors leads to a phospholipase C-mediated breakdown of phosphatidylinositol 4,5-bisphosphate with subsequent formation of the two putative second messengers: inostiol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG).

In agreement with the observation in mouse calvarial bones, treatment of primary mouse calvarial osteoblasts with des-Arg^9-BK results in a delayed enhancement of PGE_2 formation that can be observed at and after 24 hr (Ljunggren and Lerner, 1990). The effect of des-Arg^9-BK is inhibited by des-Arg^9-[Leu^8]-BK, indicating that des-Arg^9-BK exerts its effect via B1 receptors. Similar observations have been made in MG-63 cells using des-Arg^9-BK, des-Arg^{10}-Lys-BK, [Tyr-Gly-Lys-Aca-Lys]-des-Arg^9-BK, and Sar[D-Phe8]-des-Arg^9-BK as agonists. Stimulation caused by these B1 receptor agonists is inhibited by a variety of B1 receptor antagonists, but not by B2 receptor antagonists. In addition, MG-63 cells display specific binding sites using [^{3}H]-des-Arg^{10}-Lys-BK as ligand and mRNA expression of human B1 receptors (Bernhold and Lerner, in preparation). The delay in the action of B1 receptor agonists (as compared to B2 receptor agonists) could be due to different postreceptor signal-transducing mechanisms (indicated by findings that des-Arg^9-BK does not stimulate IP3 formation, intracellular calcium, and translocation of protein kinase C) or differencies in the mechanism by which prostaglandin biosynthesis is stimulated. An alternative explanation could be that B1 receptors are not constitutively expressed on osteoblasts, but are rather gradually induced in culture (Phagoo *et al.,* 1999).

The observation that BK can stimulate depletion of calcium from intracellular stores and enhance inositol phosphate production in the human osteoclast-like cell line GCT23 indicates that preosteoclasts, and possibly also multinucleated osteoclasts, may be equipped with BK receptors (Seuwen *et al.,* 1999). However, whether BK may be able to directly stimulate osteoclast formation and/or activity is not known. Our finding that BK can stimulate osteoclastogenesis in mouse bone marrow cultures does not reveal if stromal cells or osteoclast progenitor cells are the target cells (Lerner *et al.,* in preparation).

Interactions among Kinins, Cytokines, and Neuropeptides

Although much interest has been focused on the role of cytokines, growth factors, hormones, kinins, and neuropeptides in bone metabolism and cell activities in general, the approach has often been to study the effect of these substances in systems in which they have been tested one by one. It is apparent that cells are exposed *in vivo* to several agonists simultaneously and therefore the ultimate cell and tissue response will be dependent on interactions among a variety of hormones, cytokines, growth factors, kinins, and neuropeptides.

The possibility that kinins may interact with cytokines is supported by the findings that BK can stimulate IL-1 and TNF production in murine macrophages (Tiffany and Burch, 1989), cytokine release in rat spleen mononuclear cells (Reissmann *et al.,* 2000), and IL-6 formation in human bone cells (Rahman *et al.,* 1992). The capacity of BK to affect cytokine production has also been demonstrated in human gingival fibroblasts; in these cells, BK does not affect IL-1 production, but potentiates the stimulatory effect of TNF on the biosynthesis of IL-1α and IL-1β (Yucel-Lindberg *et al.,* 1995). Interestingly, hyperalgesia induced by BK is blocked by the IL-1 receptor antagonist and is enhanced by antiserum neutralizing the IL-1 receptor antagonist (Cunha *et al.,* 2000). Such observations should prompt future studies on the possible role of cytokines in BK-induced bone resorption.

Although the role of cytokines, including the recently discovered receptor activator of NF-κB (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) (Martin *et al.,* 1998; Suda *et al.,* 1999; Hofbauer *et al.,* 2000; Lerner, 2000a; Teitelbaum, 2000), in the mechanism by which BK stimulates bone resorption is elusive at present, data suggest that kinins and cytokines may act in concert. It has been shown that BK synergistically potentiates the stimulatory effects of IL-1α and IL-1β on bone resorption and prostanoid biosynthesis in mouse calvarial bones (Lerner, 1991). A similar interaction in mouse calvariae has also been observed between BK and TNF (Lerner *et al.,* in preparation). We have observed that several BK B2 receptor agonists, as well as B1 receptor agonists, synergistically potentiate IL-1- and TNF-induced biosynthesis of PGE_2 and 6-keto-$PGF_{1\alpha}$ in the human osteoblastic cell line MG-63 (Bernhold and Lerner, in preparation). The effect is associated with a cytokine-induced upregulation of binding sites for B1 and B2 receptor-specific ligands. As mentioned already, BK potentiates osteoclastogenesis in mouse bone marrow cultures induced by IL-1 (Lerner *et al.,* in preparation). The synergistic interactions between BK and cytokines are not restricted to bone cells, as it can also be obtained in human gingival and dental-pulp fibroblasts, as well as in periodontal ligament cells (Lerner and Modéer, 1991; Ransjö *et al.,* 1998; Sundqvist and Lerner, 1995). The molecular mechanism involved in the interaction between cytokines and kinins is not known, but could involve changes of receptor number or affinity, signal transduction, or arachidonic acid release and/or metabolism.

Pretreatment of human osteoblast-like cells with estrogen upregulates the subsequent stimulation of prostaglandi production induced by BK (Cissel *et al.,* 1996), suggesting the existence of steroid hormone/kinin interactions in bone.

There are indications that the skeleton may be systemically affected in patients with chronic inflammatory diseases and in rats with experimentally induced chronic inflammation (Minne *et al.,* 1984; Motley *et al.,* 1993). The systemic factor involved is not known, but could be related to the demonstration that haptoglobin, one of the acute-phase proteins induced in the liver during chronic inflammation, stimulates bone resorption in neonatal mouse calvariae (Lerner and Fröhlander, 1992). Interestingly, BK synergistically potentiates the stimulatory effect of haptoglobin on PGE_2 formation in mouse calvarial osteoblasts (Fröhlander *et al.,* 1991).

Data indicate that skeletal neuropeptides may play important roles as local mediators regulating bone metabolism (see later). The novel observations that (i) BK stimulates the expression of β-adrenergic receptors (Yasunaga *et al.,* 2000), (ii) BK enhances the release of calcitonin gene-related peptide (Averbeck *et al.,* 2000), and (iii) kinins participate in neurokinin-1 receptor-dependent neutrophil accumulation in inflamed skin (Cao *et al.,* 2000) raise the possibility of a link between neurohormonal- and kinin-regulated bone metabolism. Our finding that the skeletal neuropeptide vasoactive intestinal peptide regulates the mRNA expression of RANKL, RANK, and OPG in mouse bone marrow cultures (Mukohyama *et al.,* 2000b) and preliminary observations indicating that the same neuropeptide can affect the mRNA expression of IL-6 and its receptor components gp 80 and gp 130 (Persson *et al.,* unpublished results) suggest the possibility of a neuroimmune interplay in bone cell activities.

Neuronal Influence on Bone Tissue

The activities of and interactions between different bone cells are regulated by a variety of systemic hormones, cytokines, growth factors, and inflammatory mediators. Another proposed regulatory element is the nervous system, which, through the release of neuronal messengers, has been suggested to participate in bone metabolism. Although Hohmann *et al.* (1983) reported that the neuropeptide VIP can stimulate bone resorption in mouse calvariae, it has only been since the early 1990s that the possible role of neuroactive substances in the control of bone cell activities has been appreciated. This field of interest is based partly on the recognition of an intense network of skeletal nerve fibers and partly on the view that the neuronal systems may not only have sensory functions and regulatory roles in the control of vessel and muscle activities, but may also exert a neurohormonal control of a variety of tissues, with one example being neuroendocrine–immune interactions (Bellinger *et al.,* 1992; Besedovsky and Rey, 1996).

Innervation of Bone

During the first decades of the 20th century the presence of nerve fibers in bone and periosteum was demonstrated using routine histological techniques (reviewed by Hurrel, 1937; Sherman, 1963). The techniques applied were useful in establishing the distribution of nerves and in discriminating between myelinated and unmyelinated nerves in bone, but the information provided was limited by morphology. Three decades ago, a breakthrough in neuroscience occurred when the immunohistochemical technique was developed and made visualization of nerves according to their transmitter content possible. Numerous neuroactive substances have been demonstrated in many different tissues, but the difficulty in bone tissue was to demineralize the bone without destroying the antigenicity of the neuro-related substances. Bjurholm *et al.* (1989) and Hill and Elde (1990) developed techniques making it possible to preserve neuroactive substances in decalcified bone specimens. Following these reports, a number of neuronal messengers and their distribution in bone have been mapped extensively .

In addition to transmitter phenotyping with immunohistochemical techniques, surgical or chemical selective denervation has established the origin of the nerves in bone (reviewed by Lundberg, 2000). Both sensory and autonomic nerve fibers are present in bone tissue. Overall, a substantial part of skeletal nerve fibers are seen along blood vessels, but blood vessel-unrelated and free nerve endings have also been demonstrated. Fibers are spread in all the cell layers of the periosteum of bone and are expressed at a higher density in the epiphysis than in the diaphysis. Small branches of periosteal nerve fibers enter the cortical bone, usually associated with blood vessels located in Volkmann's canals or in Haversian canals (Bjurholm, 1989; Hill and Elde, 1991a; Hukkanen *et al.,* 1992). Entering the inner compartments of bone, nerve fibers are spread in the bone marrow and richly innervate the osteochondral junction of the growth plate. Interestingly, the epiphyseal part of the growth plate is intensively supplied by peptidergic nerves, whereas the metaphyseal part is innervated more poorly (Hukkanen *et al.,* 1992; Hukkanen, 1994).

The immunohistochemical staining of bone tissue sections has demonstrated the presence of a wide variety of neuronal messengers, including both slowly acting transmitters, so-called neuropeptides, and rapidly acting small molecules, so-called classical neurotransmitters, in bone. Presently, the neuropeptides demonstrated in bone are substance P (SP), calcitonin gene-related peptide (CGRP), neurokinin A, and pituitary adenylate cyclase activating peptide 27 (PACAP 27) and 38 (PACAP 38), mainly representing the sensory system; vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY), mainly representing the autonomic system; and met-enkephalin, representing the opioid system. The classical neurotransmitters present in bone are the amines serotonin and the catecholamine noradrenaline (NA) and the excitatory amino acid glutamate (reviewed by Kontinen *et al.,* 1996; Lerner, 2000b; Kreicbergs and Ahmed, 1997; Lundberg, 2000). In addition to morphological demonstrations, neuroactive substances in bone have also been quantified biochemically. A technique has been developed to extract and quantify neuropeptides in bone and joints using RIA (Ahmed *et al.,* 1994). Using this technique, extracts from diaphyseal rat bone tissue, periosteum, and bone marrow have been analyzed for their contents of neuropeptides. SP, CGRP, NPY, and VIP could all be quantitated at all three localizations, with NPY exhibiting the highest concentration at all sites (Ahmed *et al.,* 1994). Moreover, neurotrophic factors such as neurotrophins, known to be important factors required for the development and maintenance of the central and peripheral nerve systems, have been demonstrated in bone tissue. Neurothrophins demonstrated in bone tissue so far are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) (Asaumi *et al.,* 2000).

A neuronal regulation of bone metabolism would not only require the presence and release of neuronal messengers in the vicinity of bone cells, but also the presence of functional receptors for such factors on bone-forming or bone-resorbing cells. Therefore, several attempts have been made to study whether different neuropeptides, neurotransmitters, and neurotrophins can affect receptor-associated functions in osteoblasts and osteoclasts.

Receptors and Effects by Neuropeptides in Bone

Vasoactive Intestinal Peptide

VIP is a member of the growing family denoted VIP/secretin/glucagon family of neuropeptides, which also includes the structurally related peptides secretin, gastric inhibitory peptide, growth hormone-releasing hormone, peptide histidine isoleucine amide (PHI), PACAP 27 and 38, and the reptilian venom peptides helodermin, helospectin, and exendine.

VIP was first discovered as a vasodilator peptide (Said and Mutt, 1969). Isolation of VIP from porcine gut (Said and Mutt, 1970) revealed an amidated 28 amino acid peptide (Mutt and Said, 1974). VIP is a cleavage product of the 170 amino acid precursor pre-pro VIP, which also is the parent molecule of PHI (Itoh *et al.,* 1983). Using RIA and the immunofluorescence technique, VIP immunoreactivity was discovered in many tissues outside the gastrointestinal tract. In 1976, the finding of VIP in the brain and in peripheral nerves (Said and Rosenberg, 1976) introduced VIP as a neuropeptide.

VIP has been investigated extensively, and a broad range of biological actions has been ascribed to this peptide, both in animal and in human studies. Important actions of VIP in the cardiovascular (Sakai *et al.,* 1998), reproductive (Ottesen and Fahrenkurg, 1995), pulmonary (Maggi *et al.,* 1995), immune (Sirinek and O'Dorisio, 1991; Ganea, 1996), and gastrointestinal systems (Schuttleworth and Keef, 1995) have been reviewed. General physiologic effects encompass vasodilatation, bronchodilatation, immunosuppression, hormonal

secretion, and increases in gastric motility. In the nervous system, VIP seems to participate in neuronal survival, maturation, and maintenance (reviewed by Gozes and Brenneman, 1993).

VIP is widely distributed in both the central (CNS) and the peripheral nervous systems (PNS). In the brain, VIP immunoreactive neurons are found in the hypothalamus and the cerebral cortex (Hökfelt *et al.,* 1982). In the PNS, VIP immunoreactive nerves, nerve plexus and terminals supplying blood vessels, nonvascular smooth muscles, glandular acinis, and ducts, in a variety of organs, have been described (Hökfelt *et al.,* 1982). VIP immunoreactivity is detected in nerve fibers with both sympathetic and parasympathetic origin and, to a minor extent, in sensory nerve fibers.

In 1986, Tashijan and collegues initially demonstrated VIP immunoreactive (IR) nerve fibers in bone tissue (Hohmann *et al.,* 1986). VIP-IR nerve fibers with a postganglionic sympathetic origin were localized in the periosteum, associated with vascular elements of bones from different species. In a large study of neuropeptide expression in rat bone nerve fibers, Bjurholm *et al.* (1988a,c) detected VIP-IR nerve fibers preferentially in the periosteum and the epiphysis. These VIP-IR nerve fibers, whose origins are uncertain, were only occasionally associated with blood vessels. In line with these data, Hill and Elde (1991a) also demonstrated VIP-IR in rat periosteum. These nerves were only partially associated with vascular elements. VIP, NPY, and dopamine-β-hydroxylase (DβH) immunoreactive nerves were reduced dramatically in guanethidine-treated animals, strongly indicating a sympathetic origin of theses nerves (Hill and Elde, 1991b).

Tashjian and co-workers provided the first *in vitro* evidence for functional neuropeptide receptors on bone cells, demonstrating that VIP stimulates calcium release in neonatal mouse calvariae (Hohmann *et al.,* 1983). In osteoblasts, the presence of functional receptors for VIP, linked to enhanced formation of cyclic AMP, has been demonstrated in a human osteosarcoma cell line (Saos-2) (Hohmann and Tashjian, 1984; Bjurholm *et al.,* 1992; Lerner *et al.,* 1994), in the rat osteosarcoma cell line UMR 106-01 cells, but not in rat osteosarcoma cell line ROS 17/2.8 cells (Bjurholm *et al.,* 1992; Lerner *et al.,* 1994). In addition, VIP has been found to stimulate cyclic AMP formation in isolated mouse calvarial osteoblasts and in the cloned, nontransformed, osteoblastic cell line MC3T3-E1 (Bjurholm *et al.,* 1992; Lerner *et al.,* 1994).

Since the early 1990s, three different subtypes of VIP receptors have been cloned. These receptors are designated VIP-1 receptors (VIP-1R), VIP-2 receptors (VIP-2R), and PACAP-receptors (PACAP-R) (reviewed by Rawling and Hezareh, 1996). They are all seven transmembrane, G protein-coupled receptors and members of the VIP/secretin/PTH receptor superfamily. All three receptors are distributed both in the CNS and the PNS and can be distinguished by comparing their relative adenylate cyclase-activating capacities and by radioligand-binding assays. PACAP-R bind PACAP with a much higher affinity (100- to 1000-fold) than VIP. VIP-1R and VIP-2R bind PACAP and VIP with similar affinities. The fact that VIP-1R bind secretin, and VIP-2R do not, can be used to distinguish between these two VIP receptors.

We have characterized the VIP-binding receptors in mouse calvarial osteoblasts. By comparing the rank order of response of peptides in the VIP/secretin/glucagon family on cyclic AMP formation, we found that PACAP 38 was 10-fold more potent than VIP (Lundberg *et al.,* 2001). A similar 10-fold difference in potency between PACAP and VIP has also been detected in the rat osteoblast-like tumor cell line UMR 106 (Kovacs *et al.,* 1996) and in the nontransformed murine calvarial cell line MC3T3-E1 (Susuki *et al.,* 1994). By comparing the relative potency of VIP and related peptides to displace ^{125}I-PACAP binding, we found a rank order of response similar to that obtained when cyclic AMP enhancement was quantified. The fact that PACAP-preferring VIP receptors do not bind secretin was confirmed by demonstrating that secretin did not elevate cyclic AMP levels and failed to displace ^{125}I-VIP or ^{125}I-PACAP 38 binding. Using atomic force microscopy (AFM), a novel technique modified recently to detect specific binding sites on cell surfaces, we have demonstrated specific binding of VIP, but not secretin, on mouse calvarial osteoblasts. Reverse transcriptase PCR further demonstrated that these undifferentiated osteoblasts express mRNA for VIP-2R, but not for VIP-1R or PACAP-R. When these osteoblasts were cultured for 20 days to induce bone noduli formation, VIP-1R, in addition to VIP-2R, were expressed when the nodules started to mineralize at 12 days (Lundberg *et al.,* 2001). Taken together, these data demonstrate that mouse calvarial osteoblasts express functional VIP-2R, with higher affinity binding for PACAP than for VIP, and that VIP-1R expression is induced during osteoblastic differentiation. Information on mRNA expression of VIP/PACAP receptors in osteoblasts is limited to observations in mouse calvarial osteoblasts and to a report by Togari *et al.* (1997), demonstrating that primary human osteoblasts and human osteosarcoma cell lines express VIP-1R, but not VIP-2R or PACAP-R. The observed differences in VIP-R expression in mouse and human osteoblasts may be a matter of differentiation discrepancies, although it cannot be excluded that it is due to species differences.

Interestingly, a differentiation-dependent manner of receptor expression in mouse osteoblasts is not only observed for VIP receptors, but also for vascular endothelial growth factors (VEGF) and their receptors in mouse osteoblasts (Deckers *et al.,* 2000). A role for VEGF in endochondral bone formation has been proposed because inactivation of VEGF inhibits endochondral bone formation via inhibition of angiogenesis (Gerber *et al.,* 1999). Therefore, the increased expression of VEGF receptors and their ligands during osteoblastic differentiation and mineralization supports the theory that VEGF plays an important role in the regulation of bone metabolism. The role of VIP-1R induction during osteoblastic differentiation remains to be elucidated. In contrast to differentiation-induced upregulation of VIP-1R and VEGF receptors, the expression of glutamate transporter in rat osteoblasts declines

when mineralization starts in rat osteoblast cultures (Bhangu *et al.,* 2000).

Whether VIP receptors on osteoblasts are coupled to anabolic actions of VIP has been evaluated *in vitro* using mouse calvarial osteoblasts (Lundberg *et al.,* 1999b). After 6 days of culture, VIP stimulates activity of the bone mineralization-associated enzyme alkaline phosphatase (ALP), and the mRNA expression of this enzyme, without affecting cell proliferation. The ALP-staining pattern in histochemical analysis demonstrated that VIP, to a minor extent, increased the number of ALP-stained cells, but mainly increased the staining of individual cells. These morphological analyses suggest that VIP treatment causes an increased differentiation of committed osteoblasts. In line with this, we found that VIP initially causes an increased accumulation of calcium in osteoblasts during the formation of mineralized bone nodules, but does not change the total amount of calcium found at the end of the culture. Our preliminary finding that VIP does not change the mRNA expression of type I collagen in osteoblast cultures further supports the view that VIP does not increase bone formation, but rather stimulates the differentiation of bone forming committed osteoblasts. The capacity of VIP to regulate osteoblastic differentiation is also indicated by the observations in mouse calvarial osteoblasts demonstrating that VIP increases the mRNA expression of osteonectin and decreases those of osteopontin and bone sialoprotein at 4 and 8 days of culture. After 20 days of treatment, the expressions of osteonectin and ALP are decreased, whereas that of osteopontin is increased (Mukohyama *et al.,* 2000a). The fact that VIP stimulates ALP activity at 6 days of culture, a time point when only VIP-2R are expressed (Lundberg *et al.,* 2001), together with the absence of effect by secretin on ALP activity, clearly suggest that VIP-2R receptors mediate the anabolic events in bone caused by VIP (Lundberg, unpublished data). Whether VIP-1R may mediate similar bone-forming effects has to be ascertained.

The first documented *in vitro* effect of a neuropeptide on bone was that of Tashjian and co-workers, demonstrating a catabolic effect by VIP on bone metabolism (Hohmann *et al.,* 1983). Thus, VIP stimulated calcium release in organ-cultured mouse calvarial bones. This stimulation of calvarial bone resorption by VIP may be due either to enhanced activity of osteoclasts or to stimulation of osteoclast formation.

Morphological studies of isolated rat osteoclasts revealed that VIP treatment caused a rapid cytoplasmic contraction along with an associated decrease in motility (Lundberg *et al.,* 2000). Functional studies using an *in vitro* resorption assay showed that VIP caused a transient inhibition of osteoclastic bone resorption. When the osteoclast incubations were extended over time and performed in the presence of marrow-derived stromal cells/osteoblasts, the osteoclasts escaped from the initial inhibition and VIP caused a delayed stimulation of osteoclastic pit formation in bone slices. Similar to VIP, the initial inhibitory effect of calcitonin (CT) was lost over time. However, in contrast to VIP, CT-treated osteoclasts never start to resorb bone more than unstimulated controls. The finding of inhibitory effects, both on osteoclast morphology and on resorptive capacity, suggests that osteoclasts are equipped with VIP receptors and that VIP might be acting directly on osteoclasts.

In order to localize binding sites for VIP on osteoclasts, we took advantage of the newly developed AFM technique. Using AFM and measurements of intracellular calcium, specific VIP-binding sites on osteoclasts were found (Lundberg *et al.,* 2000). Further evidence for the presence of VIP receptors in osteoclasts is our finding of mRNA for VIP-1R and PACAP-R in mouse bone marrow osteoclasts isolated by micromanipulation (Ransjö *et al.,* 2000). The late stimulatory effect of VIP is probably the basis of the finding that VIP stimulates resorption in calvarial organ culture (Hohmann *et al.,* 1983). When AFM was used to analyze the presence of VIP receptors in stromal cells/osteoblasts, we found that approximately 20% of the stromal cells/osteoblasts expressed specific binding sites for VIP. This was supported further by the observation that these cells also responded to VIP with a rapid enhancement of intracellular calcium (Lundberg *et al.,* 2000). These receptors might mediate the indirect bone-resorbing effect caused by VIP, both in the resorption pit assay and in the calvariae.

VIP has been reported to stimulate IL-6 production in stromal cells (Cai *et al.,* 1997) and in an osteosarcoma cell line (Greenfield *et al.,* 1996). We have confirmed these observations using mouse calvarial osteoblasts and, in addition, demonstrated that VIP-induced IL-6 production is mediated via VIP-2R (Lundberg *et al.,* 1999a). PTH also induces IL-6 production in osteoblasts. Interestingly, the stimulation of PTH on rat osteoclast pit formation is inhibited by antiserum-neutralizing IL-6 (Ransjö *et al.,* 1999). These findings suggest that the stimulatory effect of VIP on rat osteoclast pit formation may be due to VIP-induced IL-6 release.

The stimulation of bone resorption by VIP in organ-cultured mouse calvariae can be explained either by an effect on osteoclast activity or by an effect on osteoclast formation. When osteoclastogenisis was studied in mouse bone marrow cultures, VIP did not enhance the number of osteoclasts (Mukohyama *et al.,* 2000b). In contrast, VIP caused an inhibition of osteoclast formation induced either by $1,25(OH)_2$ vitamin D_3 or by PTH. The antiosteoclastogenic effect of VIP is associated with inhibitory effects of these peptides on the $1,25(OH)_2$ vitamin D_3-induced upregulation of RANK (receptor activator of NF-κB) and RANK ligand (RANKL). In addition, VIP counteracts the decrease of osteoprotegerin (OPG) caused by $1,25(OH)_2$ vitamin D_3 (Mukohyama *et al.,* 2000b). In summary, VIP inhibits osteoclast formation, probably by regulating the expression of RANK, RANKL, and OPG, three molecules known to be important for osteoclast formation.

The fact that VIP stimulates osteoclast activity and inhibits osteoclast recruitment suggests that VIP may have a unique role in bone metabolic processes by acting as a fine tuner of osteoclastic resorption.

Pituitary Adenylate Cyclase-Activating Peptide

PACAP was first isolated from ovine hypothalamus and described on the basis of its ability to increase adenylate cyclase activity in rat pituitary cells (Miayata *et al.,* 1989). PACAP occurs in two molecular forms: the 38 amino acid peptide PACAP 38 and the C-terminally truncated form PACAP 27. Both forms of PACAP share 68% amino acid homology with VIP at their N-terminal domains (reviewed by Arimura, 1991, 1992).

In addition to exhibiting extensive molecular similarities to VIP, PACAP partially shares receptors as well as functions with VIP. PACAP has been shown to be a pleiotropic neuropeptide, functioning as a hypothalamic hormone, neurotransmitter, neuromodulator, vasodilator, and a neurotropic factor. Examples of endocrine functions by PACAP are numerous. PACAP (i) stimulates the secretion of adrenaline from the adrenal medulla, (ii) stimulates insulin release from pancreatic β cells, and (iii) causes an increase of $[Ca^{2+}]_i$ in pancreatic β cells. One important developmental biological action of PACAP seems to be as a neurotrophic factor during the development of the brain (reviewed by Arimura, 1998).

PACAP immunoreactivity is detected in nearly all organs and tissues. In the brain, the highest concentration is found in the hypothalamus. In the PNS, the adrenal medulla and testis contain the highest concentrations of PACAP immunoreactivity when compared to those found in the gut and the adrenal gland. PACAP 38 is the predominant form in tissues, making up 90% of the total PACAP (reviewed by Arimura, 1998).

PACAP 27 and PACAP 38 have been demonstrated in cartilage channels in tissue sections from pigs (Strange-Vogsen *et al.,* 1997). Varicose PACAP immunoreactivity fibers were demonstrated in association with blood vessels. Nearly all PACAP immunoreactive fibers contain CGRP and SP, suggesting that these are sensory nerve fibers (Strange-Vogsen *et al.,* 1997).

In mouse calvariae and in mouse calvarial osteoblasts, the presence of functional receptors for PACAP 27 and PACAP 38, linked to the enhanced formation of cyclic AMP, has been demonstrated (Lerner *et al.,* 1994). As described earlier (see VIP), VIP and PACAP share receptors. In addition to VIP-1R and VIP-2R, which bind VIP and PACAP with equal affinity, several reports describe the cloning of a high-affinity PACAP receptor (PACAP-R). PACAP-R were, within a year, cloned from rat, human (Ogi *et al.,* 1993), and bovine tissues (Miyamoto *et al.,* 1994). Moreover, PACAP-R were demonstrated to exist in six splice variant forms (Svoboda *et al.,* 1993; Journot *et al.,* 1995). PACAP is 100- to 1000-fold more potent than VIP in binding and stimulating adenylate cyclase activity in cells transfected with PACAP-R (Rawlings and Hezareh, 1996). PACAP-R have a widespread distribution in the CNS, with the highest levels being found in the olfactory bulb, the dental gyrus of hippocampus, pituitary, cerebellum, thalamus, and hypothalamus. Messenger RNA for PACAP-R has been detected in a variety of tissues, including liver, lung, spleen, and intestine (reviewed by Christophe, 1993; Arimura, 1992; Arimura and Shioda, 1995).

Concerning skeletal cells, the presence of PACAP-R and VIP-1R mRNA has been demonstrated in microisolated mouse marrow osteoclasts (Ransjö *et al.,* 2000). Using an AFM technique, both VIP and PACAP 38 showed high-affinity binding to rat marrow osteoclasts (Lundberg *et al.,* 2000). If rat osteoclasts express the same VIP/PACAP receptor subtypes as mouse osteoclasts is unknown. However, our preliminary observations that PACAP 38, as well as VIP and secretin, causes a decrease in isolated rat osteoclastic bone resorption indicate that VIP-1R may have a functional role in the regulation of osteoclast function. Whether PACAP-R also have a functional role in the rat osteoclasts is not known.

Similar to VIP, PACAP 38 inhibits $1,25(OH)_2D_3$-stimulated osteoclastogenesis in mouse bone marrow cultures (Mukohyama *et al.,* 2000b). PACAP 38 also decreases RANKL and RANK expression and increases OPG in these bone marrow cultures.

Although PACAP seems to interact with osteoblastic bone formation by regulating the expressions of the bone mineralization associated enzyme ALP and the release of IL-6, as well as with osteoclast activity and recruitment, further studies have to be performed to ascertain the role of PACAP in bone metabolism.

Calcitonin Gene-Related Peptide

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide belonging to a superfamily of peptides including CT, CGRP-I, CGRP-II, amylin, and adrenomedullin (Wimalawansa, 1997). One domain of the insulin β chain also shares homology with these peptides, indicating that they may have diverged from a common ancestral gene during evolution. CGRP is produced by tissue-specific alternative splicing of the initial gene transcript encoding the precursor for CT. Consequently, CGRP is produced, not only in nerve fibers, but also in thyroid parafollicular C cells, together with CT. However, CGRP and CT seem not to be released in parallel, probably due to the fact that plasma levels of CGRP have a neurogenic origin. Amylin is expressed predominantly in pancreatic β cells, whereas adrenomedullin is synthesized in several different tissues and is released from endothelial cells. CGRP and amylin both have an amino-terminal ring created by a disulfide bond; this is lacking in adrenomedullin, which has a linear amino-terminal extension.

CT, CGRP, amylin, and adrenomedullin act via related heptahelical receptors. Whereas a CT receptor was cloned already 1991 (Lin *et al.,* 1991), the receptors for CGRP, amylin, and adrenomedullin have been more elusive. CGRP and adrenomedullin both bind to a CT receptor-like receptor (CRLR), originally described as an orphan seven transmembrane receptor. Interestingly, CRLR requires interaction with single transmembrane proteins called receptor activity-modi-

fying proteins (RAMP; Foord and Marshall, 1999). Three different RAMPs (RAMP1, RAMP2, and RAMP3) have been cloned and sequenced and found to be expressed in a wide variety of tissues. Cotransfection of CRLR and RAMP1 results in CGRP responsive receptors, whereas cotransfection of CRLR and RAMP2, or RAMP3, leads to expression of adrenomedullin responsive receptors (Bühlmann *et al.,* 1999). RAMPs are expressed more abundantly than CRLR, suggesting that RAMPs could be involved in the regulation of other receptors than CRLR. Intestingly, RAMP1, or RAMP3, cotransfection with a CT receptor results in a high-affinity amylin receptor (Muff *et al.,* 1999). The expression of CRLR and RAMPs has not yet been studied in bone cells.

Receptors for CGRP, as assessed by a cyclic AMP response, have been demonstrated on the rat osteosarcoma cell line UMR 106-01 (but not on ROS 17/2.8), the human osteosarcoma cell line Saos-2, the mouse calvarial osteoblastic cell line MC3T3-E1, and enzymatically isolated osteoblastic cells from chick, rat, and mouse (Michelangeli *et al.,* 1986, 1989; Thiebaud *et al.,* 1991; Bjurholm *et al.,* 1992). Expression of receptors for CGRP seems to be a feature of the osteoblastic phenotype, as the degree of cyclic AMP formation in primary osteoblasts correlates with the activity of alkaline phosphatase and to the responsiveness to PTH (in terms of cyclic AMP formation; Michelangeli *et al.,* 1989). Receptors recognizing CGRP have been demonstrated on mouse bone marrow cells using radioligand binding (Mullins *et al.,* 1993). Activation of CGRP receptors in osteoblasts also leads to stimulation of phospholipase C and a transient rise of intracellular calcium (Drissi *et al.,* 1998; Kawase *et al.,* 1995) by mechanisms separate from those stimulating cyclic AMP (Drissi *et al.,* 1999; Aiyar *et al.,* 1999). Interestingly, the activation of CGRP receptors in osteoblasts also leads to an inhibition of calcium uptake (Kawase *et al.,* 1996). Using RT-PCR, CGRP receptors have been demonstrated in human periosteum-derived osteoblastic cells and in human osteosarcoma cell lines (Togari *et al.,* 1997). The presence of receptors recognizing amylin and adrenomedullin in osteoblasts is suggested by the observations that amylin stimulates cyclic AMP formation and that both peptides stimulates [^{3}H]thymidine incorporation into osteoblasts (Tamura *et al.,* 1992; Cornish *et al.,* 1995, 1997).

CGRP receptors are coupled to the stimulation of osteoblast proliferation and enhanced bone colony formation *in vitro* (Shih and Bernard, 1997a; Cornish *et al.,* 1999). An anabolic effect of CGRP *in vivo* is demonstrated by the findings that the targeted expression of calcitonin gene-related peptide to osteoblasts, under the control of the osteocalcin promotor, results in enhanced trabecular bone density, trabecular bone volume, and increased bone formation rates in mice (Ballica *et al.,* 1999). The same group has also reported that injection of CGRP into ovariectomized rats can partly prevent the bone loss caused by estrogen deficiency (Valentijn *et al.,* 1997).

Occupancy of amylin receptors in osteoblasts stimulates anabolic activities both *in vitro* and *in vivo.* Thus, amylin stimulates cell proliferation in osteoblast cell cultures (Cornish *et al.,* 1995, 1998; Villa *et al.,* 1997); amylin is more potent than CGRP but seems to act via a common CGRP/amylin receptor (Cornish *et al.,* 1999). Treatment of mice or rats with amylin leads to enhanced trabecular bone volume as a consequence of both increased trabecular thickness and number (Romero *et al.,* 1995; Cornish *et al.,* 1998). The increase of bone volume can also be achieved by injection of the amino-terminal fragment amylin 1-8, although the effect is less than that obtained by full-length amylin (Cornish *et al.,* 2000). Treatment with amylin leads to enhanced mechanical bone strength (Cornish *et al.,* 2000).

The stimulatory effects of CGRP and amylin on osteoblast cell proliferation are shared by adrenomedullin, which in fact is more potent than the other two peptides (Cornish *et al.,* 1997). The effect of adrenomedullin, similar to that of amylin, can be blocked by the amylin receptor antagonist amylin 8-37, indicating that adrenomedullin, amylin, and CGRP act via a common receptor. Injection of adrenomedullin into mice increases bone mass, as assessed by enhanced bone formation and increased mineralized bone area (Cornish *et al.,* 1997).

The strong anabolic effects of CGRP, amylin, and adrenomedullin in the skeleton raise the possibility that these peptides, or nonpeptidergic activators of their receptors, may potentially be useful in the treatment of diseases with bone loss, including osteoporosis. It should, however, be kept in mind that the *in vivo* anabolic effects of these compounds may not only be a consequence of their effects on bone formation, but may also be attributed to inhibitory effects on bone resorption.

The interest of CGRP in bone resorption was initially prompted by the findings that CGRP shows amino acid sequence homology to CT in the amino-terminal region and that CGRP is costored with CT in thyroid C cells. Injection of CGRP into rats and rabbits causes a hypocalcemic reaction (Tippins *et al.,* 1984; Roos *et al.,* 1986). The fact that CGRP inhibits bone resorption in fetal rat long bones (Roos *et al.,* 1986; D'Souza *et al.,* 1986; Tamura *et al.,* 1992) and in neonatal mouse calvariae (Yamamoto *et al.,* 1986) indicates that the decrease of serum calcium in intact animals is due to CGRP-induced inhibition of bone resorption. In the mouse calvarial system, rat CGRP-II is slightly more potent than rat CGRP-I, which is slightly more potent than human CGRP (Lerner, unpublished results). Similar to CT, the effect of CGRP is transient both in fetal rat long bones and in neonatal mouse calvariae (Roos *et al.,* 1986; Lerner, unpublished results). Inhibition of bone resorption can be due to inhibition of osteoclast activity and/or recruitment. An inhibitory effect of osteoclast activity has been observed in isolated rat osteoclasts as assessed by decreased pit formation (Zaidi *et al.,* 1987a,b). In this system, CGRP-I and -II are equipotent. The hypocalcemic effect of CGRP, as well as the inhibitory effects on bone resorption in organ culture and on osteoclastic pit formation, is mimicked by amylin (Datta *et al.,* 1989; MacIntyre, 1989; Zaidi *et al.,* 1990; Tamura *et al.,* 1992; Pietschmann *et al.,* 1993). Interestingly, adrenomedullin does not inhibit PTH-stimulated bone resorption in neonatal mouse calvariae (Cornish *et al.,* 1997; Lerner *et al.,* unpublished results).

Calcitonin-induced inhibition of bone resorption by isolated rat osteoclasts is associated with increased levels of intracellular Ca^{2+} and and cyclic AMP, as well as with rectraction and ceased motility of osteoclasts (Alam *et al.,* 1992b). At variance, inhibition caused by CGRP and amylin is associated with enhanced cyclic AMP and ceased motility, but not with increased intracellular Ca^{2+} and retraction (Alam *et al.,* 1991, 1992a,b). These observations have prompted the speculation that the effects of CT are mediated via two separate receptors and that CGRP and amylin act only via one of these receptors (the one linked to cyclic AMP and motility). Further support for this hypothesis are the observations that CGRP 8-37 (i) inhibits the effects of amylin and CGRP on motility and (ii) inhibits the effect of CT on motility, but not on retraction. This would imply that retracted osteoclasts are still capable of motility. In contrast, Cornish *et al.* (1998) could not observe any antagonistic effect by CGRP 8-37 (or amylin 8-37) on the amylin-induced inhibition of bone resorption in neonatal mouse calvariae (an observation confirmed in the authors laboratory), although the antagonists blocked the stimulatory effect by amylin on cell proliferation in the calvarial bones. In contrast to the observations by Zaidi and collaborators, we have, in a large series of experiments, been unable to observe any differences on osteoclast motility and retraction caused by CT, CGRP, and amylin. The issue of which receptors in osteoclasts (CT receptor, CRLP/RAMP1, CRLP/RAMP2, CRLP/RAMP3) are used by CGRP (and amylin) and how these receptors are linked to the mechanism causing inhibition of bone resorption still remains an open question. The observation made by Cornish *et al.* (1998) indicates that separate amylin receptors are present on osteoblasts and osteoclasts.

The degree of bone loss is not only dependent on osteoclast activity but also on osteoclast formation. Very few studies deal with effects of CGRP on osteoclastogenesis. Owan and Ibaraki (1994) have demonstrated the presence of CGRP receptors in mouse alveolar macrophages and that osteoclast formation in $1,25(OH)_2D_3$-stimulated cocultures of mouse alveolar macrophages and mouse calvarial osteoblasts is decreased by CGRP. Concomitantly, the number of macrophages was increased substantially. Akopian *et al.* (2000) reported that CGRP inhibits GM-CSF induced CFU-GM colony formation from unfractionated, as well as $CD34^+$, bone marrow cells via receptors blocked by CGRP 8-37. It was also shown tht CGRP inhibits osteoclast formation in bone marrow mononuclear cell cultures stimulated by $1,25(OH)_2D_3$. This response may be due to an effect of CGRP on stromal cells, on preosteoclasts, or on both cell types. The fact that CGRP inhibits CFU-GM colony formation from $CD34^+$ cells indicates that the effect, at least partially, is due to inhibition of preosteoclast proliferation/differentiation.

Effects of CGRP on bone may not only be due to direct effects on osteoblasts, preosteoclasts, and osteoclasts, but may also be mediated by cytokines/growth factors released from nearby cells regulated by CGRP. Thus, CGRP has been shown to increase IGF-I, both at the mRNA and at the protein level (Vignery and McCarthy, 1996), to decrease the production of TNF-α, and to increase that of IL-6 in fetal rat calvarial osteoblasts (Millet and Vignery, 1997).

Substance P

Primary human osteoblastic cells and osteosarcoma cell lines do not express mRNA for SP receptors (Togari *et al.,* 1997). At variance from this, neurokinin-1 (NK-1) receptors recognizing SP have been immunolocalized in rat bone osteoblasts (but considerably weaker than in osteoclasts; Goto *et al.,* 1998). Osteogenesis, as assessed by bone colony formation in bone marrow cell cultures, can be stimulated by SP (Shih and Bernard, 1997b). Thus, whether SP exerts any anabolic effects on bone still remains to be shown.

The abundance of NK-1 receptors in rat bone osteoclasts (Goro *et al.,* 1998) can be reconciled with observations that SP causes an acute rise of intracellular Ca^{2+} in rabbit osteoclasts and an increased pit area (but not an increase of pit number) excavated by osteoclasts when incubated on dentine slices (Mori *et al.,* 1999). The effects were blocked by two different NK-1 receptor antagonists. These observations indicate that SP may stimulate osteoclast activity by a direct effect on terminally differentiated osteoclasts. It is not known if SP may also affect osteoclastogenesis. In this context, it is interesting that SP stimulates the proliferation of fibroblastic cells, as well as production of stem cell factor and IL-1 in bone marrow cells (Rameshwar and Gascon, 1995; Rameshwar *et al.,* 1997).

Neuropeptide Y

NPY is often costored with noradrenaline (NA) in the same nerve fibers and it may therefore be of special interest that the NA-induced cyclic AMP rise in UMR 106-01 cells is inhibited by NPY (Bjurholm *et al.,* 1988b). However, this interaction is not specific for NA and NPY, as NPY also inhibits PTH stimulated cyclic AMP formation in UMR 106-01 cells. The mechanism by which NPY interacts with the noradrenergic and PTH-induced cyclic AMP formation is not known, but seems to be distal to the level of receptor–receptor cross-talk, because NPY also inhibits forskolin-induced cyclic AMP (Bjurholm *et al.,* 1992). The presence of NPY receptors in osteoblasts has been confirmed by Togari *et al.* (1997) showing the mRNA expression of NPY receptors in human periosteum-derived osteoblastic cells and in human osteosarcoma cell lines.

The mouse NPY receptor Y1 is present in two isoforms originating from a single gene. The Y1-α receptor has been found in several tissues, whereas the Y1-β receptor is expressed in bone marrow cells and in hematopoetic cell lines (Nakamura *et al.,* 1995). Whether NPY receptors are present in cells in the osteoclastic lineage is not yet known.

Opioid Peptides

The opioid family of peptides is synthesized from three different precursor molecules: proopiomelanocortin (POMC), proenkephalin (PENK), and prodynorphin (PDYN). Due to tissue-specific processing, different opioid peptides are produced, not only in the nervous and endocrine systems, but also in several other tissues, including the skeleton (Rosen *et al.,* 1998). Opioid peptides exert their opiate-like activity via three different receptors — μ-opioid, δ-opioid, and κ-opioid receptors — all of which are seven transmembrane G protein-coupled receptors linked to inhibition of adenylate cyclase.

Prompted by their initial observation that PENK mRNA is expressed highly and transiently during embryonic development in mesenchymal tissues, including bone and cartilage (Keshet *et al.,* 1989), Rosen *et al.* (1991) reported that PENK mRNA, but not POMC or PDYN mRNAs, is highly expressed in rat calvarial osteoblasts, in rat osteosarcoma cell lines ROS 17/2.8 and ROS 25/1, and weakly in mouse MC3T3-E1 osteoblastic cells and in human Saos-2 osteosarcoma cells. The expression of PENK mRNA in osteoblasts is decreased by $1,25(OH)_2D_3$, PTH, and TGF-β (Rosen *et al.* 1991, 1995, 1998). In addition, it has been shown that osteoblasts synthesize enkephalin-containing peptides, including Met-enkephalin. Using immunohistochemistry, Met-enkephalin has been demonstrated not only in bone cells and bone marrow cells, but also in skeletal nerve fibers (Elhassan *et al.,* 1998).

The fact that Met- and Leu-enkephalin, as well as Met-enkephalin-Arg-Phe, decrease alkaline phosphatase activity in ROS 17/2.8 cells indicates that osteoblasts are equipped with opioid receptors and that opioid peptides may act as local regulators of bone cell differentiation in an auto- or paracrine manner (Rosen *et al.,* 1991). The reciprocal interrelationships between osteoblast maturation (as assessed by alkaline phophatase) and PENK expression further indicate that opioid peptide expression is linked to osteoblast differentiation. Based on these observations, Rosen and Bar-Shavit (1994) have proposed the hypothesis that the retained capacity in the adult skeleton to synthesize PENK-derived peptides, in a defined population of undifferentiated cells, may be important in local remodeling of the skeleton, including fracture repair.

In contrast to the observations in rat osteoblasts, opioid receptor agonists such as morphine and DAMGO do not affect alkaline phosphatase activity in the human osteosarcoma cell line MG-63 (Pérez *et al.,* 1997). This was not due to an absence of opioid receptors, as $1,25(OH)_2D_3$-stimulated secretion of osteocalcin was decreased by morphine and DAMGO, an effect that was abolished by naloxone. This effect was, however, seen only at very high concentrations of agonists. Also, human osteoblast-like cells isolated from cancellous bone seem to be equipped with opioid receptors; Met-enkephalin inhibits cell proliferation by a mechanism sensitive to inhibition by the opioid receptor antagonist naltrexone (Elhassan *et al.,* 1998). No analysis of alkaline phosphatase was performed in these cell cultures and therefore it is not known if Met-enkephalin affects osteoblast differentiation in human bone cells.

The effect of opioid peptides on osteoclast activity is indicated by the observation that the synthetic analgesic opioid buprenorphine inhibits rat osteoclast activity, as assessed by the pit formation assay (Hall *et al.,* 1996). However, the effect seems unrelated to opioid receptors, as it was not shared by other opioid receptor agonists nor blocked by the opioid antagonist naloxone.

Interestingly, Elhassan *et al.* (1998) found that Met-enkephalin levels are decreased significantly in ankle joints from Lewis rats with adjuvant arthritis. Using immunohistochemistry, a significant decrease was observed in synovial type A cells. If Met-enkephalin levels were affected also in bone cells was not reported.

Somatostatin

Somatostatin receptors have been immunolocalized to metaphysis immediately adjacent to hypertrophic cartilage (Mackie *et al.,* 1990). Somatostatin-binding cells stain positive for alkaline phosphatase and are probably osteoblast precursor cells, suggesting that somatostatin may be involved in the regulation of osteoblastic differentiation during enchondral ossification. Mature osteoblasts, as well as osteoclasts and chondrocytes, are negative for somatostatin receptors, which are also lacking in membranous bones. Somatostatin receptor agonists do not affect basal or PTH-stimulated bone resorption in neonatal mouse calvariae (Lerner and Feyen, unpublished observations).

Receptors and Effects by Neurotransmitters in Bone

Catecholamines

The presence of adrenergic receptors on osteoblasts is indicated by the cyclic AMP response induced by norepinephrine in rat UMR 106-01 and ROS 17/2.8 osteosarcoma cell lines, in mouse MC3T3-E1 cells, and in the human osteosarcoma cell line Saos-2 (Bjurholm *et al.,* 1992). Interestingly, estrogen inhibits isoproterenol-induced cyclic AMP in MC3T3-E1 cells (Majeska *et al.,* 1994). In human osteoblasts and osteosarcoma cell lines, mRNA expression of β_1- and β_3- but not β_2-adrenergic receptors has been observed by Togari *et al.* (1997). When screening several human osteoblastic osteosarcoma cell lines and human primary osteoblast cDNA libraries, Kellenberger *et al.* (1998) were able to find expression, to different degrees in the different cell types, of β_1, β_2, and β_3 receptors; β_2 receptors were expressed in most cell types. β_2 receptors induced c-fos gene expression. In the ROS 17/2.8 cell line, radioligand binding and RT-PCR have demonstrated β_2- but not β_1-adrenergic receptors (Moore *et al.,* 1993).

α-adrenergic receptors stimulate cell proliferation and alkaline phosphatase in MC3T3-E1 cells (Suzuki *et al.,* 1998). Stimulation of alkaline phosphatase seems to be

mediated via p38 MAP kinase and cell proliferation via the ERK pathway (Suzuki *et al.,* 1999). These observations indicate a possible anabolic effect of catecholamines. However, epinephrine does not affect osteocalcin, or type I collagen, expression in MC3T3-E1 cells (Suzuki *et al.,* 1999); in ROS 17/2.8 cells, isoproterenol inhibits osteopontin expression (Noda and Rodan, 1989).

In mouse calvariae, norepinephrine (in the presence of a phosphodiesterase inhibitor and an antioxidant) stimulates calcium release (Moore *et al.,* 1993). It is not known if this response is due to enhanced osteoclast activity or osteoclast formation or if it is caused by the stimulation of adrenergic receptors in osteoblasts, preosteoblast, or terminally differentiated osteoclasts. However, the observations that catecholamines inhibit cell proliferation, increase tartrate-resistant acid phosphatase activity, IL-6 production, mutinuclearity, and the response to CT in the human osteoclast precursor cell line FLG 29.1 indicate that catecholamines can stimulate osteoclastogenesis via the activation of adrenegic receptors on preosteoclasts (Frediani *et al.,* 1996). Alternatively, the bone resorptive response may be mediated indirectly by cytokines, as isoproterenol induces IL-6 and leukemia inhibitory factor expression in osteoblasts, two cytokines known to stimulate bone resorption (Greenfield *et al.,* 1996).

Glutamate, Glutamate Receptors, and Glutamate Transporter

The activity of excitatory amines released into synapses is controlled by a family of homologous transporters, which are responsible for the reuptake of such amines into presynaptic terminals, a mechanism for the termination of synaptic transmission. To this group belong the glutamate/aspartate transporter (GLAST), dopamine, and serotonin transporters. The expression of these transporters in the nervous system is well known, but their presence in nonneural tissues has also been recognized. The expression of GLAST, dopamine, and serotonin transporters has been demonstrated in bone cells. Mason *et al.,* (1997) reported the expression of GLAST in osteocytes and osteoblasts, both at the mRNA and protein level, and the downregulation of its expression by mechanical loading using differential RNA display in samples from rat ulnae. Immunohistochemistry demonstrated that the GLAST expression in osteocytes disappeared after loading, whereas it was upregulated in periosteal osteoblasts at sites showing enhanced cellular proliferation and bone formation. The expression of GLAST is regulated *in vitro,* being downregulated during rat osteoblatic differentiation (Bhangu *et al.,* 2000). Osteoblasts are capable of taking up glutamate and releasing glutamate by calcium-sensitive mechanisms, similar to those used by neuronal cells. Skerry (1999) has put forward the hypothesis that glutaminergic signaling may be involved in the coupling between mechanical loading and anabolic events in the skeleton. The origin of glutamate in bone is, however, not fully known. Human and mouse osteoblasts are able to actively release glutamate (Genever and Skerry, 2000), but the possibility of glutamatergic innervation in bone is indicated by the immunolocalization of glutamate in skeletal nerve fibers (Serre *et al.,* 1999).

The action of glutamate is mediated by two different types of receptors: ionotropic receptors, which use the regulation of transmembrane ion fluxes as a signal-transducing mechanism, and metabotropic receptors, which are seven transmembrane-spanning domain, G protein-coupled receptors, using either stimulation of phospholipase C or inhibition of adenylate cyclase as intracellular signaling mechanisms. Ionotropic receptors can be subdivided into three groups based on their sensitivities to *N*-methyl-D-aspartate (NMDA), AMPA, and kainate.

Using immunohistochemistry, *in situ* hybridization, and radiolabeled ligand binding, NMDA receptors have been demonstrated in rat, rabbit, and human osteoclasts (Chenu *et al.,* 1998; Patton *et al.,* 1998; Itzstein *et al.,* 2000). The presence of functional NMDA receptors in osteoclasts has been confirmed using electrophysiological patch-clamp techniques (Espinosa *et al.,* 1999; Peet *et al.,* 1999). However, controversy exists as to whether these receptors can regulate the activity of terminally differentiated osteoclasts. Chenu and collaborators have reported that an antibody directed to the NMDA receptor 1 subunit, as well as four different specific NMDA receptor antagonists (D-AP5, MK 801, DEP and L-689,560), inhibits rabbit osteoclastic bone resorption and decreases the percentage of osteoclasts with actin rings, while having no effect on osteoclast adhesion or apoptosis (Chenu *et al.,* 1998; Itzstein *et al.,* 2000). In contrast, Peet *et al.* (1999) reported that the NMDA receptor antagonist MK 801 inhibits glutamate-induced current in rabbit mature osteoclasts, but has no effect on actin ring formation in these cells nor inhibits pit formation by mature rabbit and rat osteoclasts or basal ^{45}Ca release from neonatal mouse calvarial bones. The reason why similar observations were made on mature rabbit osteoclasts concerning NMDA receptor expression and electrophysiological function, while different results were obtained by both groups regarding the regulation of bone-resorbing activity, is presently unknown.

The possibility that glutamate NMDA receptors may be involved in the regulation of osteoclastogenesis has been suggested by Peet *et al.* (1999). MK 801 inhibits osteoclast size, nuclearity, TRAP expression, and resorptive activity in cocultures of mouse bone marrow cells and calvarial osteoblasts stimulated by $1,25(OH)_2D_3$, suggesting that osteoclastogenesis is dependent on constitutive glutamate signaling. Whether this signaling is dependent on glutamate receptors expressed by stromal cells/osteoblasts or preosteoclasts is not known. Interestingly, NMDA receptor subunit 1 knockout mice do not seem to have any skeletal phenotype (Peet *et al.,* 1999), which could be due to redundancy given the expression of the many different glutamate receptor subtypes.

Immunohistochemistry and *in situ* hybridization have revealed the expression of ionotropic NMDA receptors in rat and human osteoblasts (Chenu *et al.,* 1998; Patton *et al.,* 1998; Gu and Publicover, 2000). NMDA glutamate receptors

have also been demonstrated in the human osteoblastic cell lines MG-63 and Saos-2 using radioligand binding and electrophysiological assessements (Laketic-Ljubojevic *et al.,* 1999). Activation by glutamate resulted in increased levels of intracellular Ca^{2+} via a receptor sensitive to inhibition by MK 801, suggesting the presence of active NMDA receptors in these osteoblastic cell lines. Similarly, NMDA was found to increase intracellular Ca^{2+} in rat osteoblasts (Gu and Publicover, 2000). The functional significance of NMDA glutamate receptors in osteoblasts is, however, not yet known.

Gu and Publicover (2000) reported the presence of metabotropic glutamate receptor 1b (but not 2, 3, 4, 5, or 6) in primary rat osteoblasts using RT-PCR analysis. Activation of these receptors resulted in an elevation of intracellular Ca^{2+}. Interestingly, electrophysiologic analysis and fluorometric studies on intracellular Ca^{2+} showed interactions between ionotropic and metabotropic glutamate receptors in these cells, suggesting a complex glutamatergic signaling in bone cells.

Serotonin Receptors and Serotonin Transporter

Using RT-PCR and radiolabeled ligand binding, Bliziotes and co-workers (personal communication) demonstrated the expression of the serotonin (5-HT) transporter (5-HTT) in several different rat osteoblastic cell lines and primary rat osteoblasts. The functional expression of 5-HTT was confirmed by studies on [^{3}H]5-HT uptake in ROS 17/2.8 cells. In addition, it was shown that rat osteoblasts express four different 5-HT receptors: 5-HT1A, 5-HT1D, 5-HT2A, and 5-HT2B. Interestingly, the expression of 5-HTT increased during the differentiation of rat osteoblasts (Bliziotes *et al.,* 2000a), in contrast to that of GLAST (Bhangu *et al.,* 2000). These findings show that osteoblasts can both respond to and regulate 5-HT activity. However, the presence of serotinergic skeletal nerve fibers or serotonin-expressing bone cells, as well as the role of serotonin in bone biology, remains to be elucidated. The finding that 5-HT can potentiate PTH-stimulated cyclic AMP formation in immortalized osteoblasts (Bliziotes *et al.,* 2000a) indicates a possible role of 5-HT in PTH-stimulated bone resorption.

Dopamine Transporter

The dopamine transporter (DAT) is believed to control the activity of released dopamine (DA) into presynaptic terminals (or possibly other DAT-expressing cells). Mice deficient in DAT exhibit decreased bone mass due to diminished cancellous bone volume, increased trabecular spacing, and reduced trabecular volume (Bliziotes *et al.,* 2000b). The skeletal phenotype includes a reduction in cortical thickness, cortical strength, and decreased femur length. It is not yet known whether the osteopenic phenotype is due to DAT deficiency in bone cells or is mediated by indirect mechanisms. RT-PCR in UMR 106-01 and ROS 17/2.8 cells has failed to demonstrate mRNA for DAT in these osteoblastic cell lines. No data are available regarding the possible expression of DA in skeletal nerve fibers or bone cells. The fact that serum and urinary calcium and phosphorous, as well as circulating PTH, are normal indicates that the mechanism does not involve abnormalities in calcium and phosphorous homeostasis. The possibility may exist that the pathogenesis may, at least partly, be related to the decreased body weight or the anterior pituitary hypoplasia observed in *dat*$^{-/-}$ mice.

Receptors and Effects by Neurotrophins in Bone

Neurotrophic factors, including the neurotrophins NGF, BDNF, and NT-3, are known to play important roles in development of the central and peripheral nervous systems (Levi-Montalcini *et al.,* 1987). These factors are also known to promote the differentiation and survival of various types of neurons. The *trk* protooncogenes *trk*A, *trk*B, and *trk*C have been identified as receptors, linked to the activation of tyrosine kinase, for these neurotrophins. Thus, the neurotrophins selectively recognize these receptors and NGF, BDNF, and NT-3 bind to the products of *trk*A, *trk*B and *trk*C, respectively. Several neurotrophins have been found to be expressed in bone, and it has been suggested that these factors may have a role not only in bone-associated neuronal biology, but also in bone metabolism.

In the case of bone tissues, there are several reports of osteoblastic expression of neurotrophins and neurotrophin receptors. Nakanishi *et al.* (1994a) reported that the mouse osteoblastic cell line MC3T3-E1 expresses mRNA for NGF, BDNF, and NT-3 and that the expresssion levels were upregulated during differentiation. The rat ROS 17/2.8 cell line expresses mRNA for NGF, but not for BDNF, and NGF levels are increased by $1,25(OH)_2D_3$ (Jehan *et al.,* 1996). MC3T3-E1 cells have also been demonstrated to express mRNA encoding *trk*C, the receptor for NT-3 (Nakanishi *et al.,* 1994b). A functional role of this receptor was suggested by the observations that NT-3, but not NGF, stimulated the proliferation of MC3T3-E1 cells and calcium incorporation in the cell layers. Also, ROS 17/2.8 cells have binding sites for NGF (Jehan *et al.,* 1996), although the regulatory role of NGF receptors in these cells has not been assessed. Furthermore, it has been shown that exogenous NT-3 induces DNA-binding activities in MC3T3-E1 cells at several sites, including the cyclic AMP responsive element, partly due to activation of c-fos and c-jun (Iwata *et al.,* 1996). In addition, NGF enhances cell proliferation and the biosynthesis of proteoglycans during chondrogenesis in organ culture (Kawamura and Urist, 1988). These *in vitro* findings suggest that neurotrophins may participate in the regulation of bone formation as auto- or paracrine factors.

Studies of neurotrophins and neurotrophic receptor expression during fracture healing further support the idea of neurotrophic effects in bone. Increased sensory and sympathetic innervation during fracture healing has been reported in animal experiments (Hukkannen *et al.,* 1993). NGF has been immunolocalized in normal rat bone preferentially in osteoprogenitor cells. During fracture healing, however,

osteoprogenitor cells, as well as bone marrow stromal cells, osteoblasts, young osteocytes, and most of the chondrocytes in the callus, are expressing NGF protein (Grills and Schuijers, 1998). No NGF was seen in osteoclasts. In a large study including 70 rib-fractured mice, NGF, BDNF, and NT-3 were demonstrated in bone-forming cells at the fracture callus (Asaumi *et al.,* 2000). Interestingly, expressions of the three neurotrophins were increased during the process of healing, especially those of NGF and NT-3. Messenger RNA encoding their respective receptors, *trk*A and *trk*C, were also detected in the bone-forming cells at the fracture callus. An interesting speculation, made by the authors, is that the expression of NT-3 and *trk*C in osteoblast-like cells at the fracture callus and the increasing expression of NT-3 mRNA during the week after fracture indicate autocrine loop functions for the neurotrophic factor during fracture healing (Asaumi *et al.,* 2000). Such a view is supported by the findings that local application of NGF, at the site of fractured rat ribs, results in dramatically increased levels not only of norepinephrine and epinephrine, but also in increased healing rate and bone strength (Grills *et al.,* 1997). A possible local regulation of NGF expression in fracture sites by bone-derived molecules is suggested by the observation that BMP-2 (in the presence of TNF-α) strongly upregulates NGF in fibroblasts (Hattorl *et al.,* 1996).

Leptin Control of Bone Metabolism: A Role for the Central Nervous System in Bone Biology?

Leptin, the product of the *ob* gene, is a small polypeptide hormone produced by white adipose tissue, but also by several other organs, including placenta and fetal tissues. It influences body weight homeostasis through the effects on food intake and energy expenditure by negative feedback at the hypothalamic nuclei. In addition to the effects on the central nervous system, leptin has various physiological actions on lipid metabolism, hematopoiesis, ovarian functions, thermogenesis, and angiogenesis. The leptin receptor gene is widely expressed, with several splice variants. Leptin is known to exert its central effects through several neuroendocrine systems, including neuropeptide Y, glucagon-like peptide-1, and melanocortins (reviewed by Trayhurn *et al.,* 1999; Ducy *et al.,* 2000b).

Ducy *et al.* (2000a) presented evidence that leptin, through a hypothalamic relay, may control bone metabolism. To test this hypothesis, leptin-deficient (ob^-/ob$^-$) and leptin receptor-deficient (db^-/db$^-$) mice that are obese and hypogonadic have been studied. Despite hyogonadism and hypercortisolism, increased bone mass was seen in both mutant forms of mice, independent of obesity. Similar studies have also been performed in rats with essentially identical results (Holzmann *et al.,* 2000). Histomorphometric analysis indicates that leptin exerts its effect on osteoblastic bone formation and not on osteoclastic resorption.

An intracerebroventricular infusion of leptin in leptin-deficient mouse and wild-type mice caused bone loss. Interestingly, Ducy and colleagues could not find any differences in osteoblast number in the leptin-deficient mice or in the leptin receptor knockout mice, nor could any leptin receptors in osteoblasts be found, suggesting that the inhibitory action by leptin on bone formation has to be the result of an action on osteoblast differentiation via a central regulatory role by leptin. These findings point to the existence of a neuroendocrine pathway that controls bone mass. There is no information on what mediates the central leptin signal, and it is therefore unknown which mechanisms are involved at the local level of the bone metabolic unit. In contrast to the findings by Ducy *et al.* (2000a), there are reports of both leptin and leptin receptor expression in osteoblasts. The mouse calvarial osteoblastic cell line (MC3T3-E1) and a mouse chondrocytic cell line have been demonstrated to express mRNA encoding leptin (Kume *et al.,* 2000). In both cell types, mRNA for three of the leptin receptor splice variants was expressed (Sufang *et al.,* 2000). Moreover, human mesenchymal stem cells undergoing osteogenic differentiation are demonstrated to express leptin and functional leptin receptors (Bassilana *et al.,* 2000).

Taken together, these results point to a regulatory role of leptin in the control of bone mass. Further investigations are necessary to reveal if the regulatory pathway for leptin is of central nature or if a local loop may also be involved.

Experimental Denervation

The idea that there is a close interaction among the bone neural network, the regulation of bone cell activity, and skeletal turnover is supported by experimental denervation in animals. It has been shown that developmental skeletal growth in the rat hind foot is reduced after surgical denervation. In denervated animals, CGRP and SP immunoreactive nerve fibers were not observed in the perichondrium or periosteum of the metatarsal bones. Metatarsal bones on the contralateral unoperated side exhibited a normal pattern of innervation. The skeletal phenotype could not be due to decreased physical activity, as tendectomiesed control rats exhibited normal metatarsal bone lengths (Edoff *et al.,* 1997). These results indicate that sensory nerve fibers have growth-promoting effects on immature limb bones.

The possibility that neuropeptides may also influence the metabolism of adult skeleton is suggested by studies demonstrating a significant change in osteoclast numbers in jaw bones as a consequence of sensory and sympathetic denervation (Hill and Elde, 1991b). Treatment with guanethidine results in a dramatic decrease of the immunohistochemical staining for VIP, NPY, and DβH in the periosteum of mandible and calvariae in rats, indicating a sympathetic origin of these nerve fibers. This resulted in no change of bone formation, as assessed by periosteal apposition rate in tibiae, but a 50% increase of bone surface in mandible covered by osteoclasts. This could indicate that VIP, NPY, and/or catecholamines may have an inhibitory effect on osteoclast formation and/or activity. These results are in line with our

findings that both VIP and PACAP decrease osteoclastogenesis in mouse marrow cultures (Mukohyama *et al.,* 2000b). However, capsaicin treatment results in a 20% decrease of bone surface occupied by osteoclasts and, again, no effect on the periosteal apposition rate (Hill and Elde, 1991b).

In line with the effects of sympathetic depletions demonstrated by Hill and Elde (1991b), deprived sympathetic innervation of rat mandibular alveolar bones showed an increase of osteoclast number per sockets (Sandhu *et al.,* 1987). Moreover, the periosteal and endosteal apposition and mineralization rate was reduced in the sympathectomized jaw bones (Sandhu *et al.,* 1987). Because the jaw bones are unloaded, these bone metabolic effects cannot be due to decreased loading. Together, these data indicate that sympathetic neurons modulate bone resorption and bone remodeling *in vivo.*

Clinical Observations

Skeletal pain in patients with inflammatory and neoplastic disorders clearly suggests the existence of an extensive sensory nervous system in bone tissues. An increased fracture rate in paraplegic children due to myelomeningocele, subdural hematoma, spinal fractures associated with cord lesions, lumbrosacral root avulsion, transverse myelitis, and cord tumors indicates a role of the nervous system also in skeletal metabolism. Excessive callus formation during fracture healing in paraplegic patients further suggests a role of skeletal nerve fibers in bone metabolism (for references, see Lundberg *et al.,* 1999b). The fact that the neurotoxin thalidomide induces skeletal malformation further implicates the nervous system, not only in bone turnover and fracture healing, but also in embryonic skeletal development (McCredie and McBride, 1973).

Patients with tumors producing an excess of circulating VIP may develop hypercalcemia (Dohmen *et al.,* 1991; Lundstedt *et al.,* 1994). Although the pathogenesis is not known, the possibility may exist, given the capacity of VIP to stimulate osteoclast activity (Lundberg *et al.,* 2000), that VIP-induced enhanced bone resorption may be involved.

It is well known that a high proportion of patients with hip fractures previously have had stroke (Ramnemark, 1999). Skeletal fractures are also a frequent complication in paraplegic patients during rehabilitation. Most of these poststroke fractures are on the paretic side. Although a high incidence of falls may contribute to the high incidence of hip fractures, it has been suggested that decreased bone mass in the paretic side may be an important factor. Cross-sectional studies have all demonstrated reduced bone mass in the paretic side as compared to the nonparetic side (reviewed in Ramnemark, 1999). A prospective study found a time-dependent enhanced loss of bone mineral density in the paretic side during the first year after stroke (Ramnemark *et al.,* 1999a). Another prospective study for 4 months showed similar results (Hamdy *et al.,* 1995). The development of hemiosteoporosis is independent on weight changes after stroke (Ramnemark *et al.,* 1999b). The loss of bone in paraplegic patients is highest during the first 12 months but continues at least for 36 months (Ramnemark, 1999). The fact that the bone resorption marker carboxy-terminal telopeptide of type I collagen (ICTP) is increased in patients with hemiosteoporosis (Fiore *et al.,* 1999; Ramnemark *et al.,* in manuscript) and that osteocalcin, carboxy-terminal propeptide of type I collagen (PICP), and alkaline phosphatase are normal (Ramnemark *et al.,* in manuscript) indicate that the loss of bone in the paretic side is mainly due to enhanced bone resorption. Interestingly, both osteocalcin and PICP are increased significantly over a 12-month poststroke period (Ramnemark *et al.,* in manuscript), indicating the presence of high turnover osteoporosis in the paretic skeleton.

Patients with spinal cord injuries, similar to stroke patients, lose bone mineral contents in paralyzed areas of the skeleton (Biering-Sörensen *et al.,* 1988, 1990; Garland *et al.,* 1992; Wilmet *et al.,* 1995; Dauty *et al.,* 2000). The osteopenia is fastest in trabecular bone, showing a total loss of 50% in 18 months and then reaching a plateau phase. The decrease is slower in cortical bones, but continues for longer periods of time. The loss of bone in traumatic paraplegia is associated with an increase in urinary calcium, phosphate, hydroxyproline, and deoxypiridoline (Bergmann *et al.,* 1977–1978; Dauty *et al.,* 2000), indicating that enhanced bone resorption is an important pathogenetic mechanism, similar to the observations in stroke patients. No differences in serum levels of calcium, alkaline phosphate, or osteocalcin were observed. However, serum phosphate was increased.

It may be argued that hemiosteoporosis in paretic patients is due to disuse. However, the population of stroke patients studied by Ramnemark (1999) suffered from severe stroke and was therefore substantially immobilized and still developed local bone loss. Biering-Sörensen *et al.* (1988) reported that the decrease of bone mineral content seen in the lower extremities in patients with spinal cord injuries could not be prevented by spasticity or daily use of long leg braces. In the study by Dauty *et al.* (2000), patients with spinal cord lesions showed a 41% loss of bone mineral density in sublesional areas of the skeleton. However, there was no correlation among daily duration of sitting, daily verticalization, use of long leg braces, or bone mineral density. These observations suggest that osteoporosis in paretic patients cannot simply be classified as disuse osteoporosis. Thus, hemiosteoporosis may be caused by factors unrelated to lack of loading. This raises the possiblity that loss of innervation and local control of bone metabolism by skeletal neuro-osteogenic factors may play a role. Most interestingly, Demulder *et al.* (1998) showed that osteoclast formation in $1,25(OH)_2D_3$-stimulated cultured bone marrow from iliac crest (below the lesional level) is increased significantly as compared to osteoclast formation in sternal bone marrow cultures (above the lesional level) establised from paraplegic patients with spinal cord injuries. No such differences were seen in quadriparetic, quadriplegic, or healthy patients. Differences in the *ex vivo* osteoclast formation rate were observed in cultures established both

Table II Receptor Expression and Effects on Bone Cell Functions by Neuro-osteogenic Factors

Neuro-osteogenic factor	Receptors[a]		Bone cell functions		
	Osteoblasts	Osteoclasts	Bone formation	Bone resorption	Osteoclastogenesis
VIP	+	+	↑[b]	⇵[c]	↓[d]
PACAP	+	+	↑[b]	↓[e]	↓[d]
CGRP	+	+	↑[f]	↓[g]	↓[h]
SP	(+)	+	?	↑[i]	?
NPY	+	?	?	?	?
Met-enkef.	+	?	↓[j]	?	?
Somatostatin	+	–	?	?	?
NA/A	+	?	↑[k]	↑[l]	?
Glutamate	+	+	?	↑[m]	↑[n]
Serotonin	+	?	?	?	?
BDNF[o]	?	?	?	?	?
NGF[o]	+	?	↑[p]	?	?
NT-3[o]	+	?	↑[q]	?	?

[a]The presence of receptors has been indicated by mRNA expression, a rise of cyclic AMP/Ca^{2+}i in individual cells, or immunohistochemistry.

[b]VIP and PACAP stimulate ALP; VIP increases calcium accumulation in bone nodules.

[c]VIP causes an initial, transient , "calcitonin-like" inhibition followed by delayed stimulation of rat osteoclasts; VIP stimulates calcium release from mouse calvariae.

[d]VIP and PACAP inhibit osteoclast formation in mouse bone marrow cultures.

[e]PACAP inhibits rat osteoclast pit formation; a possible delayed stimulation has not been assessed.

[f]CGRP stimulates osteoblast proliferation and increases bone mass *in vivo.*

[g]CGRP inhibits bone resorption *in vitro* and causes hypocalcemia *in vivo.*

[h]CGRP inhibits osteoclast formation in human bone marrow cultures.

[i]SP stimulates rabbit osteoclast pit resorption area.

[j]Met-enkephalin, Leu-enkephalin, and Met-enkephalin-Arg-Phe decrease alkaline phosphatase activity in ROS 17/2.8 cells; Met-enkephalin inhibits human osteoblast proliferation.

[k]Epinephrine stimulates cell proliferation and alkaline phosphoatase in MC3T3-E1 cells.

[l]Norepinephrine stimulates calcium release in mouse calvariae.

[m]Glutamate receptor antagonists inhibit rabbit osteoclast resorption.

[n]Glutamate receptor antagonist inhibits osteoclast formation in mouse bone marrow cultures.

[o]BDNF, NGF, and NT-3 are expressed by osteoblasts.

[p]NGF increases fracture healing.

[q]NT-3 stimulates cell proliferation in MC3T3-E1 cells.

6 weeks and 12 months after the spinal cord lesion. The authors speculated that the deficiency of skeletal neuropeptides may be responsible for the enhanced osteoclastogenesis seen in bone marrow from paralyzed skeletal areas of paraplegic patients and have demonstrated that CGRP inhibits human osteoclast formation induced by $1,25(OH)_2D_3$ (Akopian *et al.,* 2000).

Clinical observations, together with findings using experimental denervation to knock out signaling molecules in the nervous system and *in vitro* and *in vivo* data showing effects of and receptor expression for neuropeptides, neurotransmitters, and neurotrophins (see Table II), strongly suggest that skeletal metabolism is controlled by neuro-osteogenic factors. In addition, the nervous system has been suggested to play an important role in the pathogenesis of osteoarthritis as well as rheumatoid arthritis (Vilensky and Cook, 1998; Cerinic *et al.,* 1998). Interestingly, mild mental stress, such as cage change or cold exposure, similar to injection of corticosterone, decreases plasma osteocalcin in rats (Patterson-Buckendahl *et al.,* 1988), suggesting that not only dramatic changes of the neuronal influence on the skeleton, but also more subtile fluctuations, may influence skeletal metabolism.

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CHAPTER 45

The Role of Insulin-like Growth Factors and Binding Proteins in Bone Cell Biology

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Introduction

Bone remodeling, a function of bone formation and resorption, depends on many factors, one of the most important being the growth-promoting activity of insulin-like growth factors (IGFs) (Thomas *et al.,* 1999). This chapter reviews what is currently known about key components of the IGF system in bone, including IGF peptides and receptors, IGF-binding proteins (IGFBPs), and IGFBP proteases. The focus is on local regulation and action. At the end we will present a model, based on data discussed herein, for how the IGF system could be involved in the autocrine/paracrine regulation of human bone formation. The interested investigator is encouraged to read the many excellent reviews on IGFs that delve into more detail on molecular, biochemical, and clinical topics and other aspects of IGFs in bone (Baylink *et al.,* 1993; Canalis, 1993; Daughaday and Rotwein, 1989; Delaney *et al.,* 1994; Rosen *et al.,* 1994; Schmid, 1995; Wood, 1995).

IGF Peptides

The insulin-like growth factors (IGFs: IGF-I, IGF-II) are 7-kDa polypeptides that share structural homology with pro-insulin (Zapf and Froesch 1986). Both factors are found in high concentration in serum; in addition, many mammalian cells synthesize and export IGFs. The skeleton is a major source of IGF-I and IGF-II through *de novo* synthesis by bone cells and by virtue of their release from the bony matrix during active skeletal resorption. IGFs can act in an autocrine/paracrine fashion in bone to regulate differentiated cell function. It is also likely that some skeletal IGF is derived from the circulation. In general, the relative proportion of IGF-I:IGF-II is maintained in both the serum and the skeleton of various species (Bautista *et al.,* 1990)

IGF Gene Structure

The IGF-I protein is a 70 amino acid single chain peptide. In mice, rats, and humans, the IGF-I gene consists of 6 exons and 5 introns, spanning more than 80 kB of chromosomal DNA (Rotwein 1991). In mice the IGF-I gene is located on chromosome 10 and in humans chromosome 12. Most mammals produce multiple IGF-I mRNAs through a series of steps, which include differential promoter usage, various transcription start sites, differential RNA splicing, and RNA polyadenylation (Rotwein, 1986). The two promoters in the mammalian IGF-I gene reside in close proximity and include part of exon 1 and 5′ to exon 2 (hence the names P1 and P2) (Rotwein 1986; Adamo *et al.,* 1989).

Exon 1 codes the initial 21 amino acids of the IGF-I signal peptide (Tobin *et al.,* 1990). P1, upstream from exon 1, is the major IGF-I promoter and is active in all tissues. Exon 2 codes the initial 6 amino acids for an alternative signal peptide; P2 governs transcripts primarily in the liver (Adamo *et al.,* 1991). Alternative splicing between exon 1 and 3 or exon 2 and 3 results in variable leader peptides. There are no classical TATAA or CAAT elements within P1, resulting in dispersed initiation sites for transcription of mRNAs that contain exon 1 (Rotwein, 1991). Promoter 2 also lacks a TATAA box and transcription initiates from two clusters in exon 2 located 1.8 kb from the 5′ end of exon 1 (Jansen *et al.,* 1983). There is a CACCC box more proximal to one transcription initiation site that is important for basal promoter 2 activity (Adamo *et al.,* 1991).

The IGF-II protein is a 67 amino acid single chain peptide with a linear organization similar to IGF-I. Like IGF-I it is synthesized as a precursor with an extended C-terminal E domain. The IGF-II gene consists of 10 exons and has been mapped to chromosome 11 in humans. In a manner similar to IGF-I, IGF-II transcripts are produced by the interplay of mechanisms that include differential promoter use, alternative transcription initiation sites, and variable RNA polyadenylation. However, unlike IGF-I, four different promoters have been identified in the human gene and three in the mouse. Overall, IGF-II is also much more active during prenatal life than IGF-I, whereas IGF-I is the principal regulator of pubertal growth in most mammals. In rodents, serum IGF-I concentrations exceed IGF-II, while in humans, it is exactly the opposite (Bautista *et al.,* 1990).

Regulation of IGF-I Gene Expression

Understanding the molecular regulation of IGF-I by growth factors and hormones remains a major challenge, yet it is critical for defining the role of IGF-I in differentiated osteoblast function and the effects of calcitropic factors such as PTH on target bone cells. Much of the transcriptional regulation in both soft and hard tissue is obscure and illustrates the complexity of the IGF-I gene. For example, growth hormone (GH) is one of the most potent regulators of hepatic and skeletal IGF-I synthesis, yet its mode of action is unknown. There are no identifiable GH responsive protein-binding sites near P1 or P2. GH does induce a DNase hypersensitive site within intron 2 of the IGF-I gene, and Benbassat *et al.* (1999), utilizing a C6 neuroblastoma cell line cotransfected with the GH receptor, demonstrated a GH responsive region of the IGF-I gene, which included exons 1, 2, and a fragment of exon 3, as well as introns 1 and 2. However, further studies will be needed to determine the significance of this finding. Several transcription factors have been identified that bind to and enhance the activity of P1 in hepatoma cells. These include C/EBP, HNF-1, and HNF-3 (Nolten *et al.,* 1995). Response elements within the IGF-I gene have also been identified. For example, a cyclic AMP response element (CRE) and a glucocorticoid responsive region have been noted in P1 (McCarthy *et al.,* 1995). Prostaglandins, in particular PGE_2, have been shown to regulate osteoblast production of IGF-I by binding C/EBP, which, in turn, acts via CRE at a location approximately 200 bp 5′ upstream of P1 (McCarthy and Centrella, 1994). Similarly, although the IGF-I promoters lack estrogen response elements, 17β-estradiol suppresses IGF-I gene activation by acting through receptor binding to C/EBP (McCarthy *et al.,* 1997). Glucocorticoids have also been shown to downregulate IGF-I expression in osteoblasts through a steroid response element located approximately 100 bp upstream of P1 (Delany and Canalis, 1995). It seems certain that there are other tissue-specific transcription factors that regulate IGF-I gene expression, although so far none have been mapped to either the P1 or the P2 region in any tissue.

Overview of IGF Regulations in Bone Cells

Osteoblast-like (OB) cells in culture express both IGF-I and IGF-II genes under the control of systemic and local factors. However, there are important qualitative and quantitative differences in IGF expression among the various OB cell models (Table I). Human OB cells produce primarily IGF-II, whereas rodent OB cells produce primarily IGF-I. Transformation of OB cells may alter IGF gene expression, particularly IGF-I. In addition, differences in the state of OB differentiation during *in vitro* conditions will determine the degree of expression and secretion of the IGFs. Also, it is clear that there is unique genetic programming of skeletal IGF expression within a given species. Rosen *et al.* (1997) demonstrated that for two healthy inbred strains of mice (C3H and C57BL6), of the same body length and size, serum and skeletal IGF-I content differed by as much as 30%. Moreover similar interstrain differences in IGF-I expression were observed when calvarial osteoblasts were

Table I IGF Gene Expression in Osteoblastic Cells[a]

	IGF-I mRNA	IGF-II mRNA
hOB	+	++
HOBIT	−	++
TE-85	−	++
U-2	+/−	++
MG-63	−	−
SaOS-2	+/−	+
rOB	++	+
MC3T3-E1	++	+

[a] hOB, normal adult human osteoblastic cells derived from trabecular bone; HOBIT, SV40-immortalized hOB; TE-85, U-2, MG-63, and SaOS-2, human osteosarcoma cell lines; rOB, normal osteoblastic cells derived from fetal or neonatal rat calvaria; MC3T3-E1, clonal mouse osteoblastic cell line.

maintained *in vitro.* Other work also suggests that the strain differences in IGF-I expression are related to differential promoter usage such that there is a more than twofold greater P2 expression in calvarial osteoblasts from C3H mice than B6 (Rosen, 2000; Adamo, personal communication). In sum, the rodent species, the particular inbred strain, the state of OB development, the degree of confluence, and the type of media for culturing all contribute to the variance in expression of IGF-I and therefore are important aspects to consider when selecting study models or interpreting reports on IGF gene expression in bone.

IGF-I and Bone Cells

The results of numerous studies on the regulation of IGF expression in bone cells are compiled in Table II for IGF-I and in Table III for IGF-II. Radioligand assays for IGF measurement in cell-conditioned media have been problematic historically (Bang *et al.,* 1994; Mohan and Baylink, 1995); therefore, the studies discussed focus on mRNA expression and/or validated peptide production. Because of their abundant expression of IGF-I, rodent OB cells have been the principal model for studying IGF-I regulation and action in bone. Expression of IGF-I by fetal rat OB cells is clearly under hormonal control. The major hormones that regulate the skeleton, including parathyroid hormone (PTH), estrogen, glucocorticoids, and 1,25-dihydroxyvitamin D, all have significant effects on skeletal IGF-I. Substantial evidence from *in vitro* and *in vivo* studies shows that the anabolic effects of PTH on rat bone are mediated largely through increased local IGF-I expression (Canalis *et al.,* 1989; Pfeilschifter *et al.,* 1995). PTH exerts its effect on IGF-I synthesis through increased cyclic AMP (cAMP) production (McCarthy *et al.,* 1990a). PTH and other potent stimulators of cAMP in OB cells, such as prostaglandin E_2 (PGE_2), increase IGF-I synthesis via increases in gene transcription (McCarthy *et al.,* 1989a, 1995; Pash *et al.,* 1995). β-Estradiol also enhanced IGF-I synthesis at the transcriptional level in rat bone cells transfected with estrogen receptors (Ernst and Rodan, 1991). As noted previously, no consensus estrogen responsive element has been identified within the cloned promoter regions of the IGF-I gene. Hence, it is likely that estrogen acts through the cAMP-dependent C/EBP pathway either as an inhibitor in some cell lines and species or as a stimulator of IGF-I transcription in rat and human osteoblasts (Ernst and Rodan 1991; McCarthy *et al.,* 1997). $1,25(OH)_2D_3$ has been reported to inhibit IGF-I expression in MC3T3-E1 cells (Scharla *et al.,* 1991), but to reverse inhibition by dexamethasone in primary cultures of rat OB cells (Chen *et al.,* 1991b). As OB cells differentiate *in vitro,* IGF-I secretion decreases (Bimbaum *et al.,* 1995); therefore, hormones such as $1,25(OH)_2D_3$ that induce differentiation may indirectly affect IGF-I expression. Extracellular calcium may also regulate IGF-I synthesis in cultured rodent cells (Canalis and Gabbitas, 1994; Canalis *et al.,* 1993; Hurley *et al.,* 1992; Sugimoto *et al.,* 1994).

Table II Regulation of IGF-I Expression in Osteoblastic Cells

Factor	Effect	Cell model
PTH	↑	rOB, MC3T3-E1
Estrogen	↑	rOB, hOB
Glucocorticoid	↓	rOB
$1,25(OH)_2D_3$	⇵	MC3T3-E1, rOB
PGE_2	↑	rOB
cAMP inducers	↑	rOB, hOB
TGFβ	⇅	hOB, rOB
bFGF	↓	rOB, MC3T3-E1
PDGF	↓	rOB
BMP-2	↑	rOB
Ca^{2+}	↑	MC3T3-E1

Table III Regulation of IGF-II Expression in Osteoblastic Cells

Factor	Effect	Cell model
BMP-2	↑	rOB
BMP-7	↑	SaOS, TE-85
TGFβ	↓	rOB
bFGF	↓	rOB, MC3T3-E1
PDGF	↓	rOB

Skeletal growth factors and cytokines may also regulate IGF expression in osteoblasts (see Table II). BMP-2 increases IGF-I and II mRNA expression in rat osteoblasts and may be a critical factor in early osteoblast recruitment within the remodeling unit. BMP-7 also has a very potent effect on both IGF-I and II production in bone cells, and antisense oligonucleotides of both IGF-I and II can block BMP-7-induced alkaline phosphatase expression. IL-6 may upregulate IGF-I expression in osteoblasts, while its effect on hepatic expression is the opposite. Prostaglandins regulate IGF expression and are produced locally by bone cells providing a major paracrine regulatory circuit in the skeleton. Finally, mechanical loading is a major stimulus to enhanced IGF-I expression in bone cells, possibly through the induction of PGI_2 and PGE_2. Strain-induced production of PGI_2 has been shown to immunolocalize to osteocytes whereas IGF-II is released. PGE_2, also generated by strain, tends to localize to osteoblasts and can induce the generation of either IGF-I or II. Overall, IGF-I and II appear to be important in mediating osteogenic strain, with differences noted in various experimental paradigms a function of the *in vitro* conditions as well as the cell model and species.

In contrast to the extensive work in rodent osteoblastic cells, very little is known about modulation of IGF-I expression in human bone cells (Okazaki *et al.,* 1995a). Studies indicate that forskolin (a potent cAMP inducer) and

transforming growth factor-β (TGFβ) treatment increase IGF-I mRNA expression in hOB cells (Okazaki *et al.,* 1995b), as does estrogen (Kassem *et al*., 1998), suggesting similar regulation of IGF-I expression in human and in rat OB.

IGF-II and Bone Cells

IGF-II is the most abundant mitogen produced by human bone cells, but little is known about its regulation. Unlike IGF-I, skeletal IGF-II synthesis does not appear to be regulated by systemic hormones or intracellular cAMP (McCarthy *et al.,* 1992; Okazaki *et al.,* 1995b). Nonetheless, differences in IGF-II expression occur and can significantly impact bone cell function (Durham *et al.,* 1994a). Skeletal factors produced by bone cells may regulate the synthesis of IGF-II (Gabbitas *et al.,* 1994; Hurley *et al.,* 1995). Studies implicate bone morphogenic proteins (BMPs), members of the TGFβ superfamily, as likely candidates for local IGF-II regulatory factors. BMP-7 stimulated IGF-II expression coincident with increased proliferation and alkaline phosphatase activity in human osteosarcoma cells (Knutsen *et al.,* 1995), and BMP-2 had similar effects in fetal rat calvarial-derived OB cells (Canalis and Gabbitas, 1994).

Mechanical stress, be it from physiological or pathophysiological load adjustments or from electrical stimulation, appears to be an important local regulator of IGF-II expression. Electric field stimulation of TE-85 human osteosarcoma cell proliferation was associated with increases in IGF-II mRNA and protein (Fitzsimmons *et al.,* 1992, 1995).

IGF Expression in Bone Cells *in Vivo*

Messenger RNAs encoding for IGF-I and, to a lesser extent, IGF-II are expressed in osteoblasts in trabecular bone during rat and mouse skeletogenesis (Shinar *et al.,* 1993; Wang *et al.,* 1995). Rat models have also verified regulation of IGF-I expression by PTH in bone cells *in vivo.* Watson *et al.* (1995) studied ovariectomized rats treated intermittently with PTH and found an anabolic effect of PTH on bone associated with increased IGF-I mRNA expression in trabecular OB. Middleton *et al.* (1995) performed *in situ* hybridization for IGF-I and IGF-II mRNA in adult human osteophyte tissue, i.e., bone tissue from the femoral heads of patients with osteoarthritis. IGF-II mRNA abundance was greater than that of IGF-I, and expression for both was highest in active osteoblasts. There was weak or absent mRNA expression in flat cells lining the quiescent bone surface and in cells of the bone marrow. Osteocytes were also negative for IGF-I and IGF-II. Andrew *et al.,* (1993) examined IGF gene expression in the normal human fracture during healing and similarly detected genes for IGF-I and IGF-II in OB at stages of active matrix formation and remodeling with a predominance of IGF-II compared with IGF-I gene expression. Again, both IGF-I and IGF-II mRNA were expressed in plump OB on active osteoid, and flat lining cells in trabecular bone showed no apparent IGF expression. This differential expression among cells of the osteoblast lineage strongly implicates IGFs in bone remodeling.

IGF-I Knockouts, Transgenics, and Targeted Trangenics: Inferences about IGF-I and BMD

Another approach to understanding the *in vivo* role of circulating and skeletal IGF-I in the acquisition of peak bone mass is to examine transgenic and knockout mice. Previous studies have demonstrated enhanced muscle mass and selective organomegaly for both GH and IGF-I transgenic mice in which IGF-I expression is enhanced (Mathews *et al.,* 1988). However, GH and IGF-I transgenics differ in their skeletal phenotypes, primarily in that overexpression of GH results in longer and bigger bones, diffuse organomegaly, and muscle hypertrophy; in contrast, IGF-I transgenics have selective organomegaly but normal bone length and mass. In part, differences in bone size may be a function of GH levels (i.e., high level in GH transgenics, suppressed levels of GH in IGF-I overexpression animals). In contrast to transgenics, IGF-I knockouts (−/−) usually die *in utero;* the few viable animals tend to be very small so that the finding of reduced bone mass might be confounded by partial volume effects of the measurement tool (Bikle, 2000). Targeted transgenics circumvent these confounding variables and provide organ-specific information, which is potentially useful in defining the effect of a single gene on tissue growth and development. Zhao and colleagues (2000) measured spine, femur, and total body using pDXA technology and femoral and vertebral volumetric BMD using pQCT in three lines of mice overexpressing rIGF-I targeted to bone by a human osteocalcin promoter. At 6 weeks of age, there were greater total body, femur, and vertebral BMD in hOCrIGF-I transgenics than their respective littermates. This difference was also noted by pQCT of the proximal femur where both trabecular and cortical BMD were significantly greater in the targeted transgenics than their littermates. These findings were subsequently confirmed by histomorphometric analysis of both cortical and trabecular bone specimens. Similar studies are currently underway utilizing targeting of IGF-I with the Col1A1 promoter, another means of selectively enhancing bone mass *in vivo.*

IGFS in the Bone Matrix

Bone is a major reservoir for the IGFs. Species specificity is maintained in this storehouse with IGF-I predominating in rodent bone and IGF-II in human bone (Bautista *et al.,* 1990). In fact, IGF-II is the most abundant of all the growth factors stored in human bone matrix (Mohan *et al.,* 1987), which is related in part to its binding to IGFBP-5 (see IGFBPs). Other matrix-associated growth factors include TGFβ and BMPs, as well as small amounts of basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) and, as noted in Tables II and III, these are potential regulators of IGF expression in OB cells. Although found at

concentrations proportional to the levels present in serum, IGFs in skeletal matrix are likely produced and deposited by the bone cells. Pfeilschifter *et al.* (1995) reported that the anabolic effect of intermittent PTH treatment of male rats to increase bone mineral density was accompanied by increases in bone matrix-associated IGF-I content. Intermittent PTH treatment had no effect on serum IGF-I levels in these experiments and in subsequent studies utilizing subcutaneous PTH as an anabolic agent in rodent or human studies. Thus skeletal expression of IGF-I in response to PTH is almost certainly a function of changes in local synthesis and deposition, rather than alterations in circulating concentrations.

Concentrations of the IGFs, as well as other growth factors, vary with changes in architecture in bone; hence there is proportionally more IGF-I in trabecular than cortical bone (Benedict *et al.,* 1994). Moreover, physiologic and pathological conditions can alter IGF content in the bone matrix. Aging, for example, has been shown to be associated with a decline in serum IGF-I and a significant reduction in both trabecular and cortical IGF-I content (Nicholas *et al.,* 1994; Boonen, 1996), whereas bone from patients with osteoarthritis has increased IGF-I and IGF-II content (Dequeker *et al.,* 1993; Nicholas *et al.,* 1994). These changes in matrix-associated IGFs may be significant in other conditions such as osteoporosis. Studies suggest a strong correlation between serum IGF-I and bone mineral density, especially in women, and there is evidence that women with the lowest quartile of serum IGF-I have a greatly enhanced risk of hip fractures independent of bone density (Langlois *et al.,* 1998; Gamero *et al.,* 2000). These findings would be consistent with parallel changes in both circulating and matrix IGFs, leading to lower bone mass and greater skeletal fragility. Murine studies support that tenet. Thus, even though there are differences in tissue-specific regulation of IGF-I, expression patterns for the liver and bone are similar at various times such as peak bone mass or old age (Rosen, 1997; Mora *et al.,* 1999).

Further evidence that IGF-I is important in the skeletal matrix comes from work by Slater *et al.* (1994), who demonstrated that fetal OB cells derived from human trabecular bone incorporate growth factors into extracellular matrix material *in vitro.* IGF-I, IGF-II, and TGFβ were focally deposited and colocalized in extracellular matrix. The intensity of IGF-II immunogold labeling of these human OB cells exceeded that of IGF-I, consistent with the predominant synthesis of IGF-II by human bone cells. Estrogen treatment of fetal hOB cultures increased growth factor incorporation into extracellular matrix. The authors reasoned that if IGFs and other growth factors released from extracellular bone matrix during osteoclast resorption serve as active bone growth factors (Farley *et al.,* 1987), then reduced growth factor incorporation in estrogen deficiency would lead to less growth factor released upon osteoclast resorption, resulting in decreased bone formation during subsequent remodeling. In this context, both deposition and release of IGFs from bone matrix may be regarded as physiological control points in local IGF bioavailability.

IGF Receptors in Bone Cells

IGFs produced by OB or released from bone matrix have the potential to stimulate proliferation and enhance osteoblastic activity. These effects are mediated through binding of IGF peptides to specific plasma membrane receptors identified on various OB cell models (Centrella *et al.,* 1990; Conover and Kiefer, 1993; Furlanetto, 1990; Raile *et al.,* 1994; Slootweg *et al.,* 1990; Wang *et al.,* 1995). The type I IGF receptor is a tyrosine kinase signaling receptor structurally related to the insulin receptor (Nissley and Lopaczynski, 1991). This receptor has preferential affinity for IGF-I and insulin. IGF-I type I receptor number is regulated by various hormonal factors, including I GF-I that downregulates its expression. PDGF, and basic fibroblast growth factor stimulate induction of the IGF type I receptor gene. Besides the well-known action of the type I IGF receptor on cell cycle progression, the IGF–IGF receptor complex has also been implicated in malignant transformation, as shown by high levels of expression in most tumor cell lines. The receptor may also be a target of oncogenes leading to increased receptor number. However, p53, a tumor suppressor, is a potent inducer of apoptosis and directly suppresses the type I IGF receptor promoter. IGF-I, when bound to the IGF type I receptor, prevents programmed cell death (Le Roith *et al.,* 1997) and therefore may be a critical factor in the life cycle of terminally differentiated cells like osteoblasts. Because IGF-I is plentiful in bone, and osteoblasts are the principle source of this peptide, it is likely that therapeutics aimed at enhancing bone formation work through the IGF system and prevent programmed cell death of osteoblasts.

The type II IGF receptor is identical to the mannose-6-phosphate (M-6-P) receptor (Nissley *et al.,* 1991). This receptor binds IGF-II and lysosomal enzymes with high affinity and does not bind insulin. The type II IGF/M-6-P receptor has no intrinsic tyrosine kinase activity but is involved in IGF-II receptor-mediated internalization and in lysosomal enzyme sorting and trafficking; these may be interrelated functions. This receptor has been implicated in insulin- or IGF-induced inhibition of protein catabolism (Kovacina *et al.,* 1989) and could play a role in bone resorption. Rydziel and Canalis (1995) reported that cortisol inhibited type II IGF/M-6-P receptor expression in fetal rat calvarial-derived OB cultures. Cell-associated IGFBP also bind IGFs. In U-2 and MG-63 human osteosarcoma cells, ^{125}IGF-1 binding is primarily to type I IGF receptor, whereas in hOB cells the majority of ^{125}IGF-I binds to cell-associated IGFBP (Conover and Kiefer, 1993; Conover *et al.,* 1996; Furlanetto, 1990). Both cell-associated and soluble IGFBPs are likely to have a profound influence on cell responsiveness and receptor signaling (see IGFBPs).

Effects of IGF on Osteoblasts *in Vitro*

IGFs increase DNA synthesis and replication of cells of the OB lineage and play a major role in stimulating differentiated function of the mature OB. *In vitro,* human and rodent

OB and osteosarcoma cells respond to ligand-activated type I IGF receptor stimulation with increases in DNA and protein synthesis (Canalis, 1993; Jonsson *et al.,* 1993; Raile *et al.,* 1994; Wergedal *et al.,* 1990). Both IGF-I and IGF-II increase type I collagen expression and decrease collagen degradation in fetal rat OB (Canalis *et al.,* 1995; McCarthy *et al.,* 1989b). Whether bone cells register a mitogenic or a differentiated response to IGF stimulus may reflect receptor population and receptor cross-reactivity and depend on cell type and OB lineage. For many of the same reasons, differential functions for IGF-I and IGF-II in bone have been difficult to define.

IGF-I may act in a bimodal fashion. During *in vitro* development of fetal rat calvaria, IGF-I is an autocrine mitogen of pre-OB, and, as pre-OB differentiate, IGF-I secretion decreases (Bimbaum *et al.,* 1995). A second rise in IGF-I secretion occurs later in OB development during matrix formation and mineralization. If this pattern exists *in vivo,* the secondary increase in IGF-I secretion by mature OB could lead to IGF-I sequestered in the bone matrix for release and action during subsequent remodeling cycles.

Effects of Locally Expressed IGF on Bone *in Vivo*

In vivo studies also point to an anabolic role for locally produced IGF-I in rat bone. Using *in situ* hybridization, Shinar *et al.* (1993) found a close correlation between IGF-I expression and osteogenesis during rat development, and estrogen treatment of ovariectomized rats resulted in decreased calvarial IGF-I mRNA levels that preceded a reduction in bone formation (Turner *et al.,* 1992). Lean *et al.,* (1995) undertook a novel study of genes expressed in rat osteocytes after a single, acute episode of dynamic loading to reproduce physiological strains in bone. This protocol induced bone formation on trabecular surface of the loaded bone. Because osteocytes are placed strategically to sense changes in strain distribution and initiate response to such stimuli, the investigators predicted a rapid expression of specific mRNA species in osteocytes after mechanical stimulation. They found IGF-I mRNA expression in osteocytes preceded increases in IGF-I expression and matrix formation in the overlying surface OB cells, providing evidence of a role for osteocytes and IGF-I in the osteogenic response of rat bone to mechanical stimuli. Further support for the importance of the local effects of IGF-I on skeletal acquisition are clearly illustrated by targeted transgenic models (see earlier discussion).

Effects of IGF on Osteoclasts

IGFs may play a role in regulation of bone resorption, although this aspect of the remodeling cycle has been less well investigated. Middleton *et al.* (1995) found that osteoclasts actively engaged in bone resorption expressed IGF-I, IGF-II, and type I IGF receptor mRNA. Type I IGF receptor mRNA was also identified in mature rabbit osteoclasts (Hou *et al.,* 1995), suggesting that osteoclasts may directly respond to IGFs. *In vitro,* IGF-I has been shown to promote the formation of osteoclasts from mononuclear precursors and to stimulate the activity of preexisting osteoclasts (Mochizuki *et al.,* 1992; Slootweg *et al.,* 1992). However, it has been suggested that these effects represent an indirect action of IGF on osteoclast activity via its effects on OB cells (Hill *et al.,* 1995). In that same vein, it has been demonstrated that stromal cells produce osteoprotogerin (OPG) and its ligand, OPGL. OPGL is responsible for activating osteoclasts and coupling resorption to formation, whereas OPG is a member of the TNF receptor superfamily and serves as an extramembrane "dummy receptor" binding OPGL. Studies by Rubin *et al.* (2000) demonstrate that physiologic doses of IGF-I (10 ng/ml) downregulate OPG expression but do not affect OPGL. Hence, the increase in osteoclast resorption with IGF-I may be a function of both direct activation of osteoclasts/osteoclast precursors and suppression of OPG synthesis, thereby making more OPGL available to its true receptor (RANK) on the osteoclast. This may also explain why the administration of rhGH or rhIGF-I to humans has been associated with a marked increase in bone resorption. Whether IGFs participate directly or indirectly in bone resorption *in vivo* remains an important issue to be resolved if we hope to understand and preferentially exploit the stimulatory effects of IGF on bone formation.

IGFBPs

From the foregoing sections, it is clear that IGFs are critical growth factors with active roles in bone formation, renewal, and repair. All cells involved in bone remodeling produce and/or respond to IGFs (pre-OB, OB cells, and osteoclasts). In addition, IGFs influence OB function at all stages of development (proliferation, differentiation, matrix production, and mineralization). However, IGF peptides and receptors are relatively ubiquitous. Any consideration of IGF action must take into account the special binding proteins that modify IGF bioactivity. Knowledge of skeletal IGF-binding proteins, their expression, regulation, and function is fundamental to our understanding of skeletal response to IGFs.

IGFBP Expression in Bone Cells

Six distinct yet structurally homologous IGFBPs have been characterized and designated IGFBP-1 through IGFBP-6 (Shimasaki and Ling, 1991). Wang *et al.* (1995) documented that OB localized in trabecular bone of the postnatal growth plate express IGFBP-2, -4, -5, and -6 mRNAs during the course of skeletogenesis in rat and mouse *in vivo.* All six IGFBPs have been found to be expressed by bone cells *in vitro,* but, like IGF peptide expression, IGFBP expression varies depending on cell type and culture conditions (Table IV). Normal human osteoblast-like (hOB) cells derived from trabecular bone

Table IV IGFBP Expression in Osteoblastic Cells

	BP-1	BP-2	BP-3	BP-4	BP-5	BP-6
hOB	+	−	++	++	++	+
HOBIT	+		++	+++	+	
TE-85	−	+++	−	++	−	
U-2	−	−	−	−	+++	
MG-63	−	+++	+	+	−	
rOB	−	++	+	+	+	
MC3T3-E1	−	+	−	++	+	+
UMR	−	−	−	++	++	−
ROS	−	−	−	+++	−	−

and SV40-immortalized hOB cells express mainly IGFBP-3, -4, and -5. IGFBP-2 expression is abundant in rat calvarial-derived OB and in the human osteosarcoma lines MG-63 and TE-85. U-2 human osteosarcoma cells secrete IGFBP-5 as the primary IGFBP. The rat osteosarcoma cell line ROS 17/2.8 secretes IGFBP-4 exclusively. UMR-106.01 rat osteosarcoma cells express IGFBP-4 and PTH-inducible IGFBP-5. These different osteoblast-like cell lines with their unique patterns of basal secretion and specific responses to hormonal stimuli provide valuable model systems for studying particular cellular and molecular aspects of IGF/IGFBP regulation and action (Hassager *et al.,* 1992).

Biological Effects of IGFBPs

In their native or recombinant state in solution, all six IGFBPs bind IGFs with high affinity, thereby preventing interaction with receptor and effectively inhibiting IGF action. However, there is increasing awareness that there is more to the IGFBP story than simple sequestering of growth factor. Posttranslational modifications produce dramatic changes in structure/function of the IGFBPs and, hence, the fate of IGFs. Moreover, it is difficult to assign a specific physiological role to any individual IGFBP. Ultimate cell response depends on cell phenotype, presence or absence of endogenous IGFs and other IGFBPs, posttranslational alterations of the IGFBP, extracellular matrix interactions, and other growth factors and cytokines. With these caveats in mid, we will present some of the biological studies on IGFBPs in bone cells.

IGFBP-1 can inhibit or enhance IGF action dependent on its phosphorylation state (Jones *et al.,* 1991). In addition, IGFBP-1 stimulates cell migration through interaction with integrins (Jones *et al.,* 1993b). Until now, IGFBP-1 has been considered the IGFBP least likely to play a significant role in bone remodeling. However, data suggest that IGFBP-1 expression in hOB cells is directly stimulated by high-dose glucocorticoid treatment and is associated with suppressed type I collagen (Okazaki *et al.,* 1994; Lee *et al.,* 1997). Because high levels of IGFBP-1 are noted in poorly controlled diabetics and in malnourished individuals, it is conceivable that suppression of bone formation noted in these conditions can be linked to locally high levels of IGFBP-1 expression in the skeleton (Lee *et al.,* 1997; Rosen and Donahue, 1998).

IGFBP-2 is a major IGFBP secreted by rat OB cells. Addition of recombinant human IGFBP-2 inhibited the actions of IGF-I on fetal rat calvarial OB replication and matrix synthesis (Feyen *et al.,* 1991). IGFBP-3 is another Janus-faced IGFBP with both inhibitory and stimulatory potential. In the intact form, exogenous IGFBP-3 is a potent inhibitor of bone cell growth (Schmid *et al.,* 1991). However, Ernst and Rodan (1990) found that accumulation of endogenous IGFBP-3 correlated with enhanced IGF-I activity in OB cells. The ability of cell-associated IGFBP-3 to modulate IGF action and the IGF-independent effects of IGFBP-3 that have been described for various cell systems have only begun to be explored in OB cells (Slootweg *et al.,* 1995).

IGFBP-4 was originally isolated from human bone cell culture media by Mohan *et al.* (1989) as "inhibitory IGFBP." Subsequently, IGFBP-4 has been shown to inhibit IGF-stimulated effects in a variety of bone cell models (Amamani *et al.,* 1993; Kiefer *et al.,* 1992; Mohan *et al.,* 1995). Intact, soluble endogenous or exogenous IGFBP-5 inhibited IGF-stimulated bone cell growth (Conover and Kiefer, 1993; Kiefer *et al.,* 1992). However, IGFBP-5 is not normally intact or in solution in the bone cell environment. IGFBP-5 is located preferentially in the extracellular matrix due to its strong affinity for hydroxyapatite where it appears to be protected from proteases (Bautista *et al.,* 1991; Canalis and Gabbitas, 1995). Bautista *et al.* (1991) have suggested that, in this form, IGFBP-5 serves to anchor IGF-II to the crystalline matrix of human bone. In fibroblasts, IGFBP-5 in the extracellular matrix is associated with the enhancement of IGF action (Jones *et al.,* 1993a). Andress and Bimbaum (1991, 1992) have shown that truncated IGFBP-5, originally purified from U-2 cell-conditioned media, will enhance the mitogenic potency of IGF-I or -II in mouse OB cultures. Interestingly, this truncated IGFBP-5 form also possesses intrinsic mitogenic activity, and preliminary evidence has been presented for an IGFBP-5 receptor on OB cells that could mediate the IGF-independent effects of IGFBP (Andress, 1995). Studies by Mohan *et al.* (1995) also demonstrated IGFBP-5 stimulation of IGF-induced bone cell proliferation. Preliminary studies using recombinant IGFBP-5 in intact animals and *in vitro* demonstrated significant enhancement of bone formation (Richman *et al.,* 1999).

IGFBP-6 is unique among the IGFBPs in its selective affinity for IGF-II over IGF-I (Kiefer *et al.,* 1992; Shimasaki and Ling, 1991). IGFBP-6 expressed by bone cells has the potential to specifically influence IGF-II action. Addition of recombinant human IGFBP-6 preferentially blocked IGF-II-stimulated DNA synthesis in rat bone-derived PyMS cells (Schmid *et al.,* 1995).

Regulation of IGFBP Expression in Bone Cells

Production of IGFBPs by bone cells is regulated by both systemic and local effectors of bone metabolism (Table V). Critical IGFBPs in the normal bone remodeling process appear to be IGFBP-4 and IGFBP-5. Along with IGF-I, these growth factors and binding proteins play a pivotal role in bone remodeling. A summary of the factors that regulate these three peptides are noted in Table VI. However, it should be pointed out that the regulation of IGFBP expression in human and animal model systems differs in various OB systems according to the species (or inbred strain) and the *in vitro* conditions for culturing. So, for example, retinoic acid may enhance IGFBP-5 expression in rat osteoblasts, but decrease it in human osteoblasts. Although the significance of these findings is not clear at present, conclusions from *in vitro* studies must always be viewed with some caution.

IGFBP-4 gene expression in OB cells appears to be a favored target for hormones that regulate the skeleton. PTH increases IGFBP-4 mRNA and protein expression in hOB and UMR-106.01 cells via a cAMP-dependent pathway (Conover *et al.,* 1993a; La Tour *et al.,* 1990). Gao *et al.* (1993) and Strong *et al.* (1993) have characterized cAMP responsive elements in rat and human IGFBP-4 promoter regions, respectively, suggesting that intracellular cAMP modulates IGFBP-4 gene transcription. $1,25(OH)_2D_3$ and estrogen also increase, whereas cortisol decreases, IGFBP-4 mRNA expression in human osteoblastic cells (Kassem *et al.,* 1996; Okazaki *et al.,* 1994; Scharla *et al.,* 1993), but the mechanisms are uncertain. Some of these hormonal effects on IGFBPs may be secondary to effects on pre-OB differentiation. Proliferation and differentiation correlate with changes in IGFBP expression and secretion. In fetal rat calvarial cultures, maximum IGFBP-2 and IGFBP-5 expression is characteristic of proliferating pre-OB, and maximum expression of IGFBP-3, -4, and -6 is associated with the mature differentiated OB phenotype (Bimbaum and Wiren, 1994).

Table V Regulation of IGFBP mRNA Expression in Osteoblastic Cells

Factor	Effect	Cell model
PTH	↑ BP-4, ↑ BP-5	hOB, UMR
Estrogen	↑ BP-4	hOB
$1,25(OH)_2D_3$	↑ BP-3, ↑ BP-4	MG-63, hOB, TE-89, SaOS
Glucocorticoid	↑ BP-1, ↓ BP-2, ↓ BP-3, ↓ BP-4, ↓ BP-5	hOB, rOB, MG-63, TE-89
Insulin	↓ BP-1	hOB
Growth hormone	↑ BP-3, ↑ BP-5	rOB
Retinoic acid	↑ BP-5, ↑ BP-6	rOB, SaOS
PGE_2	↑ BP-3, ↑ BP-4, ↑ BP-5	rOB
TGFβ	↓ BP-4, ↓ BP-5	hOB, rOB
BMP-2	↓ BP-5	rOB
BMP-7	↑ BP-3, ↓ BP-4, ↑ BP-5	MG-63, TE-85, SaOS
bFGF	↓ BP-4, ↓ BP-5, ↓ BP-6	MC3T3-E1, rOB
PDGF	↓ BP-5	rOB
IGF	↓ BP-1, ↑ BP-5	hOB, rOB, UMR

PTH also induces IGFBP-5 mRNA expression in UMR-106.01 rat osteosarcoma cells by a cAMP-dependent mechanism (Conover *et al.,* 1993a). In these bone cell cultures, PTH and IGF-I interact to increase extracellular IGFBP-5 through distinct mechanisms: PTH increases *de novo* synthesis of IGFBP-5 and IGF-I enhances accumulation of the secreted protein. Stimulation of cAMP production increased IGFBP-5 expression in hOB cells, although PTH had no significant effect presumably due to the weak cAMP response generated by PTH in these cells. As discussed in a preceding section, IGF-I is responsible for the stimulatory effect of PTH on rodent bone collagen synthesis when the hormone is delivered in an intermittent fashion. However, continuous administration of PTH overrides IGF-dependent stimulation (Canalis *et al.,* 1989). Perhaps PTH-induced

Table VI Summary of Critical Factors Regulating IGF-I and IGFBPs in Bone Cells

Regulatory agent	Effect on IGF-I	Effect on IGFBP-4	Effect on IGFBP-5
PTH	Increase	Increase	Increase
Estrogen	Increase	Increase	Not determined
PGE_2	Increase	Increase	Increase
Glucocorticoids	Decrease	Decrease	Decrease
TGFβ	Increase/Decrease	Decrease	Decrease
FGF	Decrease	Decrease	Decrease
PDGF	Decrease	Not determined	Decrease
BMP-7	Increase	Decrease	Increase/decrease
IL-6	Increase	Not determined	Increase
IGFs	Decrease	Decrease	Increase

increases in IGFBPs partially explain why prolonged treatment with PTH inhibits collagen synthesis, despite an increase in local IGF-I.

Glucocorticoids decrease expression of IGFBP-3, IGFBP-4, and IGFBP-5 and increase expression of IGFBP-1 in hOB cells (Okazaki *et al.,* 1994). Similar glucocorticoid-induced decreases in IGFBP-2 through -5 expression have been reported for other OB cell models (Chen *et al.,* 1991a; Nakao *et al.,* 1994). In contrast, glucocorticoid regulation of IGFBP-1 in bone cells is specific for untransformed hOB cells (Conover *et al.,* 1996). Furthermore, insulin inhibits both basal and glucocorticoid-induced IGFBP-1 expression in hOB cells. Growth hormone stimulates IGFBP-3 and IGFBP-5 production in rat osteoblasts without affecting IGF-I expression (McCarthy *et al.,* 1994; Schmid *et al.,* 1994), and new data indicate upregulation of IGFBP-5 and IGFBP-6 by retinoic acid (Dong and Canalis, 1995; Zhou *et al.,* 1995).

Evidence is accumulating regarding the regulation of IGFBPs by local skeletal factors such as PGE_2, TGFβ, BMP, bFGF, PDGF, and IGFs (Canalis and Gabbitas, 1995; Conover *et al.,* 1993a; Dong and Canalis, 1995; Durham *et al.,* 1994b; Gabbitas and Canalis, 1995; Hassager *et al.,* 1992; Hurley *et al.,* 1995; Knutsen *et al.,* 1995; McCarthy *et al.,* 1994; Schmid *et al.,* 1992). Canalis and co-workers (1995) have observed that local growth factors with mitogenic properties inhibit IGFBP-5 expression in cultured rat OB cells and agents that induce rOB differentiated function enhance IGFBP-5 expression. In addition, it could be speculated that the resistance to IGF induced by skeletal unloading involves mechanical stimulation of IGFBP expression (Bikle *et al.,* 1994).

IGFBP Proteases

IGFBP bioavailability is determined not only by gene expression, but also through limited proteolysis of the secreted IGFBP. Indeed, local IGF action may be largely controlled by this mechanism. IGFBP proteases that alter the high-affinity binding between IGFs and individual IGFBPs and are activated by particular physiological states have been identified in several human bone cell systems.

IGFBP-4 Proteolysis in Bone Cells

It had been noted by several investigators that IGF-I treatment of normal hOB cells results in a loss of IGFBP-4 in serum-free media, as determined by ligand blot analysis (Durham *et al.,* 1994). Further investigation of this phenomena revealed that the IGF-induced decrease in IGFBP-4 was not due to a decrease in IGFBP-4 mRNA expression or secretion. Rather, the effect could be reproduced in a cell-free assay, suggesting that hOBs secreted a protease that could cleave IGFBP-4, thereby enhancing the biologic activity of the bound IGFs. A novel IGFBP-4-specific protease was subsequently identified in media condition by hOB cells in 1994 (Durham *et al.,* 1994; Kanzaki *et al.,* 1994). This protease was a calcium-requiring metalloprotease that cleaves IGFBP-4 at a single site, attenuating inhibition of IGF action by IGFBP-4 (an inhibitory IGFBP) (Conover *et al.,* 1993; Conover *et al.,* 1995). The IGFBP-4 protease was dependent on IGFs for its functional activity, with IGF-II being more effective than IGF-I. Overexpression of IGF-II conferred constituitive IGFBP-4 protease activity in a subset of hOB cells (Durham *et al.,* 1995). Subsequently, it was found that TGFβ also regulated IGFBP-4 protease in hOB cells (Durham *et al.,* 1994). However, unlike IGF-II, TGFβ did not directly affect proteolysis in cell-free assay, but rather treatment with TGFβ in hOB cells enhanced IGF-dependent IGFBP-4 protease activity in the condition media. TGFβ may also stimulate hOB cell expression and/or secretion of the enzyme. In 1999, Conover and colleagues isolated the protease synthesized by human fibroblasts and osteoblasts as pregnancy-associated plasma protein-A (PAPP-A) (Lawrence *et al.,* 1999). PAPP-A is generated in various osteoblastic cell lines but its greatest expression is in osteoprogenitor cells. Interestingly, IGFBP-4 is the only IGFBP substrate for this protease, which is active in a broad pH range of 5.5–9.0. Estrogen has been shown to decrease IGF-dependent protease IGFBP-4 proteolysis in estrogen responsive cells, although it is unclear whether it works to decrease protease expression or increase inhibition (Kassem *et al.,* 1996). IGFBP-4 proteolysis can also be controlled by inhibitors produced by bone cells. Treatment of hOB cells with phorbol ester tumor promoters or transfection with SV40 T antigen induces a cycloheximide-sensitive inhibitor of the IGFBP-4 proteolytic reaction (Durham *et al.,* 1995b), suggesting an association with early transformation processes. As representative of the fully transformed OB phenotype, U-2, MG-63, and TE-85 human osteosarcoma cells secrete neither IGFBP-4 protease nor protease inhibitor. Thus, transformation appears to alter the IGFBP-4 protease system in bone cells.

IGFBP-5 Proteolysis in Bone Cells

U-2 osteosarcoma cell-conditioned medium readily degrades exogenous and endogenous IGFBP-5 due to a cation-dependent serine protease specific for IGFBP-5 (Conover, 1996; Conover and Kiefer, 1993; Kanzaki *et al.,* 1994). In contrast to their stimulatory role in IGFBP-4 proteolysis, IGFs attenuate IGFBP-5 proteolysis in U-2 cells (Conover and Kiefer, 1993). IGF-regulated IGFBP-5 proteolysis has also been identified in hOB cell-conditioned media (Durham *et al.,* 1994a), and IGFBP-5 protease activity varied during murine OB development (Thraikill *et al.,* 1995). In its various forms, IGFBP-5 may have numerous functions in bone. When intact and soluble, IGFBP-5 inhibits IGF-I action in bone cells *in vitro.* In osteoblasts, in which the IGFBP-5 protease has been identified, a truncated form of IGFBP-5 possesses intrinsic mitogenic activity, possibly acting through a putative IGFBP-5 receptor (Kanzaki *et al.,* 1994; Andress, 1998). In addition, secreted

IGFBP-5 that is not immediately proteolyzed appears to be localized preferentially in the extracellular matrix as the intact form, and in this state is associated with enhanced IGF action. IGFBP-5 also serves the unique function of fixing the IGFs in the bone matrix by virtue of its high affinity to hydroxyapatite.

Other IGFBP Proteases in Bone Cells

MG-63 human osteosarcoma cells secrete an acid-activated IGFBP-3 protease identified as the aspartic protease, cathepsin D, based on acidic pH optimum, inhibition by pepstatin, distinctive proteolytic fragment pattern, and immunoreactivity with cathepsin D antisera (Conover and De Leon, 1994). Acid-activated cathepsin D is not IGFBP specific and will proteolyze IGFBP-1 through -5 (Conover *et al.,* 1995b). IGFs may influence this system as well, as IGF-II modulates type II IGF/M-6-P receptor-mediated binding and uptake of cathepsin D (Nissley *et al.,* 1991). Plasmin is another highly active IGFBP protease in MG-63 osteosarcoma cells. Lalou *et al.,* (1994) demonstrated that IGF-I treatment of MG-63 cells decreased protease activity toward IGFBP-3 via inhibition of plasminogen conversion to plasmin. Plasmin will also degrade IGFBP-5 (Campbell *et al.,* 1995).

Other IGFBP proteases identified in bone cell models include matrix metalloproteases, also under IGF control (Delany *et al.,* 1995; Thrailkill *et al.,* 1995).

It is interesting that we are finding IGFs themselves to be major regulators of IGFBP proteolysis, acting at different levels and by various molecular mechanisms. By modulating IGFBP-specific proteases, skeletal IGFs may autoregulate their biological activity. These highly regulated positive and negative feedback systems could ensure temporal and spatial specificity of the bone response to critical growth factors.

Model

It is generally accepted that IGFs have a defining role in bone remodeling, but just what that role is still remains to be defined. Figure 1 represents a conceptualization of how the various components of the IGF system might interact in the bone cell microenvironment. This model is based on data

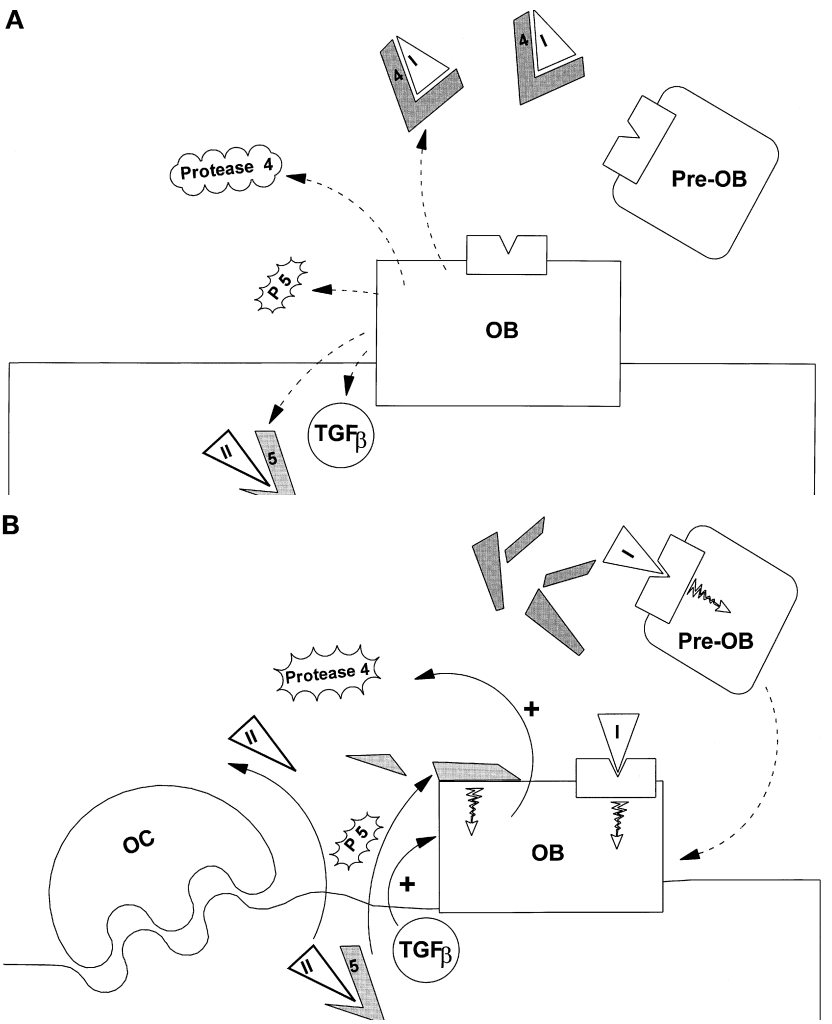

Figure 1 Model of the IGF system in human bone: (A) basal state and (B) osteoclast resorption.

derived from human bone cell studies and incorporates the following data presented in this chapter. (1) hOB cells contain cell surface receptors for IGF-I and respond to receptor activation with increased DNA synthesis and matrix production. IGF-I is more potent than IGF-II in activating type I IGF receptor signaling. (2) hOB cells secrete IGFBP-4 and functionally dormant IGFBP-4 protease. IGF-II is more effective than IGF-I in activating IGFBP-4 proteolysis. (3) hOB cells secrete IGFBP-5 and active IGFBP-5 protease. (4) IGFBP-5 is targeted to matrix and binds IGF-II. (5) IGF-II is the most abundant and TGFβ the second most abundant growth factor stored in human bone matrix.

According to this model, hOB cells are relatively unresponsive to IGF-I stimulation in the basal state (Fig. 1A), but perturbations at any point could result in major changes in response characteristics. Matrix resorption is an example (Fig. 1B). Release of IGFBP-5 during bone resorption by osteoclasts provides a substrate for the IGFBP-5 protease secreted by hOB cells, abolishing the inhibitory activity of intact IGFBP-5 and perhaps generating mitogenic forms of IGFBP-5. Proteolyzed IGFBP-5 has reduced affinity for IGF-II, thereby freeing this growth factor to initiate IGFBP-4 proteolysis. IGFBP-4 cleavage increases IGF-I availability to receptors on OB and pre-OB cells, resulting in stimulation of collagen synthesis and proliferation. TGFβ, released from the matrix in concert with IGF-II and IGFBP-5, could amplify this response by stimulating hOB cell expression/secretion of IGFBP-4 protease. TGFβ increases IGF-I expression by hOB cells as well. Thus, bone resorption initiates an IGF-dependent process, culminating in site-specific bone replacement during remodeling.

This model offers a molecular mechanism for the coupling process that has been proposed by several investigators (Farley *et al.,* 1987; Parfitt, 1984; Rodan and Martin, 1981) and extends previous models put forth by Mohan and Baylink (1991) and Bautista *et al.,* (1991) that include IGF-II and IGFBP-5 as important components in this coupling mechanism. A corollary to this model is that the amount and nature of growth factors stored in bone are determining factors, and alterations in the deposition of growth factors influence the remodeling cycle. Other scenarios can be derived from this model. Changes in synthesis of IGF-II could impact bone formation by its direct autocrine activity, through its deposition in matrix for future action, and in its role to activate IGFBP-4 proteolysis increasing IGF-I available for autocrine/paracrine stimulation. Also, differentiation signals could use this system by altering IGF and IGFBP gene expression to a affect a switch in osteoblast behavior from proliferation to differentiation.

Concluding Remarks

IGFs are abundant in the bone microenvironment. They are produced by bone cells and released from bone matrix to act as autocrine/paracrine regulators of bone formation. Synthesis of skeletal IGFs is regulated by hormones, growth factors, and mechanical stress. Deposition and resorption are also physiological points of regulation of IGF availability that need to be further explored. Activity of the local IGFs can be modified by IGFBPs, which are produced by bone cells and regulated by some of the same agents that modulate IGF synthesis, as well as by specific proteases. Interestingly, proteolytic modification of IGFBP structure/function can be regulated by IGFs. This IGF regulation of IGFBP availability is essential to our overall understanding of IGFs in bone metabolism and growth and of practical importance when considering IGFs as therapeutic agents. Finally, an understanding of the interaction among all the components of the IGF system is likely to be important regarding IGF action in bone cells and needs to be fit into the complex interplay among systemic skeletal factors and locally acting growth factors, cytokines, and extracellular matrix that undoubtedly occurs *in vivo.*

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CHAPTER 46

Platelet-Derived Growth Factor and the Skeleton

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Platelet-derived growth factor (PDGF) is a polypeptide with a molecular mass of 30 kDa. PDGF is composed of two polypeptide chains, which may be combined in a homodimeric or heterodimeric form. The chains are the products of distinct but related genes, PDGF A and PDGF B, thus PDGF can exist as either a PDGF AA or BB homodimer or as a PDGF AB heterodimer (Heldin and Westermark, 1989; Westermark and Heldin, 1993). The PDGF B gene shares extensive sequence homology with p28-*sis,* the oncogene product of the simian sarcoma retrovirus (Doolittle *et al.,* 1983). The PDGF A gene is localized to chromosome 7 and is related to the PDGF B gene so that mature PDGF A and B chains share 56% homology in their amino acid sequence (Betsholtz *et al.,* 1986). The homo- and heterodimeric forms of PDGF have the same biological activities, although PDGF BB is more potent than PDGF AA and PDGF AB has intermediate effects in most biological assays.

PDGF was initially isolated from human platelets, but its presence was subsequently found in selected tissues, including bone. In the circulation, PDGF is transported in platelet granules, where its composition is 70% PDGF AB heterodimer, 25% PDGF BB, and 5% PDGF AA homodimer (Hammacher *et al.,* 1988; Hart *et al.,* 1990). PDGF A and B genes are expressed in normal and malignant cells, where they act as local regulators of cell growth. As a local factor, PDGF is expressed primarily in endothelial cells, where it is considered to act as a local regulator of vascular cell function. Because PDGF is present in platelets and is expressed by various cell types, it is considered to act both as a systemic and as a local regulator of cell metabolism. PDGF may become available to a variety of tissues following platelet aggregation, whereas the expression and regulation of the locally synthesized PDGF are specific and cell type dependent.

Synthesis of PDGF by Skeletal Cells

PDGF is synthesized by osteoblasts and osteosarcoma cell lines, and these cells express the PDGF A and B genes (Betsholtz *et al.,* 1986; Sejersen *et al.,* 1986; Rydziel *et al.,* 1992, 1994; Graves *et al.,* 1989). Experiments in normal osteoblasts revealed that the synthesis of PDGF A chains is enhanced by other growth factors produced by skeletal cells (Table I). Transforming growth factor (TGF) β1 and PDGF itself increase PDGF A mRNA and PDGF AA polypeptide levels in cultured rat osteoblasts (Rydziel *et al.,* 1992). This autoregulation of the PDGF A gene is not unique to the osteoblast, as it has also been reported in nonskeletal fibroblasts and mesangial cells (Bhandari *et al.,* 1994). Furthermore, growth factor autoregulation is not specific to PDGF, and other genes, such as TGF β1, are autoregulated (Kim *et al.,* 1989). Mechanisms involved in the synthesis of PDGF A and B in osteoblasts have not been established. In endothelial cells, both phorbol esters and TGF β1 regulate the PDGF A gene at the transcriptional level (Starksen *et al.,* 1987; Kavanaugh *et al.,* 1988). Posttranscriptional regulation of the PDGF A gene in skeletal cells has not been reported, and the rapid induction of PDGF A transcripts following stimulation suggests that transcriptional mechanisms are involved in its synthesis. However, modest reductions in the half-life of PDGF A and B transcripts were reported in mesangial cells following exposure to phorbol esters (Bhandori *et al.,* 1994). The promoter region of the PDGF A gene contains multiple

Table I Stimulation of PDGF A and B Gene Expression in Osteoblasts

PDGF A	PDGF B
PDGF	TGF β
TGF β	
Phorbol esters	

GC-rich regions, a TATA box, multiple Sp-1 and Egr-1-binding sites, and negative regulatory or silencer elements (Takimoto *et al.,* 1991; Lin *et al.,* 1992, 1993). The promoter region of the PDGF A gene contains a serum response element located at base pair −477 to −468, and this element appears to be responsible for the induction of the PDGF A gene by PDGF (Lin *et al.,* 1992). Sites responsible for the regulation of PDGF A by TGF β1 have not been defined. A second PDGF A promoter has been described, and the use of alternate promoters and alternative splicing, as well as multiple polyadenylation signals, results in the expression of multiple species of PDGF A transcripts (Rorsman *et al.,* 1988, 1992). In contrast to the regulation of the PDGF A gene by selected growth factors in osteoblasts, polypeptide, steroid, and thyroid hormones do not modify its synthesis in skeletal cells. This indicates that growth factors, but not hormones, regulate the synthesis of PDGF A in osteoblasts (Rydziel *et al.,* 1992). However, the systemic administration of estrogens enhances expression of the PDGF A and B genes in murine uterus and vagina, indicating that PDGF in selected cells could be a potential mediator of estrogen action (Gray *et al.,* 1995). This effect has not been demonstrated in bone cells.

Normal rat osteoblasts express the PDGF B gene, and TGF β1 increases the expression of PDGF B transcripts in a time- and dose-dependent fashion (Rydziel and Canalis, 1996). In contrast, other growth factors, such as PDGF, insulin-like growth factor I (IGF I), and fibroblast growth factor (FGF) -2, do not modify PDGF B mRNA levels in osteoblasts. At the present time, there is no evidence of hormonal regulation of PDGF B expression in skeletal cells. TGF β enhances PDGF B expression at the transcriptional level in nonskeletal cells (Daniel and Fen, 1988), and a similar level of regulation may be involved in osteoblasts. The PDGF B gene promoter contains a classic TATA box, Sp-1-binding sites, and a transcriptional silencer region (Pech *et al.,* 1989; Jin *et al.,* 1993). A proximal element at bp -58 to -39 relative to the PDGF B transcription start site is essential for the phorbol ester-induced activation of the PDGF B gene (Jin *et al.,* 1993). At the present time there is limited information about the elements responsible for the regulation of the PDGF B gene by TGF β1 in skeletal cells, and studies on the transcriptional regulation and promoter activity of the PDGF B gene in osteoblasts have not been reported. It is possible that specific osteoblast nuclear transcription factors modify the expression of the PDGF B gene in a cell-specific fashion.

Biological Actions of PDGF on Skeletal Cells

PDGF AA, BB, and AB have similar biological actions for cells of the osteoblastic lineage. In skeletal as well as in nonskeletal cells, PDGF BB is more potent than PDGF AA, and PDGF AB has intermediate activity (Centrella *et al.,* 1991). PDGF stimulates bone cell replication and DNA synthesis, both in intact calvariae and in isolated rat osteoblasts. It appears that the primary effect of PDGF in bone is related to its mitogenic activity (Table II) (Canalis *et al.,* 1989). As a consequence of its effect on cell replication, PDGF increases the number of cells capable of synthesizing collagen. However, on a cellular basis, PDGF does not increase collagen synthesis and it may have a modest inhibitory effect on bone collagen production by the osteoblast. In accordance with this observation, PDGF inhibits matrix apposition rates in intact calvariae (Hock and Canalis, 1994). Although acute exposure to PDGF does not enhance the differentiated function of the osteoblast, it is likely to increase the pool of cells of the osteoblastic lineage, which eventually differentiate and express the osteoblastic phenotype. In fibroblasts, PDGF is a competence factor and its presence is required for the effect of IGF I on cell replication (Stiles *et al.,* 1979; Olashaw *et al.,* 1987). This does not seem to be the case in osteoblasts, and the mitogenic effect of PDGF in bone cells is observed in the absence of exogenously added IGF I (Centrella *et al.,* 1991; Canalis *et al.,* 1989). This may be due to the fact that osteoblasts synthesize significant amounts of IGF I and II, which may allow them to progress through the cell cycle.

IGF I and II and bone morphogenetic proteins (BMP) 2, 4, and 6 are synthesized by skeletal cells known to increase the differentiated function of the osteoblast. IGFs stimulate bone collagen synthesis and matrix apposition rates, whereas BMPs induce the differentiation of immature cells into cells of the osteoblastic lineage and stimulate the function of mature osteoblasts (Gazzerro *et al.,* 1998; Zhao *et al.,* 2000). Because PDGF tends to inhibit the differentiated function of the osteoblast, we examined whether it modified the synthesis of IGF I and IGF II and BMPs in skeletal cells (Table III). PDGF caused a time- and dose-dependent inhibition of IGF I and II mRNA and polypeptide levels (Canalis *et al.,* 1993; Gabbitas *et al.,* 1994; Gangi *et al.,* 1998) and inhibited BMP-4 expression in osteoblasts (Pereira *et al.,* 2000). The inhibition of IGF I and II and BMP-4 expression correlate with the inhibitory effect of PDGF on the differentiated function of the osteoblast, although this does not prove

Table II Effect of PDGF on Skeletal Cells

Increases cell replication
Inhibits the differentiated function of the osteoblast
Decreases collagen synthesis
Decreases bone matrix apposition rates
Increases bone resorption
Increases collagen degradation and collagenase expression

Table III Effect of PDGF on Cytokine Expression in Osteoblasts

Decreases IGF I and IGF II synthesis
Increases IGF I receptor binding
Decreases IGFBP-5 synthesis
Decreases BMP-4 expression
Increases IL-6 transcription
Increases HGF/SF expression

that the decrease in IGF I and II or BMP-4 mediates the effect of PDGF on osteoblastic function. PDGF BB decreases the rates of IGF II transcription in osteoblasts without modifying the decay of IGF II in transcriptionally arrested cells. The synthesis of IGF II in osteoblasts is driven by IGF II promoter 3 and its expression is inhibited by PDGF BB (Gangi *et al.,* 1998). Some of the effects of PDGF on the osteoblast, such as the inhibition of type I collagen transcription, occur following a short exposure to PDGF, suggesting direct effects on the differentiated function of the osteoblast. However, persistent or chronic exposure of skeletal cells to PDGF could result in a significant reduction of IGF I and BMP levels, which may be ultimately responsible for a decrease in osteoblastic function and bone formation. The inhibitory effect of PDGF on IGF expression is not related to the mitogenic activity of PDGF, as it is observed when cells are exposed to PDGF in the presence of the DNA synthesis inhibitor hydroxyurea. In addition to the decrease in IGF I and IGF II synthesis, PDGF inhibits the transcription of IGF-binding protein (IGFBP) 5, a binding protein known to stimulate bone cell growth and to enhance the effects of IGF I on this process (Canalis and Gabbitas, 1995). The interactions of PDGF with IGF I in skeletal cells appear relevant to the effects of PDGF in bone. PDGF modifies the synthesis of IGF I and II and of IGFBPs, and it also opposes the effects of IGF I on collagen synthesis in calvariae (Canalis *et al.,* 1989). Surprisingly, PDGF increases IGF I receptor binding in skeletal and nonskeletal cells.

In addition to its effects on bone formation, PDGF increases bone resorption and collagen degradation (Canalis *et al.,* 1989; Walther *et al.,* 1992; Cochran *et al.,* 1993). This effect is likely secondary to an increase in the expression of the matrix metalloproteinases (MMP), collagenase 1 and 3. An increase in MMP expression by PDGF has also been observed in nonskeletal fibroblasts. Collagenases can initiate the cleavage of collagen fibrils at neutral pH (Knauper *et al.,* 1996). Consequently, they are considered central to the process of collagen degradation and matrix breakdown. Three collagenases have been described: collagenase 1, 2, and 3. Human osteoblasts express collagenase 1 (MMP-1) and collagenase 3 (MMP-13), whereas rat osteoblasts secrete collagenase 3, but do not express collagenase 1 (Freije *et al.,* 1994; Rajakumar and Quinn, 1996; Varghese *et al.,* 1996; Ståhl-Bäckdahl *et al.,* 1997). Collagenases 1 and 3 degrade type I collagen fibrils with similar efficiency, but collagenase 3 is more effective on type II collagen (Knauper *et al.,* 1996). Although other proteases, particularly those biologically active at acid pH, are relevant to the bone resorptive process, the importance of collagenase 3 is documented by studies demonstrating that collagenase-neutralizing antibodies inhibit bone resorption (Holliday *et al.,* 1997). Furthermore, studies in mice with a mutation of the type I collagen gene that causes resistance to collagenase 3 cleavage fail to resorb bone following exogenous parathyroid hormone (PTH) (Zhao *et al.,* 1999).

The synthesis of collagenases 1 and 3 by osteoblasts is regulated by hormones and by cytokines present in the bone microenvironment. PDGF enhances collagenase 3 expression by transcriptional and posttranscriptional mechanisms (Varghese *et al.,* 1996). PDGF BB increased the activity of collagenase 3 promoter fragments transiently transfected into osteoblasts. Deletion analysis of the collagenase promoter revealed regions that impaired the induction of collagenase 3 by PDGF BB, but these were not characterized. A collagenase 3 construct spanning bp -53 to 28 in relation to the start site of transcription was fully responsive to PDGF BB when transiently transfected into osteoblasts. Targeted mutations of an AP-1 site in this fragment decreased basal collagenase promoter activity and the responsiveness to PDGF BB. Electrophoretic mobility shift assay revealed AP-1 nuclear protein complexes that were enhanced in extracts from PDGF BB-treated osteoblasts, and supershift assays demonstrated interactions of c-Fos, Fos B, Fra-2, c-Jun, Jun B, and Jun D with AP-1 sequences in extracts from PDGF-treated cells. Furthermore, PDGF caused a rapid induction of these transcription factors known to interact with the AP-1 site (Rydziel *et al.,* 2000). The elements and cytosolic proteins responsible for posttranscriptional regulation have not been reported.

The induction or activation of members of the Fos and Jun family of transcription factors by PDGF BB is consistent with the mitogenic activity of the growth factor in skeletal cells and with its stimulatory effects on bone resorption (Angel and Karin, 1990). PDGF increases osteoclast number in calvariae; this could be due to an increase in osteoclastogenesis or a decrease in osteoclast apoptosis. Mice lacking c-Fos develop osteopetrosis and decreased bone remodeling, and c-Fos appears to be essential for osteoclast differentiation (Wang *et al.,* 1992; Grigoriadis *et al.,* 1994). PDGF may have direct and indirect effects on bone resorption, as it increases the expression of interleukin (IL)-6 in osteoblasts, and IL-6 enhances the recruitment of osteoclasts and is critical to the process of bone resorption (Jilka *et al.,* 1992; Masiukiewicz *et al.,* 2000). The effect of PDGF on IL-6 expression occurs at the transcriptional level and PDGF increases the activity of IL-6 promoter fragments transiently transfected into osteoblasts. Deletion analysis revealed two responsive regions: one containing an AP-1 site located between bp -276 and -257 and a second, less well defined, downstream of -257. Targeted mutations of a cyclic AMP-responsive element (CRE) in the bp -257 to 20 region revealed that this site contributes to IL-6 promoter induction by PDGF BB. Electrophoretic mobility

shift assay revealed nuclear protein complexes, forming with the AP-1 and CRE sites, that were enhanced by PDGF BB. Supershift assays revealed that classic members of the Fos and Jun families of transcription factors interact with AP-1, as well as with CRE sequences in the IL-6 gene, whereas the related activating transcription factor-2 (ATF-2) interacted only with CRE sequences, the known target sequence for this transcription factor (Meyer and Habener, 1993). ATF-2 is known to form heterodimers with members of the Jun family of proteins, which may also be the case in osteoblasts exposed to PDGF BB (Hai and Curran, 1991; Morooka *et al.,* 1995). The induction of IL-6 by PDGF involves a CRE, but PDGF does not increase cyclic AMP in osteoblasts, and ATF-2 is not dependent on cyclic AMP pathway activation.

Changes in IL-6 expression could play a role not only in the actions of PDGF in bone resorption, but also in its effects on collagenase expression and matrix breakdown, particularly as IL-6 is one of the most potent inducers of collagenase 3 in osteoblasts (Franchimont *et al.,* 1997). The actions of PDGF on bone cell replication would suggest a possible role in fracture healing similar to that reported in wound healing (Pierce *et al.,* 1991; Lynch *et al.,* 1987). In addition, PDGF A and B genes are expressed by cells present in normal healing fractures, and reduced expression of PDGF and its receptors results in impaired would healing (Andrew *et al.,* 1995; Beer *et al.,* 1997). PDGF could play a role as a systemic and local regulator of fracture repair. Because TGF β and PDGF are present in platelet granules, it is possible that when these factors are released following platelet aggregation, they induce the expression of PDGF A and B at the fracture site. Along these lines, PDGF, like FGF-2, induces the expression of hepatocyte growth factor/scatter factor (HGF/SF) in osteoblasts (Blanquaert *et al.,* 1999, 2000). HGF/SF is a polypeptide composed of a 69-kDa α chain and a 34-kDa β chain with a serum protease-like sequence linked by disulfide bonds (Nakamura *et al.,* 1989; Strain, 1993). HGF/SF stimulates mitogenesis in hepatic and extrahepatic cells, enhances angiogenesis, and plays a role in tissue repair (Strain, 1993). HGF/SF signals via the product of the protooncogene c-met, a tyrosine kinase-activated receptor, and is mitogenic for cells of the osteoblastic and osteoclastic lineage (Grano *et al.,* 1996). Therefore, it was postulated to have a function in bone remodeling and repair. Interestingly, the stimulatory effect of PDGF and FGF-2 on HGF/SF expression is blunted by glucocorticoids, which are known to delay wound and possibly fracture healing (Blanquaert *et al.,* 2000; Beer *et al.,* 1997).

Although PDGF appears to play a role in fracture repair, this occurs following release, and possibly local *de novo* synthesis of the factor. However, the function of basal concentrations of PDGF and its role in normal skeletal physiology are uncertain. Studies using knockout mice have revealed, following disruption of the PDGF B or the PDGF β receptor genes, serious renal, cardiovascular, and hematological abnormalities (Leveen *et al.,* 1994; Soriano, 1994). Mutant mice developed a bleeding disorder with thrombocytopenia and lacked glomerular development because of absent mesangial cells. The animals had cardiomegaly and dilatation of major vessels. No abnormalities in the musculoskeletal system were reported. However, the embryos died prior to birth, and it is possible that skeletal abnormalities, if they were to occur, could require longer periods of time and postnatal growth. PDGF A knockout mice are phenotypically distinct; about half die *in utero,* and the surviving mice are severely growth retarded and develop emphysema (Bostrom *et al.,* 1996).

PDGF Receptors and Binding Proteins

There are two PDGF receptors: α and β. The two receptors have a 44% overall amino acid sequence homology and have immunoglobulin-like domains in the extracellular region and a tyrosine-kinase domain in the intracellular region (Qiu *et al.,* 1988; Claesson-Welsh *et al.,* 1989a,b; Gronwald *et al.,* 1988; Lee *et al.,* 1990; Matsui *et al.,* 1989; Yarden *et al.,* 1986). PDGF receptors are closely related to the receptor for colony-stimulating factor-1 as well as to the c-*kit* oncogene (Qui *et al.,* 1988). PDGF A chains bind primarily to the α receptor, whereas PDGF B chains bind to either the α or the β receptor (Seifert *et al.,* 1989). PDGF induces dimerization and activation of PDGF receptors, and PDGF also increases PDGF α and β receptor expression (Heldin *et al.,* 1989; Eriksson *et al.,* 1991; Kelly *et al.,* 1991). Skeletal cells express PDGF α and β receptors, which are regulated by other cytokines and growth factors. IL-1 and tumor necrosis factor α enhance PDGF AA binding to rodent osteoblasts, but not to human osteoblasts, by increasing PDGF α receptor transcripts and number (Centrella *et al.,* 1992; Gilardetti *et al.,* 1991; Tsukamoto *et al.,* 1991). The enhanced PDGF AA binding to osteoblasts results in increased biological activity, and after treatment with IL-1, PDGF AA becomes as biologically effective as the more potent PDGF BB. FGF-2 and epidermal growth factor also increase PDGF α receptors in the MC3T3 osteoblastic cell line, whereas TGF β1 has little effect and decreases the binding of PDGF AA in normal rat osteoblasts (Tsukamoto *et al.,* 1991). In addition, TGF β1 inhibits PDGF β receptor expression and PDGF mitogenic activity in fibroblasts (Vaziri and Faller, 1995; Gronwald *et al.,* 1989). The signal transduction pathways involved in the various PDGF effects in bone cells include protein kinase-C pathways, resulting in an induction of Jun and Fos expression, activation of phospholipase C-γ and phosphatidylinositol kinase 3, and changes in intracellular calcium (Okazaki *et al.,* 1992). The same pathways are used in nonskeletal cells (Valius and Kazlauskas 1993; Epstein *et al.,* 1992; Okazaki *et al.,* 1992).

Although there is considerable information about the existence and function of specific binding proteins for many growth factors, including IGFs and TGF β, less is known about specific binding proteins for PDGF. PDGF BB binds to α_2-macroglobulin and osteonectin or SPARC (secreted protein acidic and rich in cysteine) (Crookston *et al.,* 1993; Lane and Sage 1994; Raines *et al.,* 1992), and PDGF is stored in the extracellular matrix by association to osteonectin, as well

Table IV Effect of PDGF on Bone Matrix Proteins

Decreases type I collagen synthesis
Decreases osteonectin expression
Increases osteopontin expression

as to other extracellular matrix proteins (Kelly *et al.,* 1993). The interactions between PDGF and osteonectin may be important, as osteonectin, by binding PDGF B chains, may modify its binding to specific receptors and activity or may prolong the half-life of PDGF. Skeletal cells secrete substantial amounts of osteonectin, and osteonectin plays a critical role in bone remodeling and angiogenesis. Studies in osteonectin null mice have clarified the function of this matrix protein in bone (Delany *et al.,* 2000). Osteonectin −/− mice have decreased bone formation and decreased osteoblast and osteoclast surface and number, leading to decreased bone remodeling with a negative bone balance and osteopenia. These data indicate that osteonectin supports bone remodeling and the maintenance of bone mass in vertebrates, but it is not known whether these findings relate to the binding of PDGF B chains. It is possible that changes in the expression of osteonectin in skeletal cells play a role in the regulation of PDGF activity. In chondrocytes, PDGF increases osteonectin expression, whereas in osteoblasts, PDGF BB and FGF-2 decrease the expression of this matrix protein (Chandrasekhar *et al.,* 1994; Delany and Canalis, 1998) (Table IV). FGF-2 acts by destabilizing osteonectin transcripts, but the mechanisms involved in the action of PDGF have not been explored. PDGF also increases osteopontin expression in osteoblasts, a matrix protein necessary for normal bone resorption. (Sodek *et al.,* 1995).

Role and Clinical Relevance of PDGF

The mitogenic activities of PDGF and its release by platelets suggest a role in wound healing and fracture repair. This is supported further by the demonstration of PDGF A and B gene expression at fracture sites. It is also possible that PDGF plays a role in acute bone repair following inflammation, particularly since the mitogenic actions of PDGF are enhanced in the presence of cytokines released during this process. It is less likely that PDGF A or B plays a role in the maintenance of bone mass in view of its inhibitory effects on the differentiated function of the osteoblast. Because the PDGF A and B genes are expressed by a variety of malignant cells, including osteosarcoma, PDGF may play a role in tumorigenesis. This possibility is supported by the near identity of the PDGF B gene and p28-*sis.* In addition, studies reported that the Wilms' tumor gene product WT1 represses transcription of the PDGF A gene. Consequently, a loss of WT1 or related repressor activities could contribute to the pathogenesis or growth of Wilms' tumors (Wang *et al.,* 1992; Gashler *et al.,* 1992). In view of the mitogenic activity of PDGF, it is unlikely that it will be a suitable agent for the systemic therapy of skeletal disorders. It could, however, be useful in the topical treatment of bone disorders. Systemic administration of PDGF to estrogen-deficient rats prevents bone loss of the spine (Mitlak *et al.,* 1996). Bone histomorphometric analysis of these animals revealed that PDGF increases the number of osteoblasts and, as a consequence, bone formation. These results are consistent with the mitogenic effects of PDGF *in vitro,* which increases the replication of preosteoblasts, resulting in an increased number of osteoblastic cells capable of forming bone. Surprisingly, systemic PDGF did not change osteoclast number in ovariectomized rats, but this may be due to the model chosen, as estrogen-deficient rats display a substantial increase in bone resorption and remodeling that may preclude an additional effect. PDGF administered systemically caused fibroblast replication and fibrosis in extraskeletal tissues, pointing to potential problems if the growth factor were to be used for the treatment of clinical disorders. Topical application of PDGF to rat craniotomy defects results in increased soft tissue repair, but not increased osteogenesis (Marden *et al.,* 1993). This may be due to nonspecific effects of PDGF. The effects of PDGF on endothelial cell proliferation and angiogenesis are likely beneficial to the process of wound and fracture repair, as appropriate vascularization is required for healing (Battegay *et al.,* 1994). Although PDGF enhances the early process of wound healing, the effect is short-lived, and there is less information regarding the possible effectiveness of PDGF in fracture repair.

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CHAPTER 47

Fibroblast Growth Factor (FGF) and FGF Receptor Families in Bone

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Introduction

The seminal work that began in the 1970s (Armelin, 1973; Gospodarowicz, 1974, 1975) and early 1980s (Bohlen *et al.,* 1984) with the purification of a single polypeptide possessing a novel bioactivity has evolved dramatically with the discovery of 23 genes encoding a family of structurally (as well as functionally) related polypeptides, the fibroblast growth factors (FGFs). As a family, FGFs act in a paracrine or autocrine manner and display a characteristic affinity for heparin (Gospodarowicz *et al.,* 1984; Shing *et al.,* 1984) which is critical for interactions with signal-transducing receptors (Ornitz, 2000; Weismann and de Vos, 1999). In addition to their property of promoting cell growth and their ability to induce a mitogenic response (Burgess and Maciag, 1989), FGFs also stimulate cell migration (Presta *et al.,* 1986), angiogenesis (Gospodarowicz *et al.,* 1979; Folkman and Klagsbrun, 1987; Klagsbrun and D'Amore, 1991; Montesano *et al.,* 1986), vasculogenesis (Krah *et al.,* 1994), hematopoietic development (Faloon *et al.,* 2000), wound healing and tissue repair (Davidson *et al.,* 1985), differentiation (Kimelman and Kirschner, 1987; Kimelman and Maas, 1992; Olwin *et al.,* 1994), transformation (Sasada *et al.,* 1988), and morphogenesis (Dono and Zeller, 1994; Niswander *et al.,* 1994; Olwin *et al.,* 1994). To mediate such multifarious activities, FGFs function in concert with a similarly complex family of cognate signal-transducing receptors (FGFRs) and nontransducing heparan sulfate-containing proteoglycans (HSPGs), the cysteine-rich receptor (CFR), and binding proteins (BPs). Furthermore, FGFs and their receptors exhibit remarkably varied mechanisms regulating their expression and/or bioavailability. These mechanisms occur at multiple levels of gene expression, including transcription, translation, intracellular protein trafficking, extracellular matrix localization, and release. For additional perspectives into the FGF family, a series of reviews can be referred to for information not provided herein (Basilico and Moscatelli, 1992; Delrieu, 2000; Martin, 1998; McKeehan *et al.,* 1998; Nugent and Iozzo, 2000; Okada-Ban *et al.,* 2000; Szebenyi and Fallon, 1999).

The Fibroblast Growth Factor Family of Bioactive Polypeptides

Fibroblast growth factors isolated from many sources act to modify the growth of cell types of epithelial, neuroectodermal, and mesenchymal origin (Burgess and Maciag, 1989). Structurally, the FGF family encompasses the polypeptides encoded by 23 distinct genes. As new members of the FGF family were identified, a standardized system for assigning nomenclature was adopted in 1991 (Baird and Klagsburn, 1991). Thus, aFGF became FGF-1 (Jaye *et al.,* 1986) and bFGF became known as FGF-2 (Abraham *et al.,* 1986). In contrast to the nearly ubiquitous tissue distribution of FGF-2 and the wide distribution of FGF-1, the expression of FGFs 3–21 is restricted both temporally and/or spatially. FGF-3/int-2 (Dickson and Peters, 1987) was identified as an activated gene product following retroviral insertion into mouse mammary tumor cells. The fourth family member is also known as the Kaposi's sarcoma FGF/K-FGF (Taira *et al.,* 1987), or the human stomach-transforming gene/HSTF-1/hst-1 (Delli Bovi *et al.,* 1987). FGF-5 was isolated as a transforming activity detected after transfection of DNA from a human bladder carcinoma into NIH/3T3 cells (Zhan *et al.,* 1988). FGF-6/hst-2, or the hst-1-related gene (Marics *et al.,* 1989), was originally identified by low-stringency screening of a human genomic library using an FGF-4-derived probe and FGF-7 [or keratinocyte growth factor (KGF)] as a selective mitogen for epithelial cells but not fibroblasts (Finch *et al.,* 1989).

Next in order of discovery is FGF-8, or androgen-induced growth factor/AIGF (Tanaka *et al.,* 1992), purified from a murine androgen-dependent carcinoma, and FGF-9, originally termed glia-activating factor (GAF), was purified from a human glioma cell line (Miyamoto *et al.,* 1993). In addition, the single copy FGF-8 gene directs the synthesis of multiple primary AUG-initiated translation products via differential mRNA processing of various 5′exons. The cDNA for FGF-10 was first isolated from human lung (Emoto *et al.,* 1997) and has been shown to modulate limb development (Sekine *et al.,* 1999). As a subgroup, FGFs 11–14 (also known as FGF homologous factors) are distinguished by the fact that when synthesized *de novo,* they appear to be preferentially targeted for direct intracellular trafficking into the nucleus (Smallwood *et al.,* 1996; Munoz-Sanjuan, 2000), as are the high molecular weight isoforms of FGF2 and FGF-3. Interestingly, there are

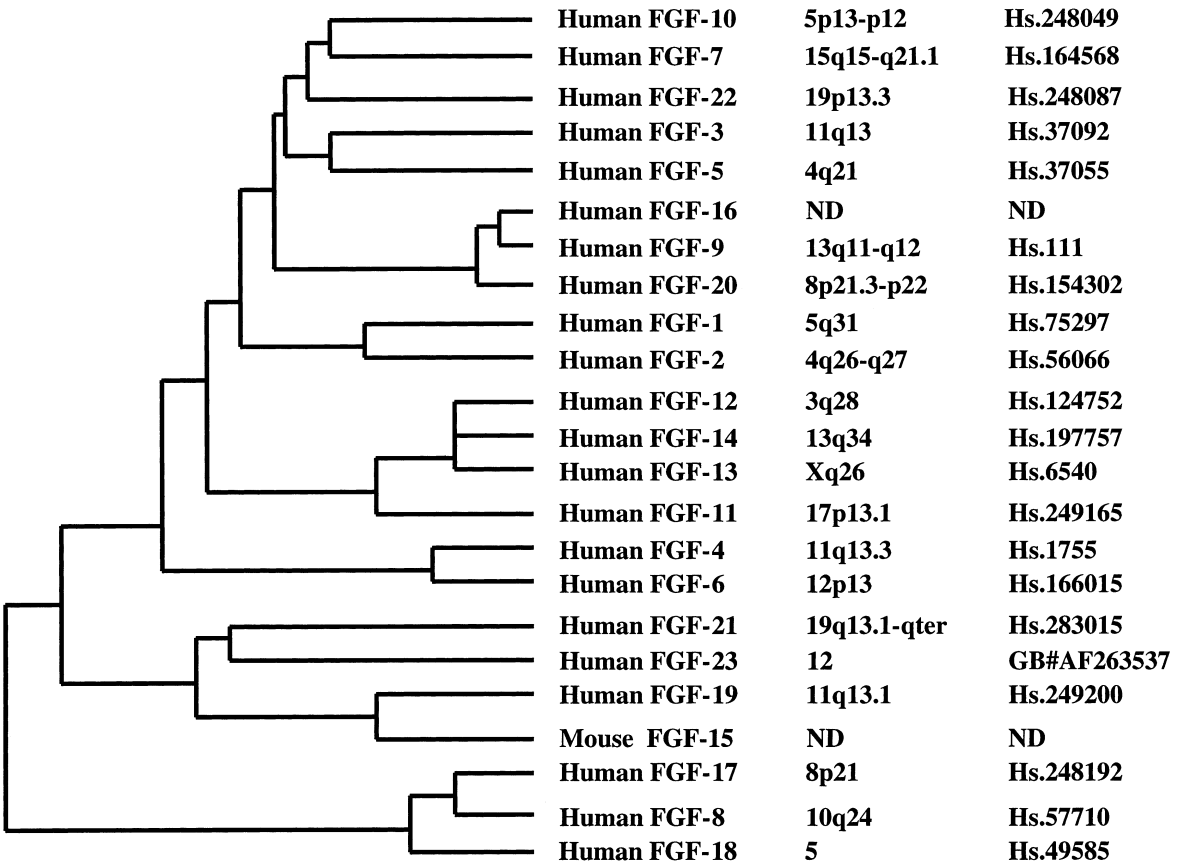


Figure 1 The human FGF gene family is made up of 23 genes. A dendrogram depicting the sequence relationship between members of the family is shown along with their human chromosomal location and corresponding unigene number. For FGF-15, no human homologue has been published and the chromosomal location of FGF-16 has not yet been determined.

alternative AUG-initiated translation products associated with the genes encoding FGFs 11–14. FGF-15 was identified in a murine model of pre-B-cell leukemia harboring a chromosomal translocation that results in the expression of a chimeric transcription factor mediating gene activation (McWhirter *et al.,* 1997); a human homologue, however, has yet to be characterized. Other publications have described cDNA cloning, chromosomal gene mapping, and embryonic patterns of expression for FGF-16 (Miyake *et al.,* 1998), FGF-17 (Hoshikawa *et al.,* 1998; Xu *et al.,* 1999), FGF-18 (Hu *et al.,* 1998; Ohbayashi *et al.,* 1998), FGF-19 (Nishimura *et al.,* 1999; Xie *et al.,* 1999), FGF-20 (Kirikoshi *et al.,* 2000), and FGF-21 (Nishimura *et al.,* 2000). The identification of FGF-22 and FGF-23 has not appeared in publication but may be found in nucleotide sequence data bases with accession numbers 10119766 and 10119773, respectively; both are likely to be classically secreted. In summary, the evolutionary relationship between the entire human FGF family (to date) and with their chromosomal locations and unigene identificarion number are shown in Fig. 1.

Distinctive Features That Regulate the *de Novo* Synthesis of FGFs

FGF Promoters

The FGF family can be divided into those with multiple or single promoters and/or transcription start sites. For example, there are at least two, possibly four, regions that functionally define the FGF-1 promoter (Chotani *et al.,* 1995; Myers *et al.,* 1995). Four mRNAs may be transcribed, each with a different transcription start site (Myers *et al.,* 1993). The four mRNAs represent alternative splice variants that place different noncoding exons 5′ of the FGF-1 translation start site. In addition, promoter choice and the appearance of different mRNAs occur in a pattern of expression that is tissue or cell-type specific. The entire FGF-1 transcription unit has been examined from rodent cells (Madiai *et al.,* 1999). Correspondingly complex mechanisms also regulate the transcription of other members of the FGF family.

The FGF-2 promoter has been functionally mapped and shown to contain both negative and positive *cis*-acting regulatory domains (Shibata *et al.,* 1991), as illustrated in Fig. 2. A novel transcription factor mediates the negative regulation of FGF-2 (Ueba *et al.,* 1999). Interestingly, in contrast to FGF-1, the human FGF-2 gene contains one core promoter region that directs the transcription of multiple mRNAs possessing the same transcription start site. Hormonal and transsynaptic receptor-mediated intracellular signaling pathways are thought to regulate the activity of the FGF-2 promoter in a fashion that may be of physiologic significance (Stachowiak *et al.,* 1992; Logan *et al.,* 1992), including astrocyte cell plasticity (Moffett *et al.,* 1998). In addition, the FGF-2 promoter has been shown to be positively regulated by the mutant p53 gene product, leading to speculation that the constitutive expression of FGF-2 may correlate with the development of some forms of cancer (Ueba *et al.,* 1994). Posttranscriptional regulation of FGF-2 gene expression through the synthesis of an endogenous antisense mRNA transcript was originally described for *Xenopus* (Kimelman and Kirschner, 1989). Expression of antisense FGF-2 mRNA is developmentally regulated, encodes a protein of 35 kDa, and modulates translation of FGF-2 (Li and Murphy, 2000). In addition, the gene promoter for antisense FGF-2 has been characterized in human cells (Gagnon *et al.,* 1999).

The FGF-3 gene is transcribed into multiple classes of mRNA with different transcription start sites, in mouse embryonal carcinoma cells (Dickson *et al.,* 1990a; Murakami *et al.,* 1993), a human colon carcinoma cell line (Galdemard *et al.,* 1995), mouse mammary tumors (Dickson *et al.,* 1990b), and in developing mouse embryos. One FGF-3 promoter region is located within intronic sequences 3′ of noncoding exon 1, the other is 5′ of the transcription start site. Further studies have functionally characterized several transcription factor-binding sites (Murakami *et al.,* 1999) and expression in tumorigenic and nontumorigenic colon carcinoma cell lines (Galdemard *et al.,* 2000). The FGF-4 promoter was initially shown to be developmentally regulated in F9 teratocarcinoma cells (Koda *et al.,* 1994; Ma *et al.,* 1992). In F9 cells, the FGF-4 promoter is regulated via binding of nuclear factor NF-Y to motifs within the core promoter region (Hasan *et al.,* 1994). Additional enhancer-like sequences are contained within the 3′ noncoding region of exon III (Curatola and Basilico,1990). Transcription factor-binding sites (Sox-2 and Sp1) within sequences defining the promoter and enhancer have been characterized (Luster *et al.,* 2000). Genomic sequence analysis indicated a region likely to contain the FGF-5 promoter (Zhan *et al.,* 1988). The location of sequences within the FGF-5 promoter that function during differentiation of retinal pigment epithelial cells has been mapped (Gelfman *et al.,* 1998). The cell type specific activation of the FGF-7 promoter has been described, including the identification of two promoter regions containing sequence elements that regulate expression negatively (Finch *et al.,* 1995), as well as corresponding transcription factors (Zhou and Finch, 1999). Differential promoter recognition and mRNA processing drive the expression of numerous isoforms of FGFs 11–14 (Munoz-Sanjuan, 2000).

A Distinctive Translation Initiation Mechanism Mediates Expression of FGF-2 and FGF-3

While all FGFs are encoded by single copy genes, multiple isoforms of both FGF-2 (Florkiewicz and Sommer, 1989; Prats *et al.,* 1989) and FGF-3 (Dickson and Acland 1990) have been detected. The novel mechanism by which this occurs distinguishes these FGFs from all other known growth factors and ultimately may prove to be a critical point from which FGF-2 and FGF-3 gene expression is qualitatively regulated. Each of the multiple isoforms is a

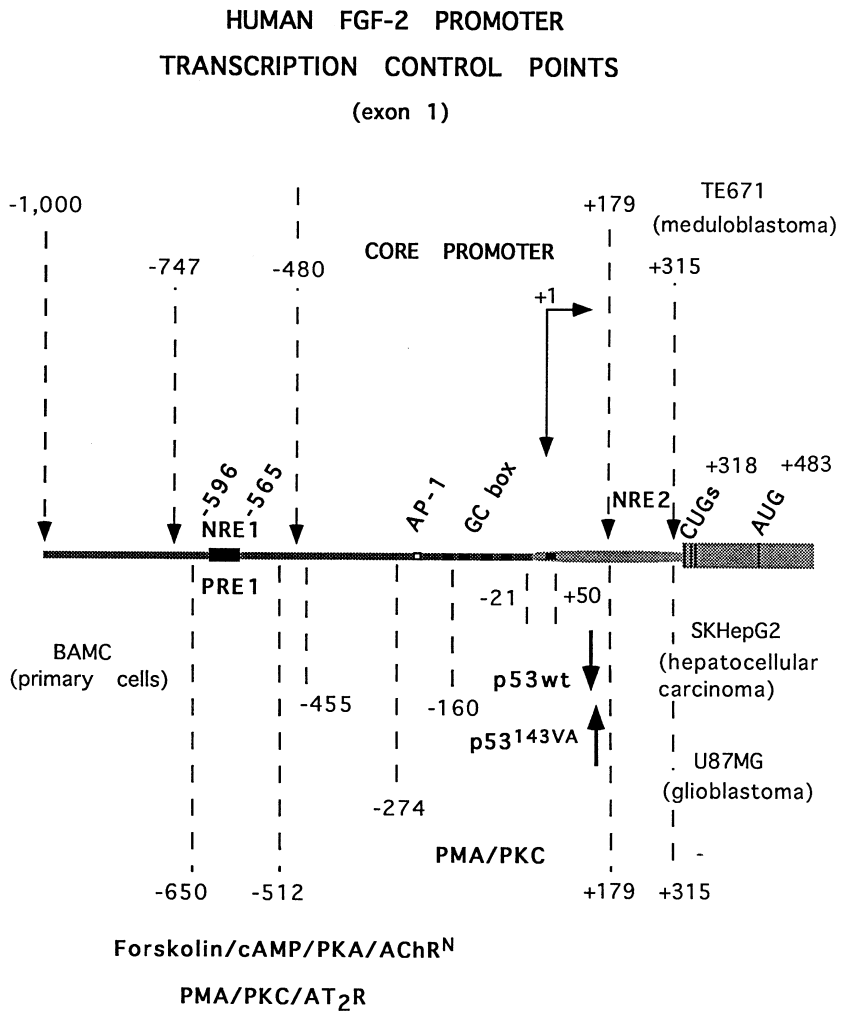


Figure 2 Summary of regulatory regions within the human FGF-2 promoter. The various cell types used and intracellular signaling pathways are labeled along with the location of the AP-1 site and GC box of the core promoter. The approximate location of p53 regulation is shown. The promoter contains positive (PRE) as well as negative regulatory regions (NRE). The sequence of the entire region has been published previously (Florkiewicz *et al.,* 1991).

primary translation product with no precursor–product relationship. The larger isoforms are colinear amino-terminal extended versions of the shorter isoforms. Moreover, translation of each of the four high molecular weight (HMW) human FGF-2 isoforms (22, 23, and 24 kDa) is initiated with an unconventional CUG translation initiation codon; a 34-kDa isoform of FGF-2 has also been described (Arnaud *et al.,* 1999). In contrast, translation of the 18-kDa isoform is initiated with a classical AUG codon located downstream of the four CUG codons. Multiple HMW isoforms of rodent FGF-2 also initiate translation with CUG codons (Powell and Klagsbrun, 1991). Curiously, the rainbow trout FGF-2 gene appears to encode only an 18-kDa translation product, even though preferential CUG translation initiation from transfected human cDNA expression vectors does occur (Hata *et al.,* 1997). Likewise, the larger isoform of FGF-3 (31.5 kDa) utilizes a CUG codon, whereas the 28.5-kDa isoform uses an AUG codon to initiate translation (Dickson and Acland, 1990). In the case of FGF-2, CUG-initiated HMW isoforms are not minor translation products, and in some cases the HMW isoforms are expressed at levels equal to or greater than the 18-kDa isoform (Coffin *et al.,* 1995). Thus, the unusual utilization of CUG codons to initiate the translation of HMW FGF-2 effectively diversifies this single copy gene.

The uncommon use of CUG codons to initiate FGF-2 translation prompted speculation that selecting a CUG, as opposed to AUG translation initiation codon, may represent a physiologically relevant mechanism regulating FGF-2 gene expression. Three observations support this possibility. The first is that the CUG-initiated translation products of FGF-2 and FGF-3 localize to the nucleus preferentially, whereas their respective AUG-initiated isoforms are predominantly cytosolic or extracellular (Florkiewicz *et al.,* 1991). Second, the HMW isoforms of FGF-2 are expressed in a tissue-specific pattern distinct from the 18-kDa isoform

that is maintained in transgenic model systems (Coffin *et al.,* 1995), as well as during development of the central nervous system (Giordano *et al.,* 1992). Third, partially purified HMW and 18-kDa FGF-2 isoforms have similar *in vitro* extracellularly mediated mitogenic activities (Florkiewicz and Sommer, 1989), but when synthesized in continuously expressing transfected cells, the HMW isoforms are transforming whereas the 18-kDa isoform is not (Quarto *et al.,* 1991). Although FGF-2 appears to be widely expressed when detected by immunohistochemical techniques and *in situ* hybridization, it is likely that the different molecular isoforms are expressed in a spatially and temporally restricted manner. Therefore, in order to put the complete FGF-2 picture into perspective, it will be necessary to determine the qualitative, as well as quantitative, patterns of expression.

As noted previously, the HMW isoforms of human FGF-2 initiate translation from CUG codons that are differentially recognized in a *de novo* pattern that is tissue specific. Regulating translation is one mechanism by which eukaryotic cells control gene expression, including start site selection followed by formation of a highly order translation initiation complex (Hershey *et al.,* 1996; Kosak, 1999). In most circumstances, ribosomes arrive at the site of translation initiation after establishing contact and assembling at the 5′ end of eukaryotic mRNAs and then scan 3′ until encountering the most favorable combination of mRNA structure and sequence to initiate protein synthesis. However, in some cases, ribosomes recognize translation initiation sites directly without scanning, termed internal ribosome entry (Le and Maizel, 1997). The CUG-mediated translation initiation of FGF-2 HMW isoforms is an example of internal ribosome entry that is also maintained in a tissue-specific manner (Creancier *et al.,* 2000; Vagner *et al.,* 1995). A number of factors are likely to be involved in this process (Kevil *et al.,* 1995; Touriol *et al.,* 2000). Although the sophisticated cellular mechanism mediating CUG-initiated translation of HMW FGF-2 is being elaborated, their physiological significance remains an enigma. Ultimately, selected expression in transgenic animal model systems will be necessary for a thorough analysis. In addition to the isoform-specific phenotypes reported previously, other publications show that 24-kDa FGF-2 modulates interleukin-6 promoter activity (Delrieu *et al.,* 1999) and cell migration (Piotrowicz *et al.,* 1999).

Export or Secretion of FGFs?

FGFs are known to be released from cells by two clearly distinct intracellular pathways: one that is or likely to be endoplasmic reticulum (ER)/Golgi dependent (FGF-3 through 10 and 15 through 23) and another that is ER/Golgi independent (FGF-1 and -2). Although considered to be prototypic members of the FGF family, FGF-1 and FGF-2 are distinctive in that they are found extracellularly but do not possess hydrophobic signal peptide sequences. Historically, this has been one of the most intriguing features of FGFs because their signal transducing receptors are extracellular (Rifkin and Moscatelli, 1989). In contrast, with the exception of FGFs 11–14, the other FGFs contain typical or noncleaved internal hydrophobic amino acid signal sequences that function as ER-targeting motifs. Consequently, FGF-3 through 10 and 15 through 23 are secreted proteins. However, an alternative exocytic ER/Golgi-independent trafficking pathway must also exist because FGF-1 and FGF-2 are detected routinely on the cell surface or in the extracellular matrix (Bashkin *et al.,* 1992; Florkiewicz *et al.,* 1991; Folkman *et al.,* 1988; Gonzalez *et al.,* 1990), in vitreous fluid (Baird *et al.,* 1985), in cell culture media (Florkiewicz *et al.,* 1995), and in the urine (Nguyen *et al.,* 1994) and serum (Dietz *et al.,* 2000) of patients with cancer. Several key publications have demonstrated unequivocally the existence of an energy and plasma membrane-dependent "export" pathway for FGF-2 pictured in Fig. 3 (Florkiewicz *et al.,* 1991, 1995; Mignatti *et al.,* 1992). Evidence has also been presented for the involvement of a heat shock response mechanism in the export of FGF-1 (Jackson *et al.,* 1992). A number of proteins have been characterized as functional components of the intracellular trafficking "export" pathway for FGF-2 and FGF-1 (Florkiewicz *et al.,* 1998; Frisel and Maciag, 1999). In addition, a critical FGF-1 and FGF-2 targeting signal(s) that mediates trafficking through the export pathway has been identified (Shi *et al.,* 1997). It is particularly interesting to note that interleukins 1α and β, which share structural homology with the FGF family, are also exported

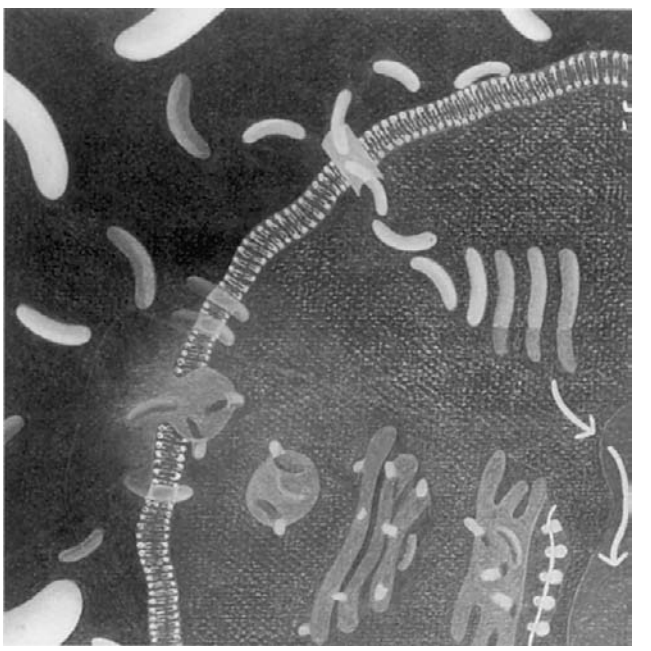


Figure 3 Two pathways traffic and release proteins from cells. One requires ER/Golgi, vesicular intermediates, and is signal sequence dependent. A second pathway exists for proteins without hydrophobic signal peptide sequences, such as 18-kDa FGF-2. These proteins are "exported" directly through a translocation apparatus located at the plasma membrane. The amino-terminally extended high molecular weight FGF-2 isoforms are targeted to the nucleus.

through a yet to be defined ER/Golgi-independent pathways (Muesch *et al.,* 1990). In any instance, it is likely that the export pathway(s) of FGF-1, FGF-2, and the interleukins is clinically important and of therapeutic value. Inhibitors of export would be predicted to interfere with the bioavailability of these potent growth-promoting polypeptides (Florkiewicz *et al.,* 1995).

Complex Interactions with Receptors, HSPGs, and Binding Proteins Regulate the Bioactivity of Extracellular FGFs

In a paracrine and/or autocrine manner, the receptor-binding and signal-transducing activities of the FGFs are initiated extracellularly. On the cell surface, at least three types of proteins may interact with the FGFs, shown conceptually in Fig. 4. The first class are high-affinity (K_d ~2–10 p*M*) integral plasma membrane proteins with three extracellular immunoglobulin (Ig)-like loop structures. These true signaling receptors contain an intracellular domain with intrinsic tyrosine kinase activity (Johnson and Williams, 1993; Givol and Yayon, 1992). Within this tyrosine kinase domain are hydrophilic amino acids that are also a site of receptor autophosphorylation.

The second type of receptor are low-affinity (K_d ~50–500 n*M*) binding sites, identified as HSPGs (Moscatelli, 1987). HSPGs do not possess intrinsic tyrosine kinase activity, but are important molecules regulating FGF bioavailability (Klagsbrun and Baird, 1991; Roghani *et al.,* 1994; Yayon *et al.,* 1991). The third type of protein, a cysteine-rich FGFR (CFR), was originally isolated and characterized from chicken embryos (Burrus and Olwin, 1989; Burrus *et al.,* 1993). Although not yet firmly established, a physiological significance is under investigation (see later). All three types of receptor proteins form dynamic interactions with the FGFs that are compensatory with their respective bioactivities.

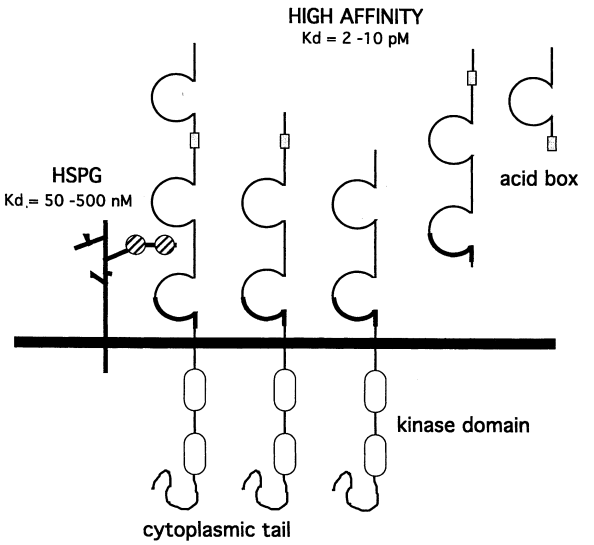


Figure 4 Cell surface FGF receptors. The low-affinity HSPG type, which protects and stabilizes FGF (hatched circles) in a conformationally dependent manner for presentation to its high-affinity receptor. Second, the high-affinity receptor containing two or three extracellular immunoglobulin-like loop structures. The characteristic location of an extracellular acid box is labeled (shaded box). Soluble splice variants of the high-affinity receptor without transmembrane domains are also diagrammed. Intracellularly, the high-affinity receptor contains a split tyrosine kinase domain and cytoplasmic tail. The third type, cysteine-rich receptor or MG-160, is found in the Golgi apparatus and is not shown here.

High-Affinity Receptors

A family of four genes encode distinct high-affinity FGFRs. The FGFRs have appeared in the literature under different names, including FGFR1/flg (Ruta *et al.,* 1988; Lee *et al.,* 1989) or cek1 (Pasquale, 1990); FGFR2/bek (Kornbluth *et al.,* 1988; Dionne *et al.,* 1990), K-Sam (Hattori *et al.,* 1990), or cek3 (Pasquale, 1990); and FGFR3 (Keegan *et al.,* 1991), flg-2 (Avivi *et al.,* 1991), or cek2 (Pasquale, 1990). The large number of dedicated reviews supports the interest and wealth of information regarding fundamental receptor–ligand interactions, developmental implications, and human diseases linked to FGFRs (Vajo *et al.,* 2000; Ornitz, 2000; Friessel and Maciag, 1999; Xu *et al.,* 1999). The most notable is the involvement of FGFRs in skeletal disorders (De Moerlooze and Dickson, 1997; Webster and Donoghue, 1997). Although structurally diverse, each of the FGFRs individually can bind different FGF family members with varying affinities (Chellaiah *et al.,* 1994; Ornitz and Leader, 1992). Mapping specific molecular interactions between FGF and FGFR suggests that multiple binding domains exist (Wang *et al.,* 1999; Venkataraman *et al.,* 1999). In some instances, expression of the FGFRs has also been shown to be tissue or cell-type specific and developmentally regulated (Patstone *et al.,* 1993). The crystal structure of the FGF–FGFR complex has been elucidated (Weismann and de Vos, 1999). Numerous subspecies of FGFR-1 and FGFR-2 can be generated via an alternative mRNA splicing mechanism (Johnson and Williams, 1993; Reid *et al.,* 1990; Yan *et al.,* 1993). As a result, alternative splicing introduces even more structural diversity into the extracellular and/or intracellular domains of the FGFRs, including secreted receptor isoforms that may then function as soluble receptor-binding proteins (Guillonneau *et al.,* 1998). In addition, certain FGFRs clearly localize in the nucleus following internalization from the cell surface (Klingenberg *et al.,* 2000). However, it is not yet known if nuclear FGFR can interact with colocalized HMW FGF-2. In some cases, it is likely that both activation of plasma membrane localized FGFR signal transduction and subsequent nuclear localization of ligand and receptor are required for biological activity (Mehta *et al.,* 1998). It is not known how the internalized, presumably transmembrane trapped, receptor actually becomes intranuclear and it is not known how many other associated proteins are contained in the translocated receptor complex. Although speculative at best, is it possible that vesicle fusion with the

nuclear membrane positions some receptor in the nuclear membrane with an orientation capable of supporting backward (nuclear to cytosolic) signaling? The diversity in structure, intracellular localization, and a regulated pattern of FGFR expression, plus their varying affinities for the FGF family members, result in a similarly complicated and delicately balanced set of intracellular signals.

High-affinity FGFRs have emerged as important regulators of bone growth and development (Trippel *et al.,* 1993). In particular, bone appears to respond rather dramatically to functional imbalances that are manifested by FGF-ligand/receptor interactions. Notably, a number of human dysmorphic syndromes have been linked genetically to mutations in different FGFRs. These syndromes include achondroplasia (Rousseau *et al.,* 1994; Shiang *et al.,* 1994) and thanatophoric dysplasia (Tavormina *et al.,* 1995), which correlate with mutations in FGFR-3; Crouzon (Reardon *et al.,* 1994) and Jackson-Weiss (Jabs *et al.,* 1994) syndromes with mutations in FGFR-2; and Pfeiffer syndrome (Muenke *et al.,* 1994), with mutations in FGFR-1. In the case of achondroplasia, ectopic overexpression of FGF-2 in transgenic animals results in a phenotypically identical syndrome (Coffin *et al.,* 1995).

Low-Affinity Receptors and Binding Proteins

A second type of FGF receptor(s) is commonly referred to as cell surface and/or extracellular matrix (ECM) low-affinity proteoglycan-binding sites (Moscatelli, 1987; Ruoslahti and Yamaguchi, 1991). As a class of ECM proteins, HSPGs are required for high-affinity FGF/receptor activation and signal transduction (Chang *et al.,* 2000; Klagsbrun and Baird, 1991; Lin *et al.,* 1999; Mason, 1994; McKeehan *et al.,* 1999; Rapraeger *et al.,* 1991; Reiland and Rapraeger, 1993; Yayon *et al.,* 1991). Perlecan and syndecan-1 are specific examples of HSPGs known to potentate FGF/receptor interactions, whereas others may actually interfere with FGF-receptor interactions (Filla *et al.,* 1998; Aviezer *et al.,* 1994; Mali *et al.,* 1993; Guimond *et al.,* 1993). HSPG-binding sites are purported to "reduce the dimensionality" of cell surface FGF diffusion from three to two dimensions (Schlessinger *et al.,* 1995) while presenting a conformationally dependent multivalent FGF–heparan sulfate complex to high-affinity FGFRs, inducing dimerization. It is likely that HSPGs in the ECM also function as an extracellular storage compartment protecting FGF from degradation (Saksela and Rifkin, 1988) until becoming bioavailable, possibly following modification such as phosphorylation (Vilgrain and Baird, 1991) or proteolytic processing of the ECM (Saksela *et al.,* 1990). The bioavailability of FGF in the ECM may also be regulated by secreted binding proteins (FGF-BPs). One such secreted FGF-BP, not derived from an alternatively spliced FGFR mRNA, is postulated to displace FGF-2 sequestered in the extracellular matrix (Wu *et al.,* 1991; Czubayko *et al.,* 1994) and may participate in the development of certain forms of cancer (Kurtz *et al.,* 1997; Harris *et al.,* 2000). There are at least three members of this emerging family of FGF-Bps (Lametsch *et al.,* 2000), others are sure to follow.

The Cysteine-Rich FGF Receptor

The third class of FGF receptor-like protein, CFR, is not found complexed with high-affinity receptors and is not known to transduce an intracellular signal (Burrus and Olwin, 1989; Burrus *et al.,* 1992). An FGF-binding domain on CFR has been characterized by Zhou *et al.,* (1997). Another protein named MG-160 also binds FGF-2 (Gonatas *et al.,* 1995) and shares greater than 90% sequence homology with the CFR. However, MG-160 is an intracellular transmembrane sialoglycoprotein retained in the medial cisternae of the Golgi apparatus (Gonatas *et al.,* 1989) and is not found on the cell surface of PC-12 cells or cells of developing chick embryos (Stieber *et al.,* 1995). Furthermore, the mouse protein named ESL-1, a ligand for E-selectin, has been shown to be 94% identical to CRF (Steegmaier *et al.,* 1995), and a *Drosophila* protein named sprouty also encodes a cysteine-rich membrane protein that appears to function as a FGF antagonist (Hacohen *et al.,* 1998). Although the function of MG-160/CFR in the Golgi apparatus is still unknown, one intriguing possibility is that it serves to regulate the posttranslation processing and/or classical secretion of the FGFs. Alternatively, MG-160/CFR may represent a retrieval mechanism removing unwanted FGF-1 or FGF-2 inadvertently localized in the ER/Golgi secretory pathway; overexpression and forced targeting of FGF-1 and -2 result in cell transformation (Blam *et al.,* 1988; Rogelj *et al.,* 1988). Thus, intracellular interactions with MG-160/CFR may play a role in regulating FGF bioavailability; recent publications support this hypothesis (Fayein *et al.,* 1996; Kohl *et al.,* 2000).

Transgenic Animal Model Systems

The use of transgenic animal model systems (ectopic and targeted overexpression as well as gene ablation) has added significant insight into the physiologically relevant activities of the FGFs and FGFRs. Most surprising has been the mild phenotypic effects following FGF-1 (Miller *et al.,* 2000) or FGF-2 (Dono *et al.,* 1998; Ortega *et al.,* 1998; Zhou *et al.,* 1998) single gene knockouts. A more obvious impact might have been expected because of the wide tissue-specific pattern of expression for both prototypic members of the FGF family, the tendency of FGF-1 to interact with all FGFRs, and the fact that the FGF-2 gene encodes and differentially expresses multiple primary translation products in a pattern that is tissue specific. In fact, the phenotypic consequence of simultaneously ablating FGF-1 and FGF-2 is again most remarkable because of the absence of an overt phenotypic change. However, although it has been rather challenging to characterize, the FGF2 knockout can be distinguished from wild-type controls. The most exciting consequences were decreased trabecular bone formation observed in 4.5-month-old mice (Figs. 5 and 6) and a profound impairment of mineralization in stromal cell-derived cell cultures (Fig. 7) from FGF2 knockout mice (Montero

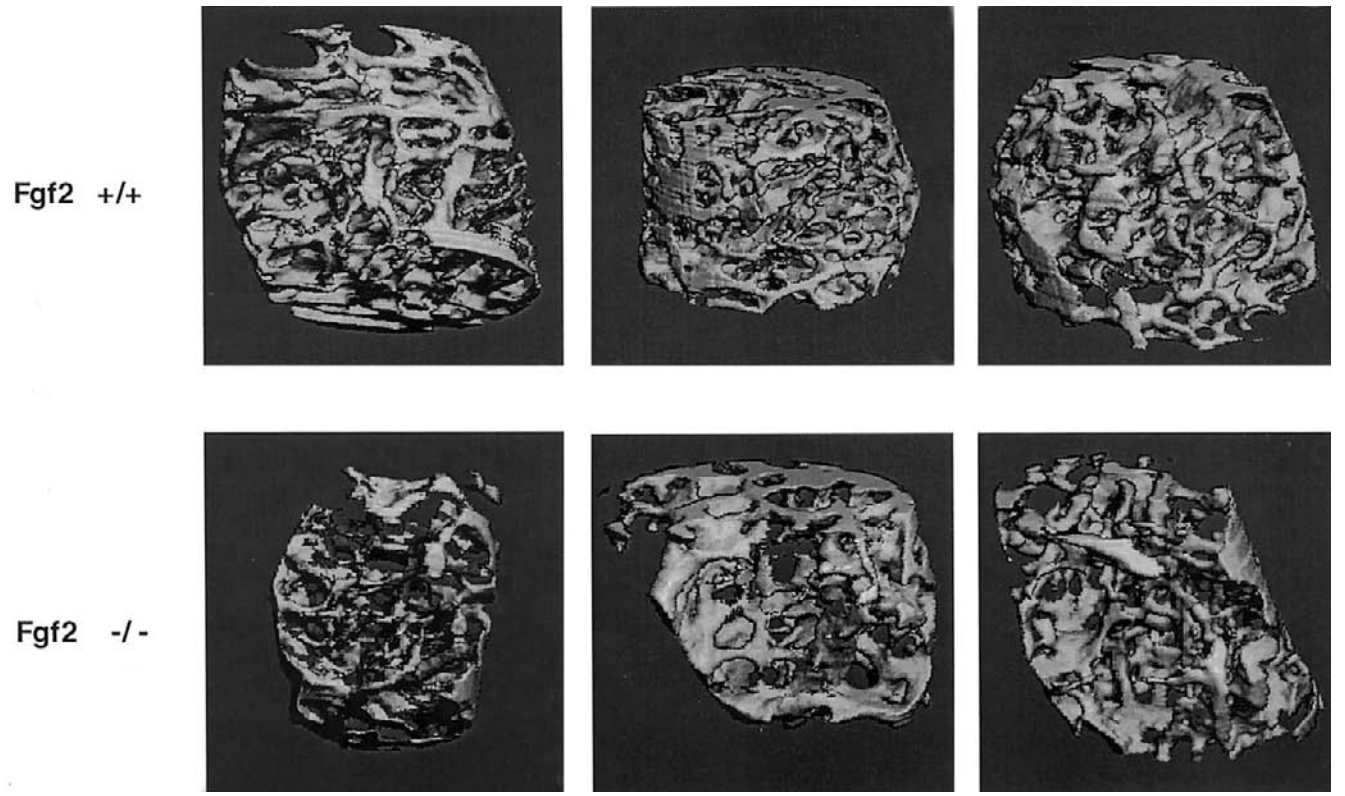


Figure 5 Morphological study by micro-CT scanning of trabecular bone of femurs of 4.5-month-old Fgf2+/+ and Fgf2−/− mice. The three-dimensional trabecular bone architecture of distal femoral metaphysis of 4.5-month-old male Fgf2+/+ and Fgf2−/− mice was analyzed by micro-CT. Note that the plate-like architecture of the trabecular bone is reduced markedly and the connecting rods of trabeculae are disrupted in Fgf2−/− mice compared with Fgf2+/+.

et al., 2000). As these mice aged, there is a progressive decrease in trabecular number in the secondary spongiosa of the distal femur (Fig. 8, Table I).

Other studies have also demonstrated that mice with Fgf2 gene knockouts exhibit a slower rate of soft tissue wound repair, neuronal defects, abnormal regulation of blood pressure, and a defect in the stromal cell-mediated formation of myeloid progenitor cells (Miller *et al.,* 2000). In addition, transgenic overexpression (gain of function) has also served to emphasize several FGF-mediated biological activities. Ectopic overexpression of FGF2 clearly results in abnormal bone growth in which the principal affect is manifested as chondrodysplasia (Coffin *et al.,* 1995) and an amplified angiogenic response (Fulgham *et al.,* 1999). Specific myocardial expression of FGF-1 increases the density of coronary arteries (Fernandez *et al.,* 2000).

Regarding FGFRs, targeted disruption of both FGFR1 (Deng *et al.,* 1994; Yamaguchi *et al.,* 1994) and FGFR2

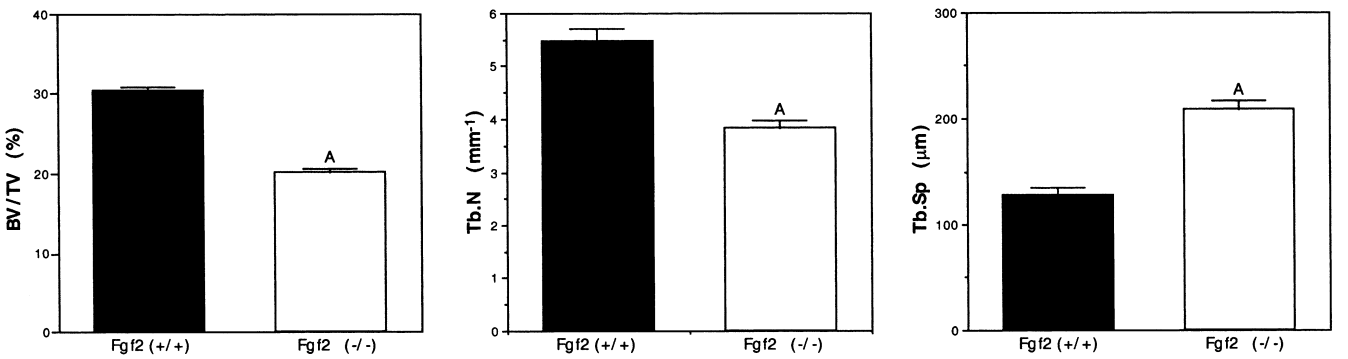
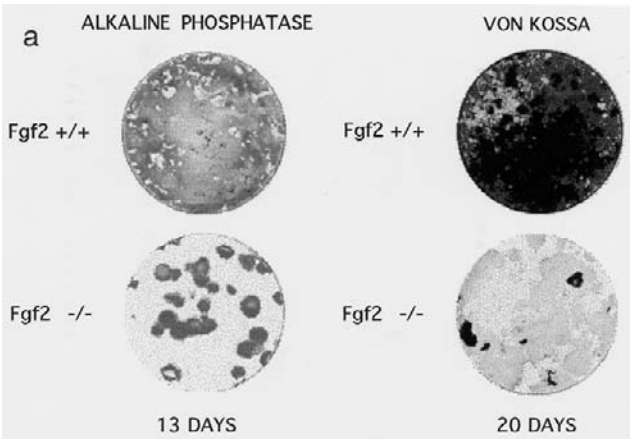


Figure 6 Three-dimensional microstructural parameters calculated using two-dimensional data obtained from micro-CT of femoral bones from 4.5-month-old Fgf2+/+ and Fgf2 −/− mice. Calculated morphometric indices included bone volume density [bone volume (BV)/trabecular volume (TV)] trabecular number [Tb.N=(BV/TV)/Tb.Th], and trabecular separation [Tb.Sp=(1/Tb.N)-Tb.Th]. [A]Significantly different from Fgf2+/+ group; $P<0.05$, Tukey–Kramer multiple comparison test (ANOVA).



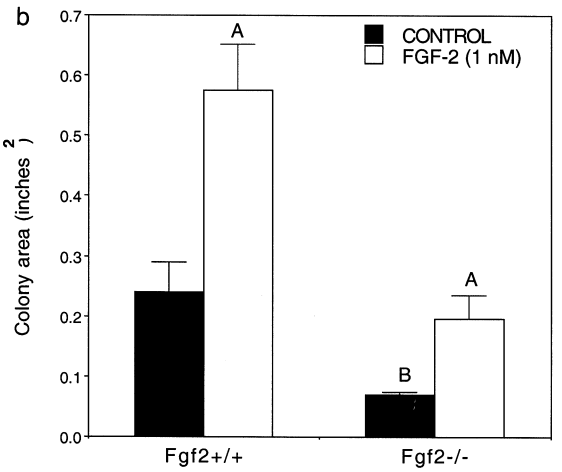


Figure 7 (a) Comparison of the ability to form ALP colonies and mineralized nodules as determined by von Kossa staining in mouse bone marrow cultures from Fgf2+/+ and Fgf2−/− mice. Cells were plated at a density of 20 million cells per well in αMEM containing penicillin/streptomycin and 10% heat-inactivated fetal calf serum (FCS). On day 3, media were changed and cells were cultured in differentiation media (αMEM, 10 n*M* dexamethasone, 10% FCS, 8 m*M* β-glycerophosphate, 50 μg/ml ascorbic acide) for the indicated times. (See also color plate.) (b) Effect of FGF-2 on colony area in mouse bone marrow cultures from Fgf2+/+ and Fgf2−/− mice. Cells were plated at a density of 1 million cells per well in αMEM containing penicillin/streptomycin and 10% heat-inactivated FCS in the absence or presence of FGF-2 (10 n*M*). On day 3, media were changed and cells were cultured in differentiation media (αMEM, 10 n*M* dexamethasone, 10% FCS, 8 m*M* β-glycerophosphate, 50 μg/ml ascorbic acid) for another 11 days. [A]Significantly different from control cultures; $P < 0.05$. [B]Significantly different from Fgf2+/+; $P < 0.05$.

(Arman *et al.,* 1998, 1999; Xu *et al.,* 1998) has been shown to be embryonic lethal, whereas disruption of the FGFR3 gene results in an abnormal bone phenotype (Colvin *et al.,* 1996; Deng *et al.,* 1996). Analysis of FGFR3 in transgenic model systems identified a single point mutation in the transmembrane domain responsible for the bone phenotype (Naski *et al.,* 1998). Furthermore, the mutation identified in FGFR3 is consistent with a naturally occurring autosomal-dominant negative (gain of function) FGFR3 mutant responsible for human dwarfism (Vajo *et al.,* 2000). The phenotypically dramatic human skeletal disorders characterized as being linked genetically to FGFR3-activating mutations, as well as overexpression of FGF ligands in transgenic model systems, provide the fundamentals necessary for a full understanding of the underlying biochemical events taking place.

Table I Three-dimensional Parameters of Micro-CT of Femora from 8-Month-Old Fgf2+/+ and Fgf2−/− Mice

Genotype	*n*	Bone volume (%)	Trabecular number (mm^{-1})	Trabecular thickness (μm)	Trabecular separation (μm)
Fgf2+/+	3	22.0 (4.8)	3.9 (0.3)	57.0 (11.1)	203 (22)
Ffg2−/−	3	7.4 (2.4)[a,b]	1.8 (0.7)[a,b]	41.7[a,b] (3.2)	564[a,b] (190)

[a]$P < 0.05$, significantly different from Fgf+/+ group, Mann–Whitney *U* test.
[b]$P < 0.05$, significantly different from Fgf+/+ group, Tukey–Kramer multiple comparison test (ANOVA).

Fibroblast Growth Factors and Bone

Studies have suggested an important role for members of the heparin-binding fibroblast growth factor family and their receptors in normal limb development, bone growth, and fracture repair and the inherited human chondrodysplastic syndromes with their attendant skeletal abnormalities. FGF-1 and FGF-2 are potent agonists with stimulatory effects on bone resorption and both stimulatory and inhibitory effects on bone formation. The inhibitory effects on collagen synthesis have been demonstrated in cell and organ culture and appear to involve inhibition of transcription of the collagen gene and to be independent of effects on cell replication. The resorptive effects of FGFs appear to be both prostaglandin dependent and independent and also involve the replication and differentiation of osteoclasts. *In vivo,* exogenous FGF-1 and FGF-2 increase bone formation by some yet unknown mechanisms. However, the effects of FGF *in vivo* may be mediated in part by transforming growth factor β (TGFβ) and prostaglandins (PGs), as the production of these factors can be regulated by FGFs. Fibroblast growth factor production in bone can be regulated by TGβ as well as by FGF itself.

Expression of FGF Receptors in Bone

The expression of FGFs and FGF receptors (FGFRs) is regulated temporally and spatially during skeletal development. Several FGF members are expressed during early stages of development in mammals (Yamaguchi and Rossant, 1995; Niswander, 1996) and their expression is related to skeletal development (Johnson and Tabin, 1997; Naski and Ornitz, 1998). During intramembranous bone formation, FGF-2 transcripts are found in mesenchymal cells and in osteoblasts (Mehara *et al.,* 1998; Rice *et al.,* 2000), together with FGF-9 (Kim *et al.,* 1998). During intramembranous bone formation, FGF-2 is produced by cells located

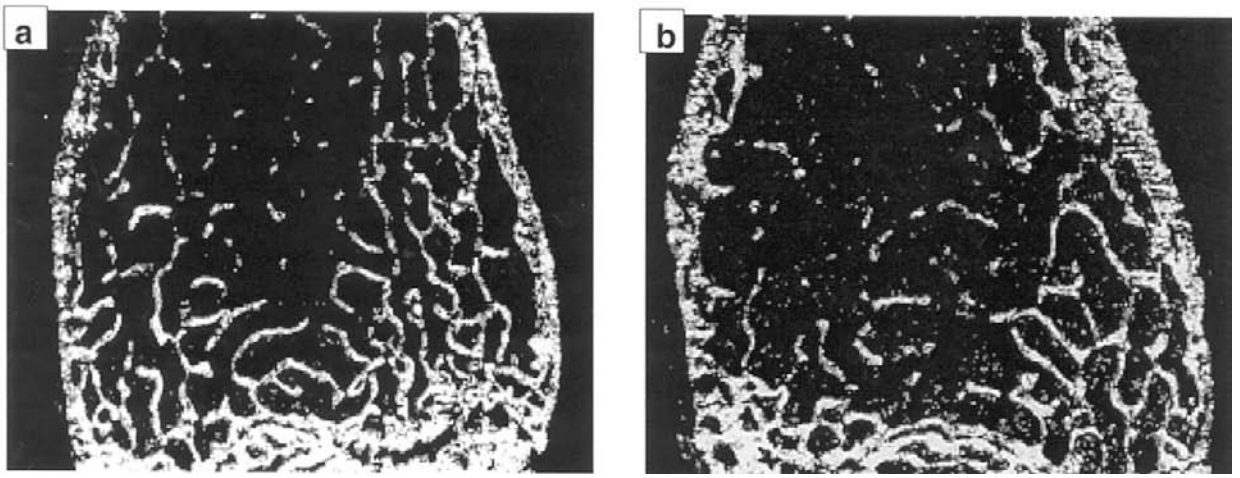


Figure 8 Undecalcified sections of the distal femur of (a) an 8-month-old Fgf2+/+ mouse and (b) a FGF−/− mouse (dark-field illumination). Trabecular number is decreased in the secondary spongiosa of the Fgf2−/− mouse.

in the resting and proliferating zones of the epiphyseal growth plate (Twal *et al.,* 1994; Tajima *et al.,* 1998). At later stages of skeletal development, FGF-1 or FGF-2 is expressed in osteoblasts and chondroblasts (Gonzalez *et al.,* 1996). FGF-2 is produced by mature osteoblasts and is stored in the extracellular matrix (Globus *et al.,* 1989; Hurley *et al.,* 1994).

FGFs actions are dependent on the expression and interactions with FGFRs. During skeletal tissue development, the spatiotemporal pattern of FGFR-1, -2, and -3 expression differs (Hughes *et al.,* 1997; Orr-Urtreger *et al.,* 1991; Wanaka *et al.,* 1991; Peters *et al.,* 1992; Patstone *et al.,* 1993; Wilke *et al.,* 1997; Delezoide *et al.,* 1998). During endochondral development, FGFR-1 transcripts are expressed in the mesenchyme and later in hypertrophic cartilage and osteoblasts, whereas FGFR-2 is expressed at sites of ossification and later in cartilage, perichondrium, and periosteum (Peters *et al.,* 1992; Delezoide *et al.,* 1998). In contrast, FGFR-3 transcripts are found mainly in cartilage rudiment and then in proliferating and prehypertrophic chondrocytes (Naski *et al.,* 1998) and are not found in mesenchymal cells or perichondral or periosteal cells until late stages of development (Peters *et al.,* 1993, Delezoide *et al.,* 1998). Consistently, the expression of FGFR-3 increases during the development of chondrogenesis *in vitro* (Szebenyi *et al.,* 1995; Moltni *et al.,* 1999a). In the human cranial suture, FGFR-1 immunostaining is localized in proliferating mesenchymal cells and preosteoblasts, whereas FGFR-2 and FGFR-3 immunostaining is found mainly with FGF-2 in preosteoblasts and osteoblasts (Delezoide *et al.,* 1998; Marie *et al.,* 2000). In the mouse, FGFR-2 transcripts are present at the osteogenic fronts of the cranial suture (Iseki *et al.,* 1997; Kim *et al.,* 1998, Johnson *et al.,* 2000), and FGFR-2 expression is associated with endogenous FGF-2 expression (Mehara *et al.,* 1998; Most *et al.,* 1998; Rice *et al.,* 2000). *In vitro,* FGFR-1 and FGFR-2 immunostaining is found in neonatal rat calvaria cells (Molténi *et al.,* 1999a) and in human calvaria osteoblastic cells (Debiais *et al.,* 1998). Although the spatiotemporal pattern of expression of FGF and FGFRs suggests that skeletal development is controlled by FGF/FGFR interactions, the FGF effects on bone cells may be modulated by the various affinity and specificity of FGF binding to the various alternative splice forms of FGFRs (Ornitz *et al.,* 1996).

FGF Receptors and Human Chondrodystrophies

Skeletal abnormalities are characteristic of human chondrodystrophies. High-affinity FGFRs have emerged as important regulators of bone growth and development (Globus *et al.,* 1988; Trippel *et al.,* 1993). In particular, bone appears to respond rather dramatically to functional imbalances that are manifested by FGF-ligand/receptor interactions. Notably, a number of human dysmorphic syndromes have been linked genetically to mutations in different FGFRs. These syndromes include achondroplasia (Rousseau *et al.,* 1994; Shiang *et al.,* 1994) and thanatophoric dysplasia (Tavormina *et al.,* 1995), which correlate with mutations in FGFR-3; Crouzon (Reardon *et al.,* 1994) and Jackson–Weiss (Jabs *et al.,* 1994) syndromes with mutations in FGFR-2; and Pfeiffer syndrome (Muenke *et al.,* 1994) with mutations in FGFR-1.

FGF Receptors and Human Craniosynostosis

The important role of FGFRs during cranial development is supported by genetic evidence that FGFR mutations induce abnormal ossification of the cranial sutures (craniosynostosis) in humans (Park *et al.,* 1995; Muenke and Schelle, 1995). Several mutations in FGFRs are associated with heritable human skeletal disorders involving craniofacial abnormalities, causing Apert, Crouzon, Pfeiffer, and Jackson–Weiss syndromes (Wilkie, 1997; Webster

and Donoghue, 1997). Mutations in the Ig III domain or in the linker between the Ig II and Ig III domain of FGFR-1 and FGFR-2 induce constitutive activation of the receptor (Neilson and Friesel, 1995, 1996; Galvin *et al.,* 1996; Robertson *et al.,* 1998; Plotnikov *et al.,* 2000). In Crouzon syndrome, the C342Y mutation in FGFR-2 results in the activation of FGFR-2 signaling and decreased binding of FGF-2 to the receptor (Mangasarian *et al.,* 1997). In Apert syndrome, FGFR-2 mutations enhance receptor occupancy by FGF ligands or prolongation of the duration of receptor signaling (Park *et al.,* 1997; Anderson *et al.,* 1998).

In mice, a secreted soluble dominant-negative FGFR-2 causes skull abnormalities resembling those induced by FGFR mutations (Celli *et al.,* 1998), suggesting that decreased or decreased FGFR signaling induces skeletal abnormalities. The phenotypes induced by FGFR genetic mutations have been studied in mice and humans. The P250R FGFR1 mutation in the mouse causes premature fusion of calvaria sutures associated with increased expression of Cbfa1, a transcription factor involved in osteoblast differentiation (Zhou *et al.,* 2000). Consistantly, implantation of FGF-2 or FGF-4 accelerates suture closure in mice (Iseki *et al.,* 1997; Kim *et al.,* 1998).

In humans, FGFR-2 mutations in Apert syndrome activate osteoblast differentiation without affecting cell proliferation or the response to FGF-2 (Fragale *et al.,* 1999; Lomri *et al.,* 1998; Lemonnier *et al.,* 2000c; Marie, 1999). The S252W FGFR-2 Apert mutation constitutively increases osteoblast marker genes in calvaria preosteoblasts through activation of PKC (Lemonnier *et al.,* 2000b). This accelerated osteoblast differentiation program induced by the mutation implicates an increased cell–cell adhesion and N-cadherin overexpression (Lemonnier *et al.,* 2000b). Consistent with this effect induced by the FGFR-2-activating mutation, exogenous FGF-2 was found to increase N-cadherin expression in human calvaria osteoblasts through activation of the PKC pathway (Debiais *et al.,* 2000). Additionally, there is a selective and PKC-independent downregulation of FGFR-2 in Apert mutant preosteoblasts and osteoblasts *in vitro* and *in vivo* (Lemonnier *et al.,* 2000c), which may contribute to increased osteoblast differentiation. This is consistent with the decreased expression of FGFR-2 in Crouzon syndrome (Bresnick *et al.,* 1995) and with the downregulation of FGFR-2 transcripts induced by FGF-2 in the mouse suture (Iseki *et al.,* 1997). In addition to affecting osteoblast differentiation, the Apert S252W FGFR-2 mutation promotes apoptosis in human calvaria osteoblasts. The constitutive activation of PKC in mutant osteoblasts leads to overexpression of IL-1 and Fas receptor, activation of caspase-8, increased Bax/Bcl-2 ratio, and premature apoptosis (Lemonnier *et al.,* 2000a). Consistently, mouse calvaria cells transfected with the S252W Apert FGFR-2 mutation show apoptosis, and FGF-2 increases osteoblast apoptosis in differentiated mouse calvaria osteoblasts (Mansukhani *et al.,* 2000). The premature ossification induced by activating FGFR mutations may thus result from alterations of both differentiation and apoptotic programs in osteoblasts.

FGF and Cartilage

FGF-2 is expressed in growth plate chondrocytes *in vivo* (Twal *et al.,* 1994) and may be an autocrine growth factor (Luan *et al.,* 1996). Exogenous FGF-2 stimulates chondrocyte cell growth and inhibits cell differentiation (Kato *et al.,* 1990; Wroblewski *et al.,* 1995), indicating that FGF/FGFR interactions control cartilage cell growth and differentiation. There is multiple *in vivo* evidence that FGFs play an important role in chondrogenesis (Ornitz, 2000a). Excess exogenous FGF-2 causes chick limb developmental abnormalities (Cohn *et al.,* 1995), and FGF-2 overexpression induces chondrodysplasia and shortening of long bones in mice (Coffin *et al.,* 1995; Lightfoot *et al.,* 1997). The negative role of FGFR-3 in endochondral growth is emphasized by the finding that FGFR-3−/− mice show excessive long bone growth, associated with increased chondrocyte proliferation (Colvin *et al.,* 1996; Deng *et al.,* 1996). In humans, mutations in FGFR-3 cause constitutive activation of FGFR-3, resulting in chondrodysplasia syndromes, including achondroplasia, the most common form of dwarfism, hypochondroplasia, and thanatophoric dysplasias (Bellus *et al.,* 1995; Rousseau *et al.,* 1994; Shiang *et al.,* 1994; Supert-Furga *et al.,* 1995; Naski *et al.,* 1996; Webster *et al.,* 1996; Tavormina *et al.,* 1995; Rousseau *et al.,* 1995), which demonstrates a role for FGFR-3 in the control of long bone cartilage growth. Several FGFR-3 mutations cause abnormal chondrogenesis in the mouse (Chen *et al.,* 1999; Segev *et al.,* 2000). Overexpression of activated FGFR-3 in mice reduces chondrocyte cell proliferation (Naski *et al.,* 1998; Ornitz, 2000), showing that activated FGFR-3 signaling inhibits chondrocyte proliferation and decreases differentiation (Li *et al.,* 1999; Naski, 2000). In addition, constitutive activation of FGFR-3 induced by the R248C mutation triggers premature apoptosis, which is mediated by activation of the STAT1 signaling pathway (Legeai-Mallet *et al.,* 1998). The expression in the growth plate of Ihh and BMP4 was found to be downregulated in mice overexpressing FGFR-3 and increased in mice lacking FGFR-3, suggesting that FGFR-3 may act on multiple signaling molecules to control endochondral bone growth (Naski *et al.,* 1996; Ornitz, 2000b).

Fibroblast Growth Factor Production and Regulation

Although there are 23 members of the fibroblast growth factor family, only FGF-1 and FGF-2 have been studied extensively in bone. FGF-2 and FGF-1 are found in the bone matrix (Hauschka *et al.,* 1986) and are produced by bone cells, with FGF-2 being 10-fold higher than FGF-1 when measured by radioimmunoassay (Seyedin *et al.,* 1985; Globus *et al.,* 1989).

In nonosseous tissues Weich *et al.* (1991) reported that there is transcriptional regulation of FGF-2 gene expression in capillary endothelial cells, which are known to synthesize

and store FGF-2. FGF-2 induces its own mRNA in these cells and there is a similar increase in FGF-2 mRNA in response to thrombin and phorbol esters. In more recent studies, Stachowiak *et al.* (1994) found that stimulation of adenylate cyclase with forskolin, or protein kinase C with PMA, increases FGF-2 protein in adrenal medullary cells. The induction of FGF-2 by forskolin and PMA is due to transcriptional activation of the FGF-2 gene. In addition, the regulation of FGF-2 protein content also involves posttranscriptional mechanisms. FGF-2 expression in the anterior pituitary of rats is decreased by castration and can be restored by testosterone. Neither adrenalectomy nor chemical thyroidectomy alters FGF-2 gene expression (Yoshimura *et al.,* 1994).

There are few data on the regulation of FGF expression in bone. In cultures of mouse osteoblastic MC3T3-E1 cells, exogenous FGF-2 increases its own mRNA (Hurley *et al.,* 1994). TGF-β also increases FGF-2 mRNA and protein in MC3T3-E1 cells (Hurley *et al.,* 1994). TGF-β increases FGF-2 protein in both the cytoplasm and the nucleus. However, there is no measurable FGF-2 in the conditioned medium. The significance of TGF-β regulation of FGF-2 in osteoblasts is not clear. Amplification of the responses to both FGF and TGF-β could occur by an increase in endogenous FGF. In addition, parathyroid hormone (PTH) (Hurley *et al.,* 1999) and prostaglandins (Sabbieti *et al.,* 1999) increase FGF-2 mRNA and protein levels in bone cells, suggesting that anabolic factors for bone may act in part by stimulating endogenous FGF-2 in osteoblasts.

Fibroblast Growth Factors and Bone Formation

Osteoblast Proliferation

FGF-1 and FGF-2 stimulate bone cell replication (Canalis and Raisz, 1980; Rodan *et al.,* 1987; Globus *et al.,* 1988, 1989; McCarthy *et al.,* 1989; Shen *et al.,* 1989; Hurley *et al.,* 1992). Canalis and Raisz (1980) demonstrated that partially purified fibroblast growth factor stimulated fetal rat calvarial cell proliferation. Subsequent studies using purified or recombinant FGF-1 and FGF-2 confirmed this initial report (Rodan *et al.,* 1987; McCarty *et al.,* 1989; Shen *et al.,* 1989; Hurley *et al.,* 1992), not only in bone organ culture but also in osteoblast-enriched cells from fetal rat calvaria. In osteosarcoma-derived ROS 17/2.8 cells, which express osteoblastic features, Rodan *et al.* (1989) found that FGF-2 stimulated cell proliferation when cells are seeded at low density in serum-free media but not when seeded at high density in the presence of serum. In contrast in mouse osteoblastic MC3T3-E1 cells, FGF-2 stimulates cell proliferation in the presence of serum at both high and low density (Hurley *et al.,* 1993). FGF-1 and FGF-2 also activate the growth of rat and human stromal bone cells (Noff *et al.,* 1989; Pitaru *et al.,* 1993; Locklin *et al.,* 1995; Hanada *et al.,* 1997; Berrada *et al.,* 1995; Pri-Chen *et al.,* 1998). In human calvaria cells, FGF-2 also promotes cell proliferation, although this was observed only at an early stage of differentiation (Debiais *et al.,* 1998). It is noteworthy that the mitogenic effect of FGF-2 on rat calvaria cells decreases with age (Kato *et al.,* 1995; Tanaka *et al.,* 1999).

Osteoblast Differentiation

In vitro FGFs inhibit markers of the osteoblast phenotype. FGFs reduce differentiation-related markers such as alkaline phosphatase (Rodan *et al.,* 1989; Shen *et al.,* 1989). FGF-2 inhibits parathormone responsive adenylcyclase activity in part via a pertussis toxin-sensitive G protein (Rodan *et al.,* 1989). Type I collagen is the major product of the osteoblast and FGF-2 affects its production. Both stimulatory and inhibitory effects of FGF-2 on collagen synthesis in osteoblasts have been reported (Canalis *et al.,* 1988; McCarty *et al.,* 1989). Similar to FGF-2, FGF-1 has stimulatory and inhibitory effects on collagen synthesis in fetal rat calvariae (McCarty *et al.,* 1989; Shen *et al.,* 1989). Short-term treatment with FGF-2, which is 24 hr of treatment followed by removal of FGF from the osteoblast cultures, results in the stimulation of collagen synthesis. However, continuous or chronic FGF treatment inhibits type I collagen synthesis. FGF-2 appear to have independent effects on osteoblast proliferation and differentiation. In bone organ cultures, the inhibitory effect of FGF-2 on collagen synthesis is similar in the presence or absence of aphidicolin, an inhibitor of cell replication (Hurley *et al.,* 1992). Rodan *et al.* (1989) found that under conditions where FGF-2 had no effect on the proliferation of ROS 17/2.8 cells, there is a reduction in alkaline phosphatase activity, osteocalcin synthesis, and type I collagen mRNA. A similar dissociation is observed in chondrocytes where terminal differentiation and calcification are inhibited by FGF-2 independent of cell proliferation (Kato and Iwamoto 1990). In myoblasts, FGF inhibits differentiation independent of effects on cell proliferation (Clegg *et al.,* 1987).

The inhibitory effect of FGF-2 on collagen synthesis appears to be transcriptional (Rodan *et al.,* 1989; Hurley *et al.,* 1993) and is consistent with previous studies showing that a predominant mechanism of hormonal and growth factor regulation of collagen synthesis is at a pretranslational site (Canalis *et al.,* 1988). In studies in osteoblastic MC3T3-E1 cells, it has been shown that FGF-2 reduces the transcription of the type I collagen gene by nuclear run-on analysis (Hurley *et al.,* 1993). Our initial studies on transcription of the collagen gene suggest that a DNA locus between -3.5 and -2.3 kb of the collagen promoter is negatively regulated by FGF-2 (Hurley *et al.,* 1993). The mechanism(s) by which FGF-2 negatively regulates collagen gene transcription is unknown.

FGF-2 also affects the expression of other bone matrix proteins. FGF-2 stimulates osteopontin and modulates osteonectin in rat osteosarcoma cells (Rodan *et al.,* 1989; Shiba *et al.,* 1995; Delany and Canalis, 1998). Although FGF-1 inhibits osteocalcin expression in rat calvaria cells (Tang *et al.,* 1996), it enhances osteocalcin in bovine bone

cells (Schedlich *et al.,* 1994) and directly affects osteocalcin transcription in mouse calvaria cells (Boudreaux *et al.,* 1996; Newberry *et al.,* 1996). In these cells, FGF-2 and cAMP have synergistic effects on osteocalcin expression, and some elements conferring FGF-2 responsiveness in the osteocalcin promoter have been identifed (Schedlich *et al.,* 1994; Newberry *et al.,* 1997). FGF may also affect osteoblast function by acting on functional membranous proteins. FGFs downregulate the expression of connexin-43 associated with decreased gap junction-mediated communication (Shiokawa-Sawada *et al.,* 1997), and FGF-2 upregulates the expression of N-cadherin in osteoblasts (Debiais *et al.,* 2000). FGF-2 also increases sodium-dependent phosphate transport in mouse calvaria cells, which may contribute to regulate intramembranous calcification (Suzuki *et al.,* 1998).

FGF Regulation of Growth Factors and Cytokines in Bone

Insulin-like growth factor-I and insulin-like growth factor-II (IGF-I and IGF-II) proteins are made by bone cells (Canalis *et al.,* 1991; Delany *et al.,* 1994) and are major modulators of bone cell function (Hock *et al.,* 1988), and FGF-2 has been shown to affect the production of IGF-I and -II and their binding proteins in osteoblasts. FGF-2 decreases IGF-I mRNA and protein levels in mouse osteoblastic MC3T3-E1 cells (Hurley *et al.,* 1992) and rat bone cell cultures (Canalis *et al.,* 1993). The inhibitory effects of FGF-2 on IGF-I mRNA and protein are not specific for osteoblasts, as similar results are reported in smooth muscle cells (Bornfeldt *et al.,* 1990). FGF-2 inhibits IGF-I mRNA in the presence of aphidicolin, an inhibitor of cell replication, thereby suggesting that the effects of FGF-2 are independent of cell replication. FGF-2 decreases the mRNA and protein levels for several IGF-binding proteins *in vitro,* in MC3T3-E1 cells (Hurley *et al.,* 1995a). In contrast to the studies of Chen *et al.* (1993), 24 hr treatment with FGF-2 does not inhibit IGF-binding protein mRNA in rat osteoblast-like cells. The inhibitory effects on IGFs and their binding proteins could play a role in the inhibition of bone formation by FGF. However, the *in vivo* effects of FGF-2 on IGF-I and -II and the IGF-binding proteins have not been examined.

Both FGF-1 and FGF-2 increase the production of TGFβ *in vitro* and *in vivo* (Noda and Vogel, 1989). In addition, TGFβ and FGF-1 and FGF-2 interact to modulate their mitogenic effects in osteoblasts (Globus *et al.,* 1989). In human calvaria cells, FGF-2 decreases TGFβ2 production in immature cells but increases TGFβ2 synthesis by more differentiated cells (Debiais *et al.,* 1998). However, FGF-2 increases vascular endothelial cell growth factor (VEGF) expression in rat and mouse calvaria cells independently of TGFβ synthesis (Saadeh *et al.,* 2000). FGF-2 also induces hepatocyte growth factor expression in osteoblasts (Blanquaert *et al.,* 1999), which may play a role in bone repair. Additionally, FGF-2 increases IL-6 transcripts and protein in mouse calvarial osteoblastic cells (Hurley *et al.,* 1996), an effect that is dependent on intracellular calcium mobilization and is autoregulated by PKC activation. These factors may perhaps mediate some of the actions of FGF on bone cells.

Bone Formation *in Vitro*

Studies have shown that FGF-2 has both stimulatory and inhibitory effects on bone formation *in vitro.* In long-term culture, the effects of FGFs on osteoblastic cell differentiation are dependent on the cell culture. FGF-1 reduces bone nodule formation in the rat calvaria system (Tang *et al.,* 1996), whereas FGF-2 inhibits osteoblast differentiation markers in human marrow stromal cells (Rifas *et al.* 1995; Berrada *et al.,* 1995; Martin *et al.,* 1997). However, FGF-2 increases cell differentiation and matrix mineralization in rat bone marrow stromal cells (Noff *et al.,* 1989; Pitaru *et al.,* 1993) and in dexamethasone-treated rat (Locklin *et al.,* 1995) and human bone marrow stromal cells (Martin *et al.,* 1997; Pri-Chen *et al.,* 1998; Scutt and Bertram, 1999; Walsh *et al.,* 2000). Interestingly, Giuliani *et al.* (1995) reported that alendronate induces mineralized nodule formation in human bone marrow cultures, which is associated with an increase in endogenous FGF-2 production. In human calvaria cells, FGF-2 initially reduces osteocalcin synthesis, whereas a prolonged treatment increases both osteocalcin synthesis and matrix mineralization, indicating that the effects of FGF-2 are dependent on the osteoblast maturation stage (Debiais *et al.,* 1998).

Bone Formation *in Vivo*

As described previously, FGF-2 stimulates proliferation of osteoblastic cells, but can inhibit collagen synthesis and osteogenic cell differentiation *in vitro.* These results are in contrast to the effects of FGFs *in vivo.* Local injection of FGF-1 promotes calvaria bone formation (Mundy *et al.,* 2000). At the endosteal level, FGF-2 at low dose also stimulates bone formation in growing rats (Aspenberg *et al.,* 1991; Nakamura *et al.,* 1995; Kawaguchi *et al.,* 1994; Mayara *et al.,* 1993; Nagai *et al.,* 1995). In normal rabbits, intraosseous application of FGF-2 increases bone formation and bone mineral density (Nakamura *et al.,* 1996; Okazaki *et al.,* 1999). Exogenous FGF-1 and FGF-2 appear to act by increasing the recruitment of osteoblast precursor cells, which then differentiate into osteoblasts (Nakamura *et al.,* 1995). It is also possible that FGF-2 acts indirectly by increasing the expression of TGFβ in osteoblasts (Nakamura *et al.,* 1995; Kawaguchi *et al.,* 1994). In osteoblastic MC3T3-E1 cells, FGF-2 enhances TGFβ1 gene expression (Noda and Vogel, 1989). Moreover, TGFβ increases FGF-2 mRNA and protein in these cells (Hurley *et al.,* 1994). Thus, it is also possible that the complex biphasic effects of TGFβ may be due in part to TGFβ altering the endogenous production of other growth factors such as FGF-2. Glypican, syndican, and β-glycan are cell surface proteoglycans

that bind FGF via their heparin sulfate moieties and are believed to present FGF to the signaling receptors. β-Glycan also binds to TGFβ, thereby providing a physical link between FGF and TGFβ (Nilsen-Hamilton, 1994).

Fibroblast Growth Factors in Fracture Repair

Fibroblast growth factors also appear to play an important role in fracture repair in rats (Kawaguchi *et al.,* 1994) and in humans (Wildburger *et al.,* 1994). During fracture repair, FGF-1 and -2 are expressed in the granulation tissue (Scully *et al.,* 1990), suggesting that FGFs may increase cell migration and angiogenesis at early stages of bone repair. At later stages, FGF-1 expression increases during callus formation, whereas FGF-2 expression is stable (Joyce *et al.,* 1991). Several studies indicate that exogenous FGF-2 can accelerate bone repair (Hiroshi *et al.,* 1994; Radomsky *et al.,* 1998). Although FGF-1 increases the proliferation of chondrocyte precursors (Cuevas *et al.,* 1988) and fibrous callus, it does not enhance bone repair in rats (Jingushi *et al.,* 1990). In various animal models of bone defects, FGF-2 at low doses was found to stimulate chondrogenesis and bone formation (Wang *et al.,* 1996; Aspenberg *et al.,* 1991; Andreshak *et al.,* 1997; Inui *et al.,* 1998; Kato *et al.,* 1998; Okazaki *et al.,* 1999; Tabata *et al.,* 1999; Zellin and Linde, 2000), suggesting that FGF may improve bone regeneration (Radomsky *et al.,* 1998). Part of the effects of FGF on bone repair may involve the release of HGF (Blanquaert *et al.,* 1999) or VEGF (Saadeh *et al.,* 2000) by osteoblasts.

FGF Signaling in Bone Cells

Few data are available on the signaling mechanisms involved in the regulation of bone cells by FGF. FGF-2 activates MAP kinase signal transduction and extracellular signal-regulated kinase 2 (ERK2) in osteoblastic cells (Hurley *et al.,* 1996; Newberry *et al.,* 1997; Chaudhary and Avioli, 1997), but the role of signaling pathways in the FGF effects on osteoblastic cell function is far from being understood. The Erk signaling pathway mediates the downregulation of procollagen gene expression by FGF-2 in osteoblasts (Chaudhary and Avioli, 2000). In contrast, the FGF-2 transcriptional activation of the matrix metalloproteinase MMP1 is independent of Erk1/Erk2 MAPK activity in mouse osteoblasts (Newberry *et al.,* 1997). However, the increased IL-6 synthesis induced by FGF-2 involves p38 MAP kinase activation (Kozawa *et al.,* 1999). The PKC pathway appears to be an important pathway regulating FGF actions in osteoblasts. FGF-2 activates PKC activity in human osteoblasts, and this pathway is implicated in the expression of N-cadherin expression induced by FGF-2 (Debiais *et al.,* 2000). The PKC pathway is also involved in the growth response of FGF-2 in Py1a rat osteoblastic cells (Hurley *et al.,* 1996) and in the FGF-2-induced increase in sodium-dependent phosphate transport in calvaria cells (Suzuki *et al.,* 1998). Additionnaly, PKC signaling is constitutively activated by the S252W FGFR-2 mutation in human mutant osteoblasts and is responsible for the increased osteoblast phenotype and premature apoptosis in these cells (Lemonnier *et al.,* 2000a,b). In chondrocytes, however, FGF signaling and activating FGFR-3 mutations activate STAT1 signaling pathway, leading to growth arrest and apoptosis (Sahni *et al.,* 1999; Li *et al.,* 1999; Legeai-Mallet *et al.,* 1998; Su *et al.,* 1997). In osteoclasts, FGF-2 activates FGFR-1 and MAPK to stimulate cell function (Chikazu *et al.,* 2000). Thus, multiple pathways are involved in the FGF effects on bone cells.

Fibroblast Growth Factors and Bone Resorption

FGF and Bone Resorption

FGF-1 induces bone resorption in fetal rat long bone via a prostaglandin-mediated mechanism (Shen *et al.,* 1989). FGF-2 is also a potent bone-resorbing agent in the fetal rat long bone model (Simmons *et al.,* 1991). However, in fetal rat long bones, the effects of FGF-2 on bone resorption appear to be independent of endogenous prostaglandin production (Simmons *et al.,* 1991). In contrast to the report by Shen *et al.* (1989), Simmons *et al.* (1991) reported that FGF-1 induces calcium release only in the presence of heparin and that this effect is not blocked by indomethacin. FGF-2 increases bone resorption in neonatal mouse calvariae, which is partially blocked by indomethacin, suggesting that the resorptive effects of FGF-2 are mediated in part by prostaglandins. In the presence of both an inhibitor of DNA synthesis and prostaglandin production, FGF-2-induced bone resorption is completely blocked. These results suggest that FGF-2 has direct effects on resorption through its effects on osteoclasts precursor proliferation and an indirect effect that is mediated by prostaglandin (Kawaguchi *et al.,* 1995a). These findings are important because prostaglandins are potent local regulators of bone metabolism (Kawaguchi *et al.,* 1995b).

FGF Regulation of Prostaglandins in Bone

FGF-2 induces mRNA and protein for prostaglandin GH synthetase 2 (PGHS-2), the major enzyme in arachidonic acid conversion to prostaglandins by a transcriptional mechanism in MC3T3-E1 cells (Kawaguchi *et al.,* 1995). In these cells, FGF-2 also increases PGE_2production in the presence of the substrate arachidonic acid. In the presence of exogenous arachidonic acid, FGF-2-stimulated PGE_2 production is paralleled by the stimulaton of PGHS-2 promoter luciferase activity, indicating transcriptional regulation of PGHS-2.

FGF Regulation of the Collagenase Gene in Bone

FGF may also modulate bone matrix degradation by regulating collagenase expression and activity. Interstitial collagenase is required to initiate the degradation of type I

collagen (Delaisee *et al.,* 1988), and inhibitors of metalloproteinases inhibit bone resorption (Delaisee *et al.,* 1988). In rat calvaria bone cells, FGFs increase interstitial collagenase-1 and -3 expression by a prostaglandin- and PKC-dependent pathway (Varghese *et al.,* 1995; Tang *et al.,* 1996). In contrast, in mouse cells the effects of FGF-2 on the collagenase gene are not affected by either indomethacin or staurosporine (Hurley *et al.,* 1995b). Similar to FGF-2, FGF-1 also upregulates the collagenase gene (M. M. Hurley, unpublished observation). FGF-2 transcriptionally regulates collagenase gene expression in mouse calvaria cells, and the tyrosine kinase inhibitors genistein and herbimycin A completely block the effect of FGF-2 on the collagenase promoter (Hurley *et al.,* 1995b). FGF response elements in the collagenase promoter have been identified (Newberry *et al.,* 1997). FGF-2 stimulates collagenase-3 gene transcription through an effect on the AP-1 site on the promoter (Varghese *et al.,* 2000) and induces the expression of tissue inhibitors of metalloproteinases (TIMP) 1 and 3 (Varghese *et al.,* 1995), which regulate collagenase activity. Although FGF-2 decreases stromelysin-3 mRNA stability, it increases gene transcription (Delany and Canalis, 1998), which may contribute to the control of the bone matrix degradation.

FGF and Osteoclastogenesis

Histologic examination reveals that FGF-1 treatment increases osteoclast number in fetal rat long bones (Shen *et al.,* 1989) and in 21-day fetal rat calvariae (Hurley *et al.,* 1992) suggesting a role for FGFs in osteoclastogenesis. Osteoclasts are derived from a hematopoietic precursor that is common to the granulocyte and the macrophage. The immediate progenitor of the osteoclast is the CFU-GM (colony-forming unit for granulocytes and macrophages) (Mundy and Roodman 1995), and studies show that FGF-2 stimulates the formation of CFU-GM in human marrow cultures (Gabrilove *et al.,* 1994). The production of osteoclasts from progenitor cells is regulated by cytokines (Roodman, 1995) and requires or is enhanced by interactions between marrow progenitor cells and either osteoblastic or stromal cells (Suda *et al.,* 1992). Interstingly, FGF-2 is a potent mitogen for stromal cells (Oliver *et al.,* 1990), which are multipotential mesenchymal cells in the marrow environment that are the precursors of osteoblasts, chondroblasts, fibroblasts, and other connective tissue cells in bone. FGF-2 is produced by bone marrow stromal cells (Brunner *et al.,* 1993) and hence may be autostimulatory.

FGF-2 has been found to increase the formation of multinuclear-resorbing osteoclast-like cells in mouse bone marrow cultures, an effect mediated by PGE_2 production (Hurley *et al.,* 1998; Kawaguchi *et al.,* 2000). Indeed, FGF-2 stimulates PGE_2 production rapidly in mouse calvaria cells through the transcriptional regulation of PGHS-2 (Kawaguchi *et al.,* 1995). FGF-2 also increases osteoclast formation by a mechanism involving COX-2 stimulation and prostaglandin formation, leading to increased osteoclast differentiation factor (ODF) production (Nakagawa *et al.,* 1999). Apart from this indirect effect of FGF-2 through osteoblastic cells, FGF-2 acts directly on mature osteoclasts to stimulate bone resorption (Kawaguchi *et al.,* 2000) through the activation of FGFR-1 and MAPK activation (Chikazu *et al.,* 2000).

Interaction of Heparin and Fibroblast Growth Factors

FGF-2 and FGF-1 bind to high-affinity cell surface tyrosine kinase receptors (Sakaguchi *et al.,* 1991; Ullrich and Schlessinger, 1991). This binding may not occur in the absence of prior binding to low-affinity membrane-associated heparan sulfate proteoglycans (HSPGs) that act as low-affinity coreceptors to modulate FGF actions (Feige *et al.,* 1989; Yayon *et al.,* 1991; Ornitz, 2000; Schlessinger *et al.,* 2000). Other studies show that site-directed mutagenesis of the heparin-binding domains of the FGF-2 gene results in structural analogs of FGF-2 with greater or lesser mitogenic activity related to changes in their affinity for heparin (Heath *et al.,* 1991). Enzymatic degradation or inhibition of sulfation of heparin inhibits binding of FGF-2 to its receptor, thus blocking the mitogenic activity of FGF-2 in fibroblasts, as well as its ability to repress myoblast differentiation (Rapraeger *et al.,* 1991). Thus heparin appears to modulate the biological responses of tissues to FGF-1 and FGF-2. Heparin sulfate proteoglycans are made by osteoblasts (Beresford *et al.,* 1987), and Globus *et al.* (1989) suggested that the FGF–heparin sulfate complex is released in an active form, resulting in osteoblast proliferation and new bone formation.

Studies suggest a direct role for heparin in modulating the biological effects of FGF-1 and FGF-2 in bone (Simmons *et al.,* 1991; Hurley *et al.,* 1992). Heparin enhances the mitogenic effect, as well as the resorptive effect of FGF-1, in fetal rat long bone cultures (Simmons *et al.,* 1991). In contrast, heparin does not enhance the mitogenic effect of FGF-2 in fetal rat long bone or in fetal rat calvarial cultures (Simmons *et al.,* 1991; Hurley *et al.,* 1992). Heparin does, however, enhance the resorptive effect of FGF-2 in fetal rat long bone cultures (Simmons *et al.,* 1991), as well as the inhibitory effect on FGF-2 on percentage collagen synthesis in fetal rat calvariae (Hurley *et al.,* 1992). The effects of heparin to enhance FGF-mediated bone resorption and inhibition of collagen synthesis may be important in heparin-induced osteoporosis.

Some cell surface transmembrane HSPGs named syndecans interact with FGF binding and signaling (Yahon *et al.,* 1991; David, 1993; Rapraeger, 1993; Bernfield *et al.,* 1993). During development, syndecan-1 is expressed in mesenchymal cells (Vainio *et al.,* 1991) and limb bud (Solursh *et al.,* 1990) and affects bone cell differentiation (Dhodapkar *et al.,* 1998). Syndecan-2 is abundant in prechondrogenic cells and is expressed in the perichondrium and periosteum at the onset of *in vitro* osteogenesis (Molténi *et al.,* 1999a). Syndecan-3 is expressed in mesenchymal cells during limb bud formation, is regulated by FGFs (Gould *et al.,* 1992),

and mediates the ability of FGFs to promote mesoderm proliferation (Dealy *et al.,* 1997). Thereafter, precartilage cells and immature chondrocytes express syndecan-3, which may control chondrocyte proliferation during endochondral ossification (Shimazu *et al.,* 1996). Syndecan-4 is strongly expressed in chondrocytes (Kim *et al.,* 1994) and osteoblasts (Molténi *et al.,* 1999a). The coexpression of syndecans and FGFRs during osteogenesis suggests a role for these HSPGs in the control of bone formation. For example, rat chondrocytes coexpress FGFR-3, syndecan-2, and syndecan-4, and their expression decreases during *in vitro* differentiation. In contrast, rat calvaria osteoblasts express syndecan-1, -2, and -4 with FGFR-1 and FGFR-2, indicating that syndecans can interact with FGFRs to control FGF actions during *in vitro* chondrogenic and osteogenic differentiation (Molténi *et al.,* 1999a). Moreover, alterations of matrix- and cell-associated proteoglycan sulfation or synthesis inhibit the growth response to FGF-2 in cultured rat mandibular condyle (Molténi *et al.,* 1999b), indicating that syndecans and other HSPGs may be important coreceptors modulating FGF/FGFR interactions in bone cells.

Heparin, Fibroblast Growth Factors, and Osteoporosis

In humans, the prolonged use of heparin is associated with the development of clinical osteoporosis and spontaneous fractures (Megard *et al.,* 1982). Heparin is the predominant glycosaminoglycan in mast cells and may play a role in the osteoporosis often seen in mast cell disease (Chines *et al.,* 1991). The bone marrow of postmenopausal women who are osteoporotic show increased numbers of heparin-rich mast cells (Frame and Nixon, 1968). *In vivo* studies in rats treated with heparin for 33–65 days reveal significant reduction in bone mineral mass (Thompson 1973). In studies in bone organ culture, heparin decreases collagen synthesis (Hurley *et al.,* 1990), and this effect is reversed by exogenous IGF-I (Hurley *et al.,* 1992). Heparin also potentiates the inhibitory effect of FGF-2 on collagen synthesis and procollagen mRNA levels in fetal rat calvariae (Hurley *et al.,* 1992). In bone, heparin augments FGF-1 and FGF-2-induced bone resorption (Simmons *et al.,* 1991). Studies by Thompson *et al.* (1990) revealed that heparin infusion into rabbits results in an increase in FGF-2-like activity in plasma. The mechanisms by which heparin induces osteoporosis are not known. It is possible that modulation of the availability or action of local factors such as IGF-I and FGF-2 is important in heparin-induced osteoporosis.

The role of fibroblast growth factors in the development of osteopenia and postmenopausal osteoporosis in humans has not been defined. However, the studies of Montero *et al.* (2000) clearly documented a role for FGF-2 in bone remodeling and maintenance of bone mass. These studies show that disruption of the Fgf 2 gene in mice results in decreased osteoblast replication, decreased mineralized nodule formation in bone marrow cultures, and decreased new bone formation *in vivo.* There was a progressive decrease in trabecular number and trabecular bone volume as these mice aged. These data are the first to demonstrate an important role for endogenous FGF-2 in maintaining bone mass, as well as bone formation. These data further suggest that the redundancy of members of the FGF family cannot compensate to prevent bone loss when the Fgf 2 gene is disrupted in mice.

A therapeutic role for FGFs in osteopenic states is suggested by studies in animal models. In osteopenic mice, FGF-2 stimulates endocortical bone modeling (Nagai *et al.,* 1999). In ovariectomized rats, FGF-1 or FGF-2 administration also stimulates bone formation and restores bone volume (Nakamura *et al.,* 1997; Dunstan *et al.,* 1999; Liang *et al.,* 1999). The mechanism of this effect has not been studied. Studies to examine the effects of menapause, as well as oophorectomy on FGF expression in bones, are needed.

Conclusion

Mutiple experimental and genetic evidence indicates that FGFs and FGFRs play important roles in the control of endochondral and intramembranous bone formation (Table II). However, the cellular and molecular effects of FGFs in bone cells are not fully understood. Identification of the signal transduction pathways that are activated by FGF/FGFR

Table II Effects of FGF/FGFR Interactions in Skeletal Cells

Chondrogenesis	Bone formation	Bone resorption/degradation
Modulate chondroblast proliferation	Increase osteoblast proliferation	Promote osteoclast formation
Inhibit chondrocyte differentiation	Decrease collagen synthesis	Promote osteoclast formation
Promote chondrocyte apoptosis	Inhibit alkaline phosphatase Modulate osteopontin, osteonectin, and osteoclacin Downregulate connexin-43, upregulate N-cadherin Increases Na-dependent P_i transport Increase osteogenesis by marrow osteoprogenitors Promote osteoblast apoptosis Increase bone formation and promote bone repair *in vivo*	Regulate collagenase-1 and −3 Promote TIMP 1 and 3

interactions and that lead to the expression of specific genes in skeletal cells may help to better understand the regulatory effects of FGFs during osteogenesis *in vivo,* and may allow for the development of therapeutic approaches to stimulate bone formation and to improve bone regeneration.

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Index for Volumes 1 and 2

A

Activated leukocyte cell adhesion molecules, 278
Activator proteins, 87–88
Activin receptor-like kinases, 930–931
Adenosine triphosphate, 1367–1369
Adhesion molecules. *see* Cell adhesion molecules
Adipocytes, role in remodeling, 311
Adrenal gland
  abnormalities MEN syndrome-associated, 1049–1050
  medullary, 634–635, 649
AER. *see* Apical ectodermal ridge
Age/Aging
  effects of cortical wall thickness, 27–28
  effects of GH/IGF-I, 1442
  effects on
    bone turnover, 1550
    collagen, 214–215
    GH treatment, 1445
    previtamin D_3 photosynthesis, 587
    skeletal adaptation, 1483–1484
  stem cell changes, 73–74
Aggrecan, 226
AHO. *see* Albright's hereditary osteodystrophy
Albright's hereditary osteodystrophy
  characteristics, 42, 1140
  clinical features, 1151–1152
  molecular defect in, 1143–1145
  phenotypic variability, 1148–1149
  treatment, 1156
  vitamin D therapy, 1411–1412
Albright's hypothesis, 1079
ALCAM. *see* Activated leukocyte cell adhesion molecules
Alkaline phosphatase. *see also* Hypophosphatasia
  biology, 1231–1232
  as bone formation marker, 1544–1545
  discovery, 1229
  effects of PTH/PTHrP, 498
  effects of thyroid hormones, 709–710
  history, 1229–1231
  levels in
    metastatic bone disease, 1562–1564
    Paget's disease, 1551, 1553
    PHPT, 1551
  physiological role, 1229–1231
  replacement therapy, 1238–1239
  role in hypophosphatasia, 1240–1243
Allele-sharing studies, osteoporosis genes, 1261–1264
ALP. *see* Alkaline phosphatase
Aluminum-bone, interaction, 360–361
AMG 073, 1352–1353
Amino acids, aromatic, 382
γ-Aminobutyric acid, as Ca sensor, 383
Amylin
  characterization, 634
  distribution, 641
  effects on
    bone mass, 648
    Ca metabolism *in vivo,* 649–650
    osteoblasts, 647–648
    osteoclasts, 645–646
  extraskeletal actions, 642–643
  production, 641
  receptors, 649–650
Amyloidosis, cutaneous lichen, 1070
Anabolic steroids. *see* Androgens
Androgens
  description, 1455
  effects on
    bone
      formation, 764–766
      metabolism, 766–767
      resorption, 764–766
    bone cell apoptosis, 161
    chondrocytes, 767–769
    osteoblasts, 762–764
  mode of action, skeletal, 1462
  nuclear receptors, 1459–1461
  osteoporosis pathophysiology and, 1280

Androgens (*continued*)
receptors
characterization, 1461
coactivators, 1461–1462
effects of osteocytes, 99
expression
localization, 760–761
regulation, 761–762
function, 1462
molecular mechanisms, 757–758
response element, 1461
signaling pathway, 758–760
in women
animal models, 1458–1459
levels, 1455–1456
skeletal role, 1456–1457
treatment effects, 1457–1458
Angiofibroma, 1059–1060
Angiogenesis
effects of OPN, 243–244
effects of VEGF, 893
Animal models
androgen studies, 1458–1459
CaR therapy, 1348–1350
diabetic studies, 741–742, 746–747
FGF, 831–833
glucocorticoids, 1644–1645
gonadal hormone-deficient, 659
hyperresorptive diseases, 1362–1364
IFG-I bone effects, 1446–1448
MEN-1 syndrome, 1060
OI, 1180–1181
osteopetrosis, 1222–1223
osteoporosis
congenic/recombinant strains, 1650–1651
congenic strains, 1651–1653
convenience, 1639
criteria, 1636
glucocorticoid, 1639
inbred strains, 1648–1650
male, 1645–1647
Mg studies, 352–353
postmenopausal, 1637–1640
recombinant/congenic strains, 1650–1651
recombinant strains, 1650–1651
regulatory recommendations, 1635
specific species, 1639–1644
in vivo experimentation, 1635
ovariectomized, effects of HRT, 659–660
PTH anabolic properties, 1307–1308
skeletal effects of thyroid hormones, 712–713
vitamin D deficiency, 1205
Anorexia nervosa, 1450
Antisense, inhibition of PTHrP, 1087
AP-1. *see* Activator proteins
Apical ectodermal ridge, 38
Apoplipoprotein E, 1267
Apoptosis
chondrocytes, 155–156
effects of PTH/PTHrP, 497
general features, 151, 153
osteoblasts
glucocorticoid-induction, 161
PTH regulation, 161–162
role of
androgens, 762–763
bisphosphonates, 162–163
estrogens, 665
sex steroid regulation, 160–161
osteoclasts
description, 156–159
glucocorticoid-induction, 161
role of bisphosphonates, 162–163
sex steroid regulation, 160–161
osteocytes
description, 95–96, 159–160
glucocorticoid-induction, 161
PTH regulation, 161–162
role of bisphosphonates, 162–163
sex steroid regulation, 160–161
regulation, 151, 153–155
TGFβ-induced, 905
Arachidonic acid derivatives, 198–199
Areal moment of inertia, 27
Aromatase
deficiency in men, 1469–1472
genes, knockout models, 1472
Arthritis
osteo
stem cell changes and, 73–74
stem cell therapy, 74
tissue engineering for, 74
psoriatic, 598
rheumatoid, 895–896
Aspartic proteinases, 258
Assays
calcium phosphate, 1510
CHIP, 1514–1515
DEAE-dextran, 1510
DNAseI footprinting, 1513–1514
electroporation, 1510
EMSA, 1513
ICTP, 220
lipopfection, 1510
nuclear run-on, 1508
viral vectors, 1510–1511
in vivo footprinting, 1514
Association studies, 1262
ATP. *see* Adenosine triphosphate
ATPases, bisphosphonate inhibition, 1366–1367
AUF1 protein, 414–415
Autosomal-dominant hypophosphatemic rickets, 330–331
Avian models, 1639–1640

B

BABMI, 933
Bapx1, 86
Barakat syndrome, 1111
Basic multicellular unit
biopsy, 1628
definition, 303
in osteoporosis, 1364
remodeling at

activation, 307–309
formation, 309–310
mineralization, 310–311
origination, 304–307
osteoporosis, 314
Basic structural unit, biopsy, 1628
Bcl-2, 153–155
Biglycan (PG-I), 226–227
Biochemical markers
bone formation, 1544–1547
bone resorption, 1547–1549
clinical use
osteoporosis, 1558–1560
Paget's disease, 1551, 1553–1554, 1556
primary hyperparathyroidism, 1556–1558
induced bone metabolism changes, 1561
variability, 1549–1551
Biomechanics
properties, 17–18
special properties, 22–24
stress-strain relationships, 18–20
Biopsy. *see* Transilial bone biopsy
Bisphosphonates. *see also specific drugs*
animal toxicology, 1377–1378
chemistry, 1361
development, 1361
distribution, 1374–1375
effects on
bone cell apoptosis, 162–163
collagen fragment processing, 217
fracture risks, 1376
OI, 1185–1186
osteolysis, 1088–1089
osteoporosis
glucocorticoid-induced, 1376
postmenopausal, 1375–1376
Paget's disease, 1376
remodeling, 315
tumor bone disease, 1376–1377
efficacy, 1375
general characteristics, 1361–1362
inhibition of
calcification, 1373–1374
osteoclasts, 1372–1373
intestinal absorption, 1374
nitrogen-containing
cellular level actions, 1369–1372
description, 1367
FPP synthase as target, 1367–1369
inhibition of
mevalonate pathway, 1369
osteoclast recruitment, 1372
osteoclasts, 1372–1373
metabolism to ATP analogues, 1367–1369
protein isoprenylation blocking, mechanism, 1369–1371
sterol synthesis blocking mechanism, 1369–1371
osteoporosis therapy models, 1362–1364
pharmacological effects
action *in vivo,* evidence, 1371
inhibition of bone resorption
general concepts, 1366
models, 1362–1365
molecular level, 1366–1367
relative potency, 1365–1366
physicochemical effects, 1362
PTH therapy and, 1314
renal clearance, 1375
response, genetic determinants, 1269
Blomstrand's lethal chondrodysplasia
affected infants, 1111–1112
characterization, 1125–1127
PTH-Rc mutation in, 440
Blood-calcium homeostasis, 99
BM40, 228–229
BMD. *see* Bone mineral density
BMP. *see* Bone morphogenetic proteins
BMU. *see* Basic multicellular unit
Bone
-Al, interaction, 360–361
architecture
CT imaging
coherent-scatter, 1611
μ, 1612–1615
high-resolution, 1600–1604
peripheral quantitative, 1611
ultra-high-resolution, 1611
volumetric, 1609–1610
gonadal hormone-deficient models, 659
microradiography, 1605–1606
MRI microscopy, 1615–1618
radiography, 1600–1604
biology, cells affecting, 1497–1498
biomechanics
mechanotransduction, 25–26
properties, 17–18
special properties, 22–24
stress-strain relationships, 18–20
biopsy, transilial
in clinical research, 1633
complications, 1617
histology, 1628–1629
morphometric methods, 1629, 1631
in patient care, 1632
practical considerations, 1633
procedure, 1615–1616
reference data, 1631–1632
regions evaluated, 1629, 1631
specimen processing, 1627
timing, 1633
-boron, interaction, 361
calcium
function, 1327–1328
storage, 1326–1327
cartilage to, 12
-Cd, interaction, 361–362
cells (*see specific bone cells)*
cellular interactions, studies, 1536
composition, 3, 24
connexin expression, 292–293
cortical (*see* Cortical bone)
-Cr, interaction, 362–363
CSF-1 expression in, 947
degradation, 135–136
diabetic nephropathy in, 746

Bone (*continued*)
discs, etopically implanted, 243–244
effects of
androgens, 1458–1459
CGRP, 784–786
C-type natriuretic peptides, 1009
glucocorticoids, 731–733
GM-CSF, 950–952
IL-4, 965
IL-6, 966–971
IL-7, 966
INF-γ, 964
leukotrienes, 988–989
LIF, 966–971
mechanical loading, 1482–1483
neuropeptide Y, 786
neurotransmitters, 787–789
neurotrophins, 789–790
NSAIDs, 988
opioid peptides, 787
PACAP, 784
PTH therapy, 1315–1316
SERMs, 681
somatostatin, 787
substance P, 786
TNF, 963–964
VEGF, 893–896
VIP, 781–783
evolution, Ca and, 1325–1326, 1328–1329
formation
basic aspects, 1544
biopsy, 1628
description, 12
effects of
androgens, 764–766
FGF, 836–838
glucocorticoids, 727–729
prostaglandin receptors, 986–987
PTH/PTHrP, 499–500
factors effecting, 1550–1551
markers, 1544–1547
nodule, 711
in osteoporosis, 1277–1278
in perichondrium, 52
rates, 1631
resorption, coupling to, 1277, 1543–1544
role of PTH/PTHrP, 1120–1122
fractures (*see* Fractures)
GH mechanisms, 1442–1443
growth
appositional, 43–44
description, 9–11
effects of IGF-I, 1446–1447
homeostasis, role of CaR, 377–379
innervation, 781
intramembranous, formation, 42–43
loss
after decreased mechanical usage, 1638
animal models, 1637
effects of IL-1, 962
as fracture risk marker, 1296–1298
glucocorticoid-induced, 724–727
ovariectomy-induced, 699–702
posttransplantation, 1561–1562
prediction, biochemical markers for, 1558
rate of, 1275–1276
role of prostaglandins, 987–988
T-4 therapy, 714–715
mechanical loading, 987
metabolism
biochemical markers, 1316
effects of
androgens, 766–767
kinins, 776–778
leptin, 790
metastases
osteoblastic, 1097–1098
osteolytic, 1099–1101
role of TGFβ, 911–912
mineral phase, 359
NO in
associated pathology, 1007
isoform occurrence, 998–999
molecular targets, 1006
therapeutic implications, 1007–1008
noncollagen proteins of, 731–732
organ cultures (*see* Tissues, cultures)
organic matrix
ectopic calcifications, 235
gene expression control by, 233–235
glycoproteins, 228–232, 235
hyaluronan, 228
proteoglycans, 225–228
serum proteins, 232–233
Pb content, 359
-Pb interaction, 363–365
physiology
dynamics of, 303–304
progesterone receptors, 668–669
plasminogen activators in, 257–258
proteases, effects of PTH/PTHrP, 498
PTH in, carboxy-terminal specific receptors, 389–400
PTH/PTHrP expression in, 493–494
puriergic receptors, 296–297
remodeling (*see* Remodeling, bone)
resorption (*see* Resorption, bone)
-Si, interaction, 365
-Sr, interaction, 365–366
strength, fracture correlation, 28–29
structural macromolecules
analysis, 1491–1493
Cis regulation, 1493–1494
target tissues, role of 1α,25(OH)$_2$ vitamin D$_3$, 574–575
tissue, effects of neurons, 780
trabecular (*see* Trabecular bone)
turnover (*see* Turnover, bone)
woven, bone remodeling *vs.,* 315
Bone-forming cells
cadherin association, 290
effects of TGFfl, 905
role of connexin43 gap junctions, 294
Bone-lining cells. *see* Lining cells
Bone marrow cells, role in remodeling, 311

Bone mass
  effects of
    amylin, 648
    CGRP, 648
    IDDM, 744–745
    insulin, 743–746
    Mg, 349–350
    NIDDM, 745
    raloxifene, 682–683
    sex steroids, 658
    tamoxifen, 685
    TNF, 963–964
  hyperinsulinemia and, 745
  maintenance genes affecting, 1494–1496
  peak, 1275–1276
  regulation, inheritance patterns, 1261
Bone matrix proteins, 498
Bone mineral density. *see also* Osteoporosis
  anabolic agents, 1306
  anti-resorptive agents, 1306
  genetic determinants
    effects on
      bisphosphonate response, 1269
      calcium absorption, 1269
      clinical practice, 1269–1270
      HRT response, 1269
      vitamin D absorption, 1269
  measurement
    densitometry techniques
      clinical use, 1582–1583
      conflicting findings, 1583–1584
      descriptions, 1574–1580
      discrepancies in, 1587–1588
      DXA, peripheral, 1577–1578
      ethnic-specific databases, 1590–1593
      evaluating, 1580–1581
      gender-specific databases, 1590–1593
      for osteoporosis diagnosis, 1589–1590
      peripheral measurements, interpretation, 1583
      reference ranges, 1580–1582
      relationship between, 1580
      reporting prevalence *vs.* risks, 1589–1590
    effects of
      estrogen therapy, 1401
      GH, 1443–1446
      NSAIDs, 988
      PTH therapies, 1313
    genetic determinants
      apoplipoprotein E, 1267
      calcitonin receptor, 1267–1268
      COLIA1, COLIA2 genes, 1265–1266
      estrogen receptor genes, 1266
      evidence for, 1260–1261
      interleukin genes, 1267
      TGFβ genes, 1266
      VDR gene, 1264–1266
    -habitual loading, relationship
      athletes, 1478–1479
      estimating, 1479–1480
      nonathletes, 1479
    high-resolution CT, 1607–1609
    MRI, 1615–1618
    serial, 1593–1596
    ultrasound, 1292–1293
  osteoporosis
    associated fractures, 1292–1293
    diagnosis, 1573–1574
    risk factor
      epidemiology, 1305
      history, 1647–1648
  pQCT, 1611
  volumetric CT, 1609–1610
Bone morphogenetic proteins
  biology, 922–924
  connexin regulation by, 293–294
  extracellular regulators, 938–939
  identification, 929
  intracellular signaling
    SMADS
      in cytoplasm, 935–936
      in nucleus, 936
      regulation, 937–938
      structure, 933–935
      target genes, 936–937
  mechanism, 919–920
  osteoblastic bone metastases and, 1098
  pseudoreceptors, 933
  receptors
    activation mechanism, 932
    mutations *in vivo,* 933
    serine/threonine kinase, 929–930
    type I, 930–931
    type II, 931–932
  therapeutic applications, 920–922
Bone sialoprotein
  description, 231–232
  levels in metastatic bone disease, 1584
  levels in PHPT, 1557–1558
  as resorption marker, 1549
Boron-bone, interaction, 361
Brandykinin receptors
  characterization, 776
  effects on resorption, 778
Breast. *see also* Mammary gland
  cancer cells
    biochemical bone markers for, 1583
    effects of calcitonin, 1428
    VDR activity, 592
  effects of
    raloxifene, 684
    SERMs, 682
    tamoxifen, 686
BSP. *see* Bone sialoprotein

C

Cadherins
  association with bone-forming cells, 290
  cell–cell contact, 288
  characterization, 268–269
  role in
    cartilage, 271–272
    osteoblast functions, 278
    osteoclastic resorption, 271–272

Cadherins (*continued*)
osteogenic cells, 289–290
skeletal development, 288–289
Cadmium-bone, interaction, 361–362
Calbindin, effects of vitamin D-3, 577–578
Calcifications
ectopic, 235
inhibition by bisphosphonates, 1373–1374
Calcinosis, tumoral, 334
Calcipotriol, 1412–1413
Calcitonin
associated clinical conditions, 629–633
cellular actions, 1426–1428
discovery, 1423
effects on
osteoblasts, 147
osteoclasts, 1009
remodeling, 315
genes
bioactions, 624–628
biochemistry, 619–621
carboxyl-terminal peptides, 629
distribution, 624
family members, 634–635
postranslational processing, 621–622
related peptides I and II, 633–634
structure, 1423–1424
mature
description, 624
measuring, 628–629
molecular actions, 1431–1432
osteoporosis pathophysiology and, 1283
peptide structure, 1424–1426
pharmacology
analgesic effects, 1434
clinical applications, 1432
general concepts, 1431
side effects, 1434
therapeutics, 1434–1435
physiopathology, 619
receptor family, 1429, 1431
role in osteoclast apoptosis, 159
secretion, 622–624, 628
Calcitonin gene-related peptides
bone microenvironment access, 643
characterization, 641, 784–786
effects on
bone mass, 648
Ca metabolism *in vivo,* 649–650
osteoblasts, 646–647
osteoclasts, 643–645
extraskeletal actions, 642–643
production, 1423–1424
receptors, 649–650
regulation, 1424
structure, 1424–1426
Calcitonin receptors
downregulation, homologous, 613–614
function, 603–604
genes
bioactivity, 635
organization, 609–611
regulation, 611–613
role in BMD, 1267–1268
identification, 603
proteins
isoforms, 607–609
signaling, 604–607
structure, 604–607
Calcitriol
levels, effects of vitamin D-3, 1204
PTH therapy and, 1314–1315
Calcium
absorption, genetic determinants, 1269
balance, effects of PTH therapy, 1316–1317
-blood homeostasis, role of osteocytes, 99
in bone
evolution and, 1325–1326
function, 1326–1327
storage, 1326–1327
channels, 538–539
effects of calcitonin, 624–625
effects of fluoride, 1388–1390
effects on PTH mRNA, 414–415
environmental, 1325
excretion
regulation by PTH, 487–488
regulation by PTHR signal transduction, 488–489
extracellular, sensing receptor
biochemical properties, 373–374
cloning, 371–372
effects on Mg, 381, 382
GABA as, 383
gene regulation, 374–375
G protein-coupled receptor association, 372–373
intracellular signaling by, 374
as nutrient sensor, 381–383
predicted structure, 372–373
role in
Ca homeostasis, 375–379
Ca metabolism, 379–381
water metabolism, 379–381
homeostasis, effects of PTH, 1339
intake
manipulating, 1329–1331
nutritional factors, 1333
osteoporosis therapy and, 1335
intercellular signaling
description, 295–296
in skeletal tissue, 297–298
metabolism *in vivo,* effects of
amylin, 649–650
CGRP, 649–650
pseudohypoparathyroidism therapy, 1154–1156
reabsorption, 485–487
requirements
defining, 1331–1332
evolutionary factors, 1328–1329
insurance of, 1328
studies, interpreting, 1334–1335
role in PTH gene regulation, 412–413
serum, effects of PTH therapy, 1317
systemic plasma regulation, 1035–1036
therapy, for hypophosphatasia, 1238

toxicity, 1333–1334
transport
effects of glucocorticoids, 725–726
placental, 522–523
urinary, 1317
Calcium phosphate, 1510
Calcium receptors
description, 1340
expression, 1340
pharmacology
calcilytics, 1342–1344
calcimimetics, 1341–1342
effects on circulating PTH, 1353–1354
effects on skeletal tissue, 1355
for hyperparathyroidism, 1345
ligand selectivity, 1344–1345
primary hyperparathyroidism, 1345–1348
secondary hyperparathyroidism
animal models, 1348–1350
clinical studies, 1350–1353
Calcium-sensing receptor sensor gene
abnormalities, 1108
function, 1024
mutations, role in FBHH, 1038–1039
Calreticulin, 411–412
Camaruti-Englemann disease, 1266
cAMP, signaling response, 391–392
Canalicular fluid flow, 100–101
Cancellous bone
bone remodeling and, 313
description, 3
effects of ovariectomy, 659
Cancer
bone metastasis
pathologic models, 1536
role of TGFβ, 911–912
breast cells
effects of calcitonin, 1428
VDR activity, 592
cells, characterization, 1017–1018
medullary thyroid
calcitonin-associated, 629–630
genetic pathogenesis, 1025
MEN2 syndrome-associated, 1069
parathyroid, 1025
prostate, 1098
vitamin D therapy and, 1412
Carcinoids, MEN1 syndrome-associated, 1051
Cardiovascular system. *see also* Heart; Vasculature
effects of
amylin, 642
CGRP, 642
PTH, 473–475
SERMs, 681–682
PTH regulation, 531–532
Cartilage
articular, 51–52
to bone, 12
Ca homeostasis, role of CaR, 377–379
connexin expression, 292–293
growth capacity, 43–44
immunoglobulins, 279–281
role of nonintegrin adhesion molecules, 279–281
structural macromolecules, 1493–1494
Caspases, 153–155
CaSR. *see* Calcium-sensing receptor sensor gene
CASTing method, 1518
CAT. *see* Escherichia coli CAT gene
Catecholamines, effects on bone, 787–788
Cathepsin K, role in bone resorption, 216
Cat models, 1642–1643
Cbfa 1
characterization, 85
expression, 85–87
identification, 83–84
regulation, 69–71
regulator factors, 177–183
role in bone formation, 234
role in osteoblast differentiation, 83–85
transgenic models, 1494–1496
c-Cbl, role in osteoclast adhesion, 145–146
C cells
Ca homeostasis, 375–376
effects of calcium, 1341–1342
neoplastic transformation, 1067–1068
CD44
cartilage expression, 280
characterization, 97, 269
expression, 98
osteoblast expression, 278
Cell adhesion molecules. *see also specific molecule*
cell–cell communications, 291–295
cell–cell contact via, 288–289
effects on osteoclasts, 290–291
families, 266, 268–269
role in
cartilage, 279–281
osteoblast function, 276–279
osteoclast clear zone, 272–274
osteoclast development, 272
role of VEGF-A, 890–891
Cell cultures
coculture systems, 1536
current techniques, 1531–1534
Cell line hierarchies, 60–61
Cell organization, control of, 287
Cell proliferation, effects of PTH/PTHrP, 497
Cell shape, effects of PTH/PTHrP, 497
Cell survival, role of VEGF-A, 890
Central nervous system
effects of calcitonin, 626
PTH/PTHrP expression in, 537–540
c-fos knockout mutation, 1223
CFU. *see* Colony forming factors
CGRP. *see* Calcitonin gene-related peptides
CHIP. *see* Chromation immunoprecipitation
Chondrocytes
apoptosis, 155–156
effects of androgens, 767–769
effects of thyroid hormones, 712
growth plate, proliferation/differentiation
articular cartilage, 51–52
mediators, 46–51
intercellular Ca signaling, 297–298

Chondrocytes (*continued*)
role of integrins, 279
Chondrodysplasia. *see* Blomstrand's lethal chondrodysplasia; Jansen's metaphyseal chondrodysplasia
Chondrodystophies, FGF in, 834
Chondrogenesis
coupling, 52–53
effects of VEGF, 893
Chromatin organization, 171–174
Chromation immunoprecipitation assays, 1514–1515
Chromium-bone, interaction, 362–363
Circadian, effects on bone turnover, 1550–1551
Cloning, transcriptional regulatory molecules, 1515–1518
Collagenases
characterization, 253–257
genes in bone, 838–839
type IV (*see* Gelatinases)
Collagen-binding proteins, role in osteoclast functions, 274–275
Collagenoma, *MEN1* gene and, 1059–1060
Collagens
definition, 189
fibril-associated, 189–190
forming sheets, 190
receptors, integrin-binding, 277
type I
in bone, percentage, 3
Cis regulation, 1491
cross-links
changes, age-related, 214–215
component release *in vitro,* 213–214
formation, 211–212
immunoassays, 1548–1549
location, 213–214
spectrum, 213–214
structure, 212–213
degradative pathways, 216–217
effects of glucocorticoids, 731
effects of thyroid hormones, 709–710
fibrillognenesis, 193
genes
mutations, consequences, 193
posttranslational modifications, 192–193
prevalence, 189
role in BMD, 1265–1266
structure, 190
transcriptional regulation, 200–204
translational modifications, 192–193
metabolism, 218–220
mutant gene transgenic models, 1492–1493
phase biomechanical effects, 24
promotors
function analysis, 1494–1496
role in osteoblast differentiation, 87
sequence analysis, 1493
structural integrity, 1491
synthesis
cytokine regulation of, 197–199
description, 190–192
effects of INF-γ, 964
effects of TNF, 963–964
growth factor regulation of, 194–197
hormone regulation of, 199–200
PGE regulation of, 198–199
vitamin regulation of, 199–200
type II
function analysis, 1493–1496
promotors, role in osteoblast differentiation, 87
sequence analysis, 1493
transportation factors, 1494
type IX, transgenic models, 1493
type X, analysis, 1492–1493
Colony forming factors, CFU-F
assays, 60–61
effects of aging, 73–74
regulation, 69–71
Colony-stimulating factors. *see also* Granulocyte-colony stimulating factor
CSF-1
biochemistry, 944
description, 943–944
expression, 944–945, 947
molecular forms, 947–948
regulation, 944–945
role in
osteoclast formation, 946–947
osteoporosis, 944–945, 1279
Communications
cell–cell, 291–295
intercellular
by CaR, 374
cell–cell adhesion, 298
description, 295–298
Compression
causes, 19
forces, 26–28
Computed tomography
coherent-scatter, 1611
μ, 1612–1615
high-resolution, 1607–1609
peripheral quantitative, 1611
ultra-high-resolution, 1611
volumetric, 1609–1610
Connexins
in bone, 292–293
in cartilage, 292–293
Cx43
location, 293
modulation, 294–295
effects on bone-forming cells, 294
in osteoclastogenesis, 295
regulation, 293–294
in skeletal development, 293
Cortical bone
bone remodeling and, 313
description, 3
effects of fluoride, 1394
strength of, 22–23
thickness, effects of aging, 27–28
Corticosteroids
effects on bone turnover, 1550
induced osteoporosis, 314
role in collagen synthesis, 199
Cortisone, hypophosphatasia-associated, 1238
COX. *see* Cyclooxgygenases

Cranial sutures, 42–43
Cranial vault, 38
Craniofacial disorders, 43
Craniosynostosis, FGF in, 834–835
Creep, 23
Cre-ERT system, 1498–1499
Cre/loxP recombinase system, 1498–1499
Critical functions rule, 12
Crouzon syndrome, 43
CSF. *see* Colony-stimulating factors
c-Src
  effects on osteoclasts, 143–144
  -Pyk2, role in osteoclast adhesion, 144–147
CT. *see* Computed tomography
C-terminal peptides, as collagen formation markers, 218
C-terminal telopeptide of collagen type I
  assay, as bone resorption marker, 220
  circadian variability, 1550–1551
  description, 1549
  levels in immobilization, 1550
  levels in pregnancy, 1550
CTR. *see* Calcitonin receptors
CTX-1. *see* C-terminal telopeptide of collagen type I
Culture shock proteins, 228–229
Cyclin D1/*PRAD1,* 1019–1021
Cyclooxgygenases
  COX-2
    expression, importance, 982
    mouse model, 984–985
    transcriptional regulation, 983
  isoforms, 980–981
  nomenclature, 979–980
  substrate, 981
Cyclosporines, 1561
Cysteine
  PTH1–Rc, characterization, 439–440
  rich FGF receptors, 831
Cysteine proteinases, 258
Cytochrome P450, 1413, 1415–1416
Cytokines. *see also* specific cytokines
  effects on bone cell function, 964
  inhibitors, role in ovariectomy-induced bone loss, 699–702
  -kinins, interactions, 780
  -neuropeptides, interactions, 780
  osteclastogenic
    effects of
      estrogen, 695–697
      menopause, 697–698
      ovariectomy, 699
    role in formation, 693–695
  role in osteoblast development, 71–73
Cytoskeleton
  osteoclasts
    organization, 143–144
    in resorption cycle, 132–133
  osteocytes, 98

D

DEAE. *see* Diethylaminoethyl-dextran
Deafness, hypoparathyroidism-associated, 1110
Death receptors, 153
Decorin (PG-II), 226–227
Degenerative joint disease, 156
5α-Dehydrotestosterone. *see* Androgens
Denervation, experimental, 790–791
Dentin Matrix protein-1, 232
Dentition, hypophosphatasia-associated, 1236
Dent's disease, 333
Deoxypyridinoline
  levels in PHPT, 1548
  as resorption marker, 1548
Desitometry. *see also* specific techniques
  conflicting findings, 1583–1584
  evaluating, 1580–1581
  peripheral measurements, interpretation, 1583
  reference ranges, 1580–1582
  relationship between, 1580
  serial measurements, 1593–1596
  T scores
    definition, 1583–1584
    discrepancies in, 1587–1588
    ethnic-specific databases, 1590–1593
    gender-specific databases, 1590–1593
    for osteoporosis diagnosis, 1589–1590
    reporting prevalence *vs.* risks, 1589–1590
  types, 1574–1578
Diabetes mellitus
  associated bone disease, 750–751
  insulin dependent (type 1)
    animal models, 746–647
    effects on
      bone mass, 744–745
      fractures, 745–746
      remodeling, 743–744
      skeletal growth, 741–743
  noninsulin dependent (type 2)
    animal models, 746–647
    effects on
      bone mass, 745
      fractures, 745–746
      remodeling, 744
Diethylaminoethyl-dextran, 1510
Differentiation, sclerotome, 37
DiGeorge syndrome, 1109–1110
1,25–Dihydroxyvitamin D. *see* Vitamin D
Dlx5, role in osteoblast differentiation, 86
DMP-1. *see* Dentin Matrix protein-1
DNA
  binding
    domains, 1516–1518
    site selection, 1518–1519
  genomic, packaging, 171–174
  probes for expression library screening, 1515–1516
  -protein
    incubation, 1518–1519
    interaction at PTH mRNA, 414–417
    interactions *in vitro* and *in vivo,* 1512–1515
DNAseI footprinting assay, 1513–1514
Dog models, 1643
Dopamine transporters, 789
Dual X-ray absorptiometry
  BMD, 1291–1293

Dual X-ray absorptiometry (*continued*)
general description, 1574–1576
and other techniques, comparisons, 1580
peripheral measurements, 1577–1578, 1583
reference ranges, 1581–1583
serial measurements, 1593–1596
Ductility, definition, 22
DXA. *see* Dual X-ray absorptiometry
Dynein light chain, 415–416
Dysplasias, skeletal, 40

E

ECD. *see* Extracellular ligand binding motif
Edothelin, 1009
Ehlers-Danlos syndrome, 193
Elasticity, 21–22
Electric fields, 1483
Electrophoretic mobility gel shift assays, 1513
Electroporation, description, 1510
Embryos
extraembryonic tissues, 523–524
mammary development, 517–518
membranes, 524
skeletal development
axial, 34–38
chondrogenesis, coupling, 52–53
intramembranous, formation, 42–52
limb initiation, 38–40
mesenchymal condensation, 40–42
morphology, 33–34
osteogenesis, coupling, 52–53
patterning, 40–42
role of BMP, 922–923
EMSA. *see* Electrophoretic mobility gel shift assays
Endocrine system
tumors, *MEN1* gene and, 1055, 1058–1059
vitamin D
description, 548–549
genetics, 556–558
Endothelin-1, osteoblastic bone metastases and, 1098
Endothelin-A receptor, in bone formation, 38
Epidermal growth factor
effects on VEGF-A expression, 891
phosphate transport regulation, 324–326
Epiphysis, vascular invasion, 52–53
Epithelial cells-mesenchymal interactions, 1122
ERK. *see* Estrogen receptor knockout
Escherichia coli CAT gene, 1511
Estrogen receptor knockout, role in osteoclast apoptosis, 158–159
Estrogens
coregulators, 657
deficiency, role in
as fracture risk marker, 1295
osteoporosis pathophysiology, 1278
disruption, rodent models, 660–661
effects of osteocytes, 99
effects on
bone mass, 658
chondrocytes, 46–47
osteoblasts, 661–665
osteoclasts, 665–686
prostaglandin production, 984
PTH, 417–418
remodeling, 315
TNF, 963–964
VEGF-A expression, 892
gene expression in
bone cells, 657–658
skeletal tissue, 658
and growth spurts, 1469–1472
-mechanical loading, relationship, 1484
mechanism of action, 655–656
menstrual/estrus cyclicity, 1637
molecular actions, 1403–1405
osteoporosis pathophysiology and, 1280
pharmacology (*see also* Hormone replacement therapy)
administration routes, 1402–1403
biochemistry, 1401
BMD, 1401–1402
fracture risks, 1402
pseudohypoparathyroidism therapy, 1156
PTH therapy and, 1314
receptors
deficiency in men, 1468–1469
effects on skeletal phenotypes, 660
genes, knockout models, 1472
genes, role in BMD, 1266
isoforms, 656
response elements
mechanism, 677
molecular mechanisms, 663–664
role in bone cell apoptosis, 160–161
VDR_{nuc} response, 553–554
Ethnicity, effects on bone turnover, 1550
Evolution, bone, 1325–1326, 1328–1329
Exercise
-BMD, relationship
athletes, 1478–1479
estimating, 1479–1480
effects on bone turnover, 1551
postmenopausal bone loss and, 1484
skeletal adaptation
constraints, 1484–1485
effective mechanical loading, 1481–1482
models, 1480–1481
Extracellular ligand binding motif, 375
Extracellular matrix, 1182–1184
Extrathyroidal neuroendocrine cell hyperplasia, 629
Extrathyroidal neuroendocrine tumors, 630

F

Facial skeleton, 38
Fanconi's syndrome, 333–334
Farnesyl diphosphate synthase
cellular level actions, 1369
inhibition by N-BP, 1367
as N-BP target, 1369
protein isoprenylation blocking, mechanism, 1369–1371
sterol synthesis blocking mechanism, 1369–1371
Farwestern blot, 1516
Fatigue failure, 28

Ferret models, 1642–1643
FGF. *see* Fibroblast growth factors
Fibrillins, description, 231
Fibrillognenesis, collagen, 193
Fibroblast growth factors
  basic, role in collagen synthesis, 197
  bioactive polypeptides, 826–827
  effects on
    bone formation, 836–837
    bone resorption, 836–837
    collagenase gene, 838–839
    limb development, 39
    osteoclastogenesis, 839
  extracellular, regulation, 830–831
  FGF-23 gene, 331
  -heparin, interaction, 839–840
  osteoblastic bone metastases and, 1098
  production, 835–836
  receptors
    in chondrodystophies, 834
    in craniosynostosis, 834–835
    effects on chondrocytes, 50–51
    expression in bone, 833–834
    types, 830–831
  regulation, 835–838
  role in osteoblast apoptosis, 160
  signaling, 838
  synthesis *de nova,* 827–830
  trangenic animal model systems, 831–833
Fibronectin, description, 231
Fibrous dysplasia
  clinical presentation, 1165–1166
  description, 1165
  genetics, 1168
  management, 1173
  pathogenesis, 1170–1173
  pathology, 1169–1170
  radiological findings, 1168–1169
Fluid flow
  cell stretch *vs.,* 102–103
  in vitro, osteocytes response, 101–102
Fluoride
  characterization, 1387
  effects on remodeling, 315
  pharmacology
    BMD trials, 1306
    bone cells, 1389
    calcium effects, 1388–1390
    food effects, 1388
    GI absorption, 1387–1388
    molecular mechanisms of action, 1389
    osteoporosis prevention, 1390–1392
    renal excretion, 1388–1389
    serum levels, 1388
    side effects, 1389–1390
  stress fractures and, 1389–1390
  therapy
    combined regimes, 1394–1395
    osteoporosis
      corticoid-induced, 1394
      male, 1394
      postmenopausal, 1392–1394
      problems/perspectives, 1396–1397
  in water, 1391
Fluorochrome labels, 1629
Focal adhesion kinase, 1431
Footprinting assay, 1514
Foregut carcinoid, 1049, 1055
FosB regulation, 71
FPP. *see* Farnesyl diphosphate synthase
Fractures
  bone behavior and, 26–28
  effects of tamoxifen, 685
  effects on bone turnover, 1550
  hip, fluoride-induced, 1394
  osteoporosis-associated
    animal models, 1638
    bone turnover in, 1558–1559
    genetic factors, 1260–1261
    prediction, 1559
    risk factors
      BMD, 1292–1293
      bone turnover, 1296–1298
      clinical, 1291–1292
      combined assessment, 1298
      hormonal markers, 1293–1294
      menopause, 1294–1296
  preventing, 29–30
  repair
    BMP application, 920–921
    role of FGF, 838
    role of PDGF, 821
  risks
    bone strength correlation, 28–29
    effects of
      bisphosphonates, 1376
      estrogen therapy, 1402
      PTH therapies, 1309–1310
  role of diabetes, 745–746
  stress
    fluoride-induced, 1389–1390
    occurance, 28
  -stroke, association, 791–792
FSD. *see* Functional secretory domain
Functional secretory domain, 130–132

G

GABA. *see* γ-Aminobutyric acid
β-Galactosidase/LacZ, 1512
Galactosyl hydroxylysine, 1549
Gap junctions
  in bone, 94
  effects of PTH/PTHrP, 497
  formation by bone-lining cells, 278
  as intercellular channels, 291–292
  role in bone remodeling, 294–295
  in skeletal cells, 292–293
Gastrinoma, MEN1 syndrome-associated, 1051
Gastrointestinal tract
  bisphosphonate absorption by, 1374
  effects of calcitonin, 627
  fluoride absorption by, 1387–1388
  phosphorus absorption by, 322

Gelatinases, 252–253
GEM2, abnormalities, 1108–1109
Genes. *see also specific genes*
  chromatin organization, 171–174
  expression, nuclear architecture link, 176–179
  sequence organization, 170
Gene therapy
  BMP, 921–922
  methods, 74–76
  for OI, 1186–1187
Germline DNA-based tests, 1057
GFP. *see* Green fluorescent protein
GH. *see* Growth hormone
GHD. *see* Growth hormone, deficiency
Glucocorticoids
  effects on
    bone formation, 727–729
    bone loss, 724–727
    calcium transport, 725–726
    chondrocytes, 47
    gene expression in bone, 730–733
    osteoblasts, 727–730
    phosphorus transport, 725–726
    PTH function, 725–726
    resorption, 729–730
    vitamin D metabolism, 725–726
  induced bone metabolism changes, 1561
  induced osteonecrosis, 725–726
  induced osteoporosis
    animal models, 1639, 1644–1645
    description, first, 723
  pharmacology, 723–724
  phosphate transport regulation, 324–326
  receptors, effects of osteocytes, 99
  role in bone cell apoptosis, 161
  target cell metabolism, 730
Glucose, effects of calcitonin, 627
Glutamate
  effects on bone, 788–789
  receptors, effects on bone, 788–789
  transporter, effects on bone, 788–789
Glycoproteins
  description, 228
  RDG-containing, 229–230
  types, 228–232
GM-CSF. *see* Granulocyte-macrophage colony stimulating factor
GNA11, role in FBHH, 1038
Gonadal status, GH/IGF-I secretion and, 1442
G proteins
  characterization, 1170–1171
  gp130, role in osteoclast apoptosis, 158–159
  heterotrimeric, 1138–1140
  receptors
    CaR association, 372–373
    characterization, 981–982
  role in
    fibrous dysplasia, 1171–1172
    McCune-Albright syndrome, 1171–1172
Granulocyte-colony stimulating factor
  biochemistry, 952
  function, 952
Granulocyte-macrophage colony stimulating factor
  biochemistry, 950
  in bone, effects of PTH/PTHrP, 496
  effects on bone, 950–952
  in osteoblasts, effects of estrogen, 662–663
  role in HHM, 1095
Green fluorescent protein, 1496–1497, 1512
Growth factor receptors, 155
Growth hormone
  deficiency, effects of GH treatment, 1443–1445
  effects on
    chondrocytes, 46
    OI, 1186
    PTH, 468–469
  function, 1441
  osteoporosis treatment
    administration, 1445
    clinical trials, 1445–1446
    effects on BMD, 1443–1446
    mechanism on bone, 1442–1443
    physiology, 1441–1442
  as reporter gene, 1512
  treatment, in GHD patients, 1443–1445
Growth plate
  chondrocyte processes
    articular cartilage, 51–52
    mediators
      local, 47–51
      systemic, 46–47
  description, 45
  vascular invasion, 52
Growth spurts, estrogen and, 1469–1472
Guinea pig models, 1642–1643

H

Hageman factor, 774
HAT. *see* Histone acetyltransferases
Heart
  effects of PTHrP, 536–537
  transplantation, associated bone loss, 1561–1562
Heparin
  effects on bone turnover, 1550
  -FGF, interaction, 839–840
Hereditary hypophosphatemic, 329–331
HHM. *see* Humoral hypercalcemia of malignancy syndrome
High-affinity receptors, 830–831
Hirschsprung's disease, MEN2 syndrome-associated, 1069–1070
Histone acetyltransferases, characterization, 174
HOB-01–CI, characterization, 97
Homeodomain proteins, role in *Cbfa 1* expression, 85–87
Homeostasis, phosphorus
  clinical disorders of, 326–334
  regulation, 321–326
Hormone replacement therapy
  effects on bone, 667–668
  effects on ovariectomized models, 659–660
  fluoride therapy with, 1394–1395
  response, genetic determinants, 1269
  skeletal response, animal models, 1637
HPA. *see* Hypothalamic-pituitary-gonadal axis
HRT. *see* Hormone replacement therapy

Humoral hypercalcemia of malignancy syndrome
associated cytokines, 1094–1095
discovery, 1093–1094
estrogen series prostaglandin association, 1095
PTH association, 1094
PTH-RP association, 1093–1095
Hyaluronan, description, 228
Hydrogen excretion, 491–492
1α-Hydroxylase, 1413
25–Hydroxylase, 558, 1413
Hydroxylysine glycosides, urinary, 219
Hydroxyproline
levels in heart transplants, 1561
as resorption marker, 1547–1548
urinary, as bone resorption marker, 219
Hypercalcemia
calcitonin therapy, 1432
effects of IL-1, 962
familial hypocalciuric
biochemistry, 1033–1034
characterization, 1024
clinical studies, 1033–1035
discovery, 1031–1032
genetic studies, 1037–1040
history, 1032–1033
management, 1040
molecular bases, 1039
pathophysiologic studies, 1035–1036
history, 1079
malignancy-associated
characteristics, 1079–1080
humoral (*see* Humoral hypercalcemia of malignancy syndrome)
leukocytosis-associated, 1101
RANKL/RANK/OPG system and, 1085–1087
rickets link, 328–329
role of PTHrP
approaches to controlling, 1086–1087
cytokine mediation, 1087–1088
mechanisms of action, 1083–1086
molecular aspects, 1080–1082
osteolysis, 1088–1089
Hypercalciuria, hereditary hypophosphatemic rickets with, 329–330
Hyperinsulinemia, 745
Hyperparathyroidism
neonatal severe
animal models, 1041
clinical characteristics, 1040–1041
discovery, 1032
genetic basis, 1041
management, 1041
primary
CaR therapy, 1345–1348
characterization, 1031
MEN1 syndrome and, 1048–1049
treatment, 1050–1051
secondary
CaR therapy
animal models, 1348–1350
clinical studies, 1350–1353
role of PTH, 416–417
Hyperplasia, extrathyroidal neuroendocrine cell, 629
Hyperresorptive diseases, 1362
Hypertension, effects of PTHrP, 536–537
Hyperthyroidism
animal models, 712–713
human studies, 713–714
Hypocalcemia, clinical features, 1150–1152
Hypoparathyroidism
associated syndromes, 1109–1112
autosomal dominant, 1107
autosomal recessive, 1107–1108
isolated, 1105–1106
pluriglandular autoimmune, 1112
x-linked recessive, 1109
Hypophosphatasia. *see also* Alkaline phosphatase
adult, 1234
biochemical defects, 1237–1238
childhood, 1233
clinical features, 1232–1234
diagnosis, 1234–1237
fibroblast studies, 1242–1243
genetic defects, 1237–1238
history, 1231
infantile, 1233
odonto, 1234
prenatal, 1232–1233, 1239
prognosis, 1239
pseudo, 1234
role of ALP, 1240–1243
treatments, 1238–1239
Hypothalamic-pituitary-gonadal axis, 725
Hypothyroidism
animal models, 712–713
human studies, 713
Hypoxia, 892
Hysteresis, 22

I

Iatrogenic fluorosis, 1390
ICTP assays, as bone resorption marker, 220
IDDM. *see under* Diabetes mellitus
Immobilization, effects on bone turnover, 1550
Immune system
effects of
PTH therapy, 1318
VDR, 592–594
vitamin D_3, 577
vitamin D therapy, 1412
Immunoglobulins, 269. *see also* specific molecules
Immunophenotyping, SCM, 62–65
Implantations, bone discs, 243–244
Indian hedgehog, 47–50
Inflammation
associated bone loss, role of prostaglandin, 987–988
process, 773
-sepsis, calcitonin-associated, 630–631, 633
systemic, calcitonin-associated, 633
Inflammatory joint disease, 156
Innervation, bone, 781
Insulin
as BMD marker, 745

Insulin (*continued*)
effects of vitamin D_3, 577
effects on
bone
animal models, 746–747
cells *in vitro,* 748–749
function, 743–746
skeletal growth, 741–743
Insulin-like growth factor
in bone, effects of FGF, 837
effects of glucocorticoids, 732
effects of PTH/PTHrP, 495
effects on
chondrocytes, 46
OI, 1186
PTH, 468–469
remodeling, 810–811
genes, 801–802
osteoporosis pathophysiology and, 1279
peptides, characterization, 801
regulation in bone cells, 802–803
role in
collagen synthesis, 196–197
osteoblast apoptosis, 160
secretion, age and, 1442
type I
as anorexia nervosa treatment, 1450
function, 1441
gonadal status and, 1444
osteoporosis treatment
animal models, 1446–1448
availability, 1446
clinical trials, 1448–1449
regulation in bone cells, 803–804
type II, regulation in bone cells, 804–806
Insulin-like growth factor-binding proteins
effects of thyroid hormones, 710
effects on pituitary function, 725
expression in bone cells, 806–809
osteoporosis pathophysiology and, 1279
proteases, 809–810
Insulinoma, MEN1 syndrome-associated, 1051
Integrins
binding, role in osteoclast apoptosis, 158
chondrocyte expression, 279
fibronectin expression, 277
$\alpha_v\beta_3$
effects of vitamin D_3, 578
role in
osteoblast apoptosis, 160
osteoclast function, 142–143, 274–275
osteoclast organization/motility, 143–144
resorption, 270–271
structure, 268
survival signaling via, 155
as therapeutic target, 275–276
osteoblast expression, 277
osteoclast expression, 144–145
Interaction cloning strategies, 1516
Interferon-γ
effects on bone, 964
role in collagen synthesis, 198
Interleukins
IL-1
effects on bone, 961–962
gene clusters, role in BMD, 1267
in osteoclasts
effects of estrogen, 662–663
effects on apoptosis, 158–159
effects on differentiation, 121
osteoporosis pathophysiology and, 1278–1279
production, effects of menopause, 698–699
role in
collagen synthesis, 198
MAH, 1087–1088
ovariectomy-induced bone loss, 699–702
IL-2, role in vitamin D_3 systems, 579
IL-3 (*see* Multicolony-stimulating factor)
IL-4
effects on bone, 965
osteoporosis pathophysiology and, 1278–1279
role in collagen synthesis, 198
IL-6
effects of FGF, 837
effects of PTH/PTHrP, 495
effects on bone, 966–971
gene, role in BMD, 1267
in osteoblasts
effects of estrogen, 495
role in apoptosis, 160
osteoporosis pathophysiology and, 1278–1279
role in
HHM, 1094–1095
MAH, 1087–1088
ovariectomy-induced bone loss, 699–702
IL-7, effects on bone, 966
IL-8, role in MAH, 1087–1088
IL-10, role in collagen synthesis, 198
IL-13, osteoporosis pathophysiology and, 1278–1279
Intestine
Ca homeostasis, role of CaR, 376–377
role of vitamin D_3, 574–575
In vivo footprinting assay, 1514
Ions
channels, effects of PTH/PTHrP, 497
excretion, effects of FBHH, 1034–1035

J

Jackson-Weiss syndrome, 43
Jansen's metaphyseal chondrodysplasia
characteristics, 1122–1123
role of PTHrP, 440

K

Kallikrein-kinin system, 774–776
Kallikreins, 774–775
Kearns-Sayre syndrome, 1111
Kidney
anomalies syndrome, 1110–1111
bisphosphonate clearance by, 1375
cells, PTHR signaling, 484–485

effects of
calcitonin, 627
PTH, 1118
1α,25(OH)$_2$ vitamin D$_3$, 575–576
fluoride excretion, 1388–1389
function, effects of FBHH, 1034–1035
osteodystrophy, vitamin D therapy, 1411–1412
phosphate transport, effects of PTH, 469–471
phosphorus excretion, 322–326
PTHrP expression, 483–485, 493
role in
FBHH, 1036–1037
Mg homeostasis, 341–342
processing collagen fragments, 216–217
transplantation, associated bone loss, 1562
Kininases, 775–778
Kininogens, 775
Kinins
characterization, 775
clinical observations, 791–792
-cytokines, interactions, 780
effects on bone metabolism, 776–778
-neuropeptides, interactions, 780
receptors, 778–779

L

Lactate, effects of calcitonin, 627
Lactation, 520–522
LacZ gene-β-galactosidase, 1512
Lead-bone, interaction, 359, 363–365
Leptin, 790
Leukemia inhibitory factor, 966–971
Leukocytosis, hypercalcemia-associated, 1101
Leukotrienes
effects on bone, 988–989
osteoporosis pathophysiology and, 1279
LIF. *see* Leukemia inhibitory factor
Limb
development, 155–156
initiation, 38–42
Lining cells
biopsy, 1629
function, 5
gap junction forming by, 278
strain sensing role, 277
structure, 5
Linkage analysis, 1261, 1263–1264
Lipomas, MEN1 syndrome-associated, 1050
Lipopfection, description, 1510
Liver
role in processing collagen fragments, 216–217
vitamin D clearance, 1416
Loading
effective mechanical
cellular basis, 1482–1483
characteristics, 1481–1482
habitual (*see* Exercise)
Long bone failures, 26–27
Low-affinity receptors, 830–831
Luciferase, 1511
Lysine, derived cross-links, 214–215

M

Macrophage colony-stimulating factor
effects on osteoclasts, 121
osteoporosis pathophysiology and, 1279
role in
osteoclast apoptosis, 157–159
osteoclast formation, 693–695
osteoclastogenesis, 111–112
ovariectomy-induced bone loss, 699–702
vitamin D$_3$ systems, 579
Magnesium
content, 339
depletion
effects on
parathyroid gland, 345–346
PTH, 347–348
vitamin D metabolism, 348–349
osteoporosis association, 349–351
effect on mineral formation, 353
excretion
regulation by PTH, 487–488
regulation by PTHR signal transduction, 488–489
homeostasis, renal role, 341–342
intestinal absorption, 339–341
intracellular, 343–344
osteoporosis association
animal models, 352–353
mechanism, 353
risk factors, 351–352
treatment, 351
RDA, 340
reabsorption, sites/mechanisms, 485–487
role in PTH secretion, 344–345
Magnetic resonance imaging, 1615–1618
MAH. *see* Hypercalcemia, malignancy-associated
Mammary gland. *see also* Breast
effects of PTH/PTrP
adolescent development, 518–520
embryonic development, 517–518
lactation, 520–522
pregnancy, 520–522
MAPKs. *see* Mitogen-activated protein kinases
Marrow stromal cells cultures, 1534–1535
Mass spectrometry, transcription factor analysis, 1519–1520
Matrix metalloproteinases. *see also specific types*
activation, 251–252
classification, 251
description, 52
MMP-1, role in organic matrix degradation, 134–135
role in osteoblast apoptosis, 160
subdivisions, 252–257
McCune-Albright syndrome
characteristics, 1166–1167
genetics, 1168
management, 1173
pathogenesis, 1170–1173
pathology, 1169–1170
radiological findings, 1168–1169
MCP-1. *see* Monocyte chemotactic peptide-1
M-CSF. *see* Macrophage colony-stimulating factor
MC3T3–E1 cells, 1506–1507

Mechanosensing, role of osteocytes, 100–101
Mechanosensory cells, osteocytes as, 99–100
Mechanotransduction, 25–26, 294
Megalin, 473
Melanin, 588
Melanoma cells, 245–246
MEN. *see* Multiple endocrine neoplasia
Men
  aromatase deficiency in, 1469–1472
  α-ER deficiency in, 1468–1469
  osteoporosis, animal models, 1639, 1645–1647
  skeleton
    interventional studies, 1474–1475
    observational studies, 1472–1473
Menopause
  associated-bone loss, effects of exercise, 1484
  associated osteoporosis
    animal models, 1637–1638
    biochemical markers, 1558–1560
    characterization, 693
    effects of bisphosphonates, 1375–1376
    fluoride treatment, 1392–1394
    PTH therapy for, 1315
    role of CSF-1, 95–96
    role of PTH, 1354–1355
  effects on
    bone-resorbing cytokines, 697–698
    cytokine production, 697–698
    IL-1 production, 698–699
  fracture risks and, 1484–1486
  natural, 1637
Menstrual cycle, 1551, 1637
Mental retardation, 1148
Mesangial cells, 817–818
Meschymal stem cells
  age-associated changes, 73–74
  disease-associated changes, 73–74
  -epithelial interactions, role of PTHrP, 1122
  immunophenotyping, 62–65
  nomenclature, 59
  therapeutic use, 74
Mesenchymal condensation, 40–42
Metalloproteinases, membrane type, 253
Metastatic bone disease, markers for, 1562–1564
Mevalonate pathway, inhibition by N-BP, 1369
MG63 cells, 1506
Microprojectile particle bombardment, description, 1511
Microtubules, effects of mynein light chain, 415–416
Mineral
  homeostasis, impaired mechanism, 347–348
  opposition rate, 1631
Mineralization
  effects of Mg, 353
  lag time, 1631
  mechanism, 4–5
  osteoid, formation, 1182–1184
  remodeling-associated, 310–311
  skeleton, effects on analysis, 12–13
  surface, 1631
Mitochondrial trifunctional protein deficiency syndrome, 1111
Mitogen-activated protein kinases, 428–429
Mitogenesis, VEGF-A, 889–890
MLO-Y4, 97
MMPs. *see* Matrix metalloproteinases
Monocyte chemotactic peptide-1, 964
Mouse
  COX-2 null model, 984–985
  ER $\alpha\beta$ knockout, 660–661
  human link, 1654
  knockout, 1472
  NOS-gene deficient, 1004–1006
  NSHPT models, 1041
  OPN-deficient, 242
  osteopetrotic phenotype, resolution, 948–949
  osteoporosis models, 1640–1641
  PR gene disruption in, 661
  RANKL/RANK deficient, 115–116
  TGFβ knockout, 904
  transgenic
    bone cell analysis, 1497–1498
    bone mass maintenance, 1494–1496
    osteoblast lineage, 1496–1497
    skeletal biology, 1498–1499
    skeletal development, 1494–1496
    structural macromolecules
      analysis, 1492–1493
      Cis regulation, 1493–1494
MRI. *see* Magnetic resonance imaging
mRNA. *see* RNA, messenger
Msenchymal cells, 962
Msx-1, role in osteoblast differentiation, 85–86
Msx-2
  function, 1496
  regulation, 69–71
  role in osteoblast differentiation, 85–86
MTPDS. *see* Mitochondrial trifunctional protein deficiency syndrome
Multicolony-stimulating factor, 949–950
Multiple endocrine neoplasia syndrome
  type 1
    clinical aspects, 1047–1048
    description, 1047
    gene
      characterization, 1021–1022
      endocrine tumors and, 1058–1059
      localization, 1057–1058
      mutations, 1048, 1058
      normal, 1058
      phenocopies, 1048
      sequence, 1058
    pathophysiology
      animal models, 1060
      associated organs, 1053–1056
      biochemical/radiological tests, 1056–1057
      general considerations, 1052–1053
      germline DNA-based tests, 1057–1060
    treatments, 1050–1052
  type 2
    components, 1067–1068
    with cutaneous lichen amyloidosis, 1070
    description, 1067–1068
    gene, characterization, 1021–1022
    Hirschsprung's phenotype, 1069–1070
    role of *RET* mutations

correlations, 1070–1071
management, 1072–1073
mechanism, 1071–1072
Myeloma
bone disease, 1095–1097
multiple, 1583

N

Natriuretic peptides, C-type, 1009
N-BP. *see* Bisphosphonates, nitrogen-containing
N-CAM. *see* Neural cell adhesion molecules
Nephropathy diabetic, in bone, 746
Nervous system
central (*see* Central nervous system)
development, role of *RET,* 1072
effects of amylin, 642
effects of CGRP, 642
role in remodeling, 311
Neural cell adhesion molecules, 278
Neuroendocrine system, diffuse, 1055
Neuromodulation, 539
Neuropeptides. *see also* specific neuropeptide
-cytokines, interactions, 780
-kinins, interactions, 780
Y, 786
Neurotransmitters. *see* specific neurotransmitters
Neurotrophins, 789–790
NIDDM. *see under* Diabetes mellitus
Nitric oxide
associated pathology, 1007
characterization, 995
donors, 1003
effects on osteoclasts, &999–1003
isoform occurrence, 998–999
molecular targets, 1006
osteoporosis pathophysiology and, 1279
synthesis
inhibition, 1003–1004
mouse model, 1004–1006
therapeutic implications, 1007–1008
Nonhuman primate models, 1644
Nonneuroendocrine tumors, calcitonin-associated, 630
Nonsteroidal anti-inflammatory drugs, 988
Notch/Delta signaling, 35–36
NPS 2143, 1343–1344
NPS R-568
characterization, 1341–1342
effects on PTH, 1342
primary hyperparathyroidism, 1346–1348
secondary hyperparathyroidism
animal models, 1348–1350
clinical studies, 1351–1352
N-terminal peptides, as collagen formation markers, 218
N-terminal telopeptide of collagen type I
assays, as bone resorption marker, 220
circadian variability, 1550–1551
description, 1548–1549
levels in immobilization, 1550
levels in pregnancy, 1550
NTX-I. *see* N-terminal telopeptide of collagen type I
Nuclear
architecture
skeletal gene expression link, 176–183
three-dimensional context, 170–176
extracts, preparation, 1513
receptors, 1459–1461
run-on assays, 1508

O

OCIF. *see* Osteoprotegerin
OI. *see* Osteogenesis imperfecta
Oncogenes. *see also specific genes*
effects of VEGF-A, 891
putative, 1021, 1024
Oncostatin M, 198
OPG. *see* Osteoprotegerin
Opioid peptides, 787
OPN. *see* Osteopontin
Oral contraception, 1550
OSE1, 87
Osf1, 87
Osseous systems, 625–626
Ossification
endochondral
embryonic, 33–34, 43–45
patterning, 12
process, 8–9
role of PTH, 156
intramembranous, 8
Osteoarthritis
stem cell changes and, 73–74
stem cell therapy, 74
tissue engineering for, 74
Osteoblasts
apoptosis
description, 159–160
effects of androgens, 762–763
effects of bisphosphonates, 162–163
glucocorticoid-induction, 161
PTH regulation, 161–162
sex steroid regulation, 160–161
associated bone metastases, 1097–1098
biopsy, 1628
connexin expression, 292
cultures, current techniques, 1531–1534
development
control of, 61–62
role of
cytokines, 71–73
growth factors, 71–73
hormones, 71–73
osteoprogenitor cells, 67–69
transcription factors, 69–71
differentiation
Cbfa 1 role, 83–85
effects of androgens, 763–64
effects of FGF, 836–837
transcriptional factors, 85–88
effects of
amylin, 647–648
calcitonin, 147

Osteoblasts (*continued*)
CGRP, 646–647
estrogen *in vitro,* 661–665
FGF signaling, 838
fluoride, 1389
gap junction communication, 294–295
G-CSF, 952–953
IGF, 802–803
IGFI, 803–804
IGFII, 804–806
IL-1, 962
INF-γ, 964
insulin *in vitro,* 748–749
kinin receptors, 778–779
mechanical loading, 1482
NO, 1001–1003
OPN, 241
PDGF, 818–820
progesterone receptors, 668
PTH/PTHrP, 495–498
TGFβ, 905
VEGF, 894
estrogen distribution, 657
formation, role of estrogen, 664–665
function, role of
adhesion molecules, 276–279
cadherins, 278
CD44, 278
description, 4–5
immunoglobulins, 278–279
integrins, 277–278
hormone action in, 729–730
IGFBP expression in, 806–809
intercellular Ca signaling, 297–298
-like cell cultures, current techniques, 1531–1534
lineage, GFP to assess, 1496–1497
-stromal cells, role in
OPG expression, 116–118
osteoclast differentiation, 109–111
osteoclast regulation, 111–112
RANKL regulation, 116–118
transcriptional complexes
culture models, 1504, 1506–1507
OI
intrinsic properties, 1183
physiological demands, 1183–1184
ontogeny, 60–65
osteosarcomas, culture models, 1504
PDGF expressing by, 817–818
phenotypic markers, 709–710
precursor cells, 494–495
primary cultures, 1507
progenitor cells, 657
proliferation, effects of
androgens, 762–763
estrogens, 664
FGF, 836
thyroid hormones, 711
prostaglandin production, 982–984
PTH regulation, 465–467
regulation, 7–8
-stromal cells, M-CSF production by, 111–112
structure, 4–5
transgenic models, 1498
Osteocalcin
as bone formation marker, 1546–1547
effects of thyroid hormones, 710
effects of vitamin D_3, 578
gene, regulatory factors, 176–183
levels in metastatic bone disease, 1561, 1562–1564
levels in PHPT, 1551
as osteoporotic fracture predictor, 1559
Osteoclastogenesis
control of, 141
effects of FGF, 839
inhibitory factor (*see* Osteoprotegerin)
role of M-CSF, 111–112
Osteoclasts
activation
effects of thyroid hormones, 711
inhibition by bisphosphonates, 1373
adhesion, Pyk2/c-Src regulation of, 144–145
apoptosis
description, 156–159
effects of bisphosphonates, 162–163
glucocorticoid-induction, 161
sex steroid regulation, 160–161
biopsy, 1628–1629
cells, degradative effects, 216
clear zone, function, 272–274
cultures, current techniques, 1535
cytoskeletal organization, 143–144
development, role of adhesion molecules, 272, 290–291
differentiation, role of
osteoblasts/stromal cells, 109–111
RANKL, 114–115
differentiation factor (*see* Receptor activator of nuclear factor $\kappa\beta$ ligand)
dysfunction, in osteopetrosis, 1221
effects of
amylin, 645–646
calcitonin, 1009, 1426–1428
CGRP, 643–645
CSF-1, 948–949
edothelin, 1009
estrogens, 665–686
G-CSF, 952–953
IGF, 806
IL-1, 961–962
NO, 999–1001
OPN, 241
progesterone receptors, 668
prostaglandins, 984–986
PTHrP, 1084
sex steroids, 265
TGFfl, 905
VEGF, 894–895
estrogen distribution, 657
formation
cytokine regulation, 693–695
role of CSF-1, 946–947
function
-$\alpha_v\beta_3$ integrin-mediated, 142–143
osteoblast/stromal cell regulation by, 112

role of RANKL, 114–115
intercellular Ca signaling, 297–298
motility, $\alpha_v\beta_3$ integrin-mediated, 143–144
in resorption cycle
cytoskeletal changes, 132–133
degradation product removal, 135–136
life span, 128–129
membrane domains, 130–132
polarization, 130–132
sealing zone formation, 129–130
surface attachment, 129–130
role in
bone degradation product removal, 135–136
degradation of organic matrix, 134–135
dissolving bone mineral, 133–134
role of
integrin receptors, 270–271
nonintegrin receptors, 271–272
signal transduction, adhesion-dependent, 143–144
transgenic models, 1498
Osteocytes
apoptosis
effects of bisphosphonates, 162–163
glucocorticoid-induction, 161
PTH regulation, 161–162
sex steroid regulation, 160–161
biopsy, 1629
cell lines, 97
cell-matrix adhesion, 98
cultures, current techniques, 1535
cytoskeleton, 98
death of, 94–96
effects of
NO, 1001–1003
PTH/PTHrP, 498–499
fluid flow response, *in vitro,* 101–102
formation, 94–96
function, 3–5
hormone receptors, 98–99
isolation, 96
location, 93
markers, 97
matrix synthesis, 97–98
as mechanosensory cells, 99–100
mediated-mechanotransduction, 25
role in
blood-calcium homeostasis, 99
canalicular fluid flow, 100–101
functional adaptation, 99
structure, 5
syncytium, 93–94
Osteodystrophy. *see* Albright's hereditary osteodystrophy
Osteogenesis imperfecta
animal models, 1180–1181
characterization, 1177
coupling, 52–53
deforming
classification, 1177–1179
molecular basis, 1181–1182
diagnosis, 1180
effects of VEGF, 893–894
management, 1180
mild nondeforming
classification, 1179–1180
molecular basis, 1182
producing mutations, 1182–1185
role of BMP, 923–924
role of collagen mutations, 193
stem cell therapy, 74
tissue engineering for, 74
treatments
anabolic agents, 1186
antiresorptive agents, 1185–1186
cell/gene, 1186–1188
Osteogenic cells, 288–289
Osteolysis
focal, role in MAH, 1088
inhibition by bisphosphonates, 1088–1089
Osteomalacia
characterization, 1209
clinical features, 1195–1196
oncogenic
differential diagnosis, 1211–1212
pathophysiology, 1210–1211
treatment, 1212–1213
tumors, 1209–1210
tumor-induced, 331–333
vitamin D therapy for, 1411
Osteonecrosis, 726
Osteonectin, 228–229
Osteopenia, 1638
Osteopetrosis
adult forms, 1220–1221
animal models, 1222–1223
avian, 1223
development, 1217
infantile forms
intermediate, 1218–1220
malignant, 1217–1218
transient, 1220
pathophysiology, 1221–1222
phenotype, 12
treatment, 1224–1226
Osteopontin
cell attachment, 241
deficient mouse, phenotype, 242
-dependent intracellular signaling, 242
description, 231
effects of vitamin D_3, 578
effects on angiogenesis, 243–244
effects on resorption, 243–245
metastatic disease and, 245–246
receptors, 241
role in mediating mechanical stress, 246–248
role in postmenopausal bone loss, 242–243
structure, 239–241
Osteoporosis
animal models
congenic/recombinant strains, 1650–1651
congenic strains, 1651–1653
convenience, 1639
criteria, 1635
inbred strains, 1648–1650
postmenopausal, 1637–1640

Osteoporosis (*continued*)
recombinant/congenic strains, 1650–1651
recombinant strains, 1650–1651
regulatory recommendations, 1635
in vivo experimentation, 1635
BMD association
epidemiology, 1305
history, 1647–1648
Ca intake and, 1335
corticoid-induced, fluoride therapy, 1394
definition, 1573–1574
diagnosis, T scores, 1589–1590
effects of
bisphosphonates
mechanisms of action, 1364–1365
models, 1362–1364
remodeling, 313–314
ethnic differences, 1590–1593
fractures associated with
risks factors
BMD, 1292–1293
bone turnover, 1296–1298
clinical, 1291–1292
combined assessment, 1298
hormonal markers, 1293–1294
menopause, 1294–1296
gender differences, 1590–1593
genes
allele-sharing studies, 1261–1262
association studies, 1262
candidates
apolipoprotein E, 1267
calcitonin receptor, 1267–1268
collagen, type I, 1264
estrogen receptor, 1266
interleukins, 1267
TGFβ, 1266–1267
VDR, 1264
linkage analysis, 1261
genetic determinants
effects on
bisphosphonate response, 1269
calcium absorption, 1269
clinical practice, 1269–1270
HRT response, 1269
vitamin D absorption, 1269
evidence for, 1260–1261
identifying, 1259–1260
quantitating, 1259–1260
glucocorticoid-induced
effects of bisphosphonates, 1375–1376
pharma, animal models, 1639, 1644–1645
growth hormone therapy
administration, 1445
BMD and, 1443–1446
clinical trials, 1445–1446
mechanisms, 1442–1443
physiology, 1441–1442
idiopathic, IGF-I treatment for, 1449
IGF-I treatment
animal models, 1446
availability, 1446
clinical trials, 1448–1449
male
animal models, 1639, 1645–1647
fluoride therapy, 1394
Mg association
animal models, 352–353
epidemiological studies, 349–350
mechanism, 353
risk factors, 351–352
treatment, 351
pathophysiology
bone formation, 1277–1278
bone resorption, 1276–1277
calcitonin, 1283
cytokines and, 1278–1279
estrogen and, 1278
growth factors, 1279
leukotrienes and, 1279
local factors, 1278
nitric oxide and, 1279
parameters, 1275–1276
parathyroid function, 1281–1283
prostglandins and, 1279
systemic factors, 1280
vitamin D, 1280–1281
pharmacogenetics and, 1268–1269
postmenopausal
animal models, 1637–1640
biochemical markers, 1558–1560
characterization, 693
effects of bisphosphonates, 1375–1376
fluoride treatment, 1392–1394
PTH therapy for, 1315
role of CSF-1, 945–946
role of PTH, 1354–1355
prevalence *vs.* risks, 1590
role of
FGF, 840–841
gene-gene interactions, 1268
heparin, 840–841
IL-1, 962
PTH, 1354
TGFβ, 913
stem cell changes and, 73–74
therapies
anabolic agents, 1306
anti-resorptive agents, 1306
calcitonin, 1433
fluoride, 1390–1392
$\alpha_v\beta_3$-intergin as target, 275–276
stem cell, 74
vitamin D, 1411
tissue engineering for, 74
WHO classification, 1573–1574
Osteoprogenitor cells
age-associated changes, 73–74
differentiation, 67–69
disease-associated changes, 73–74
function, 65–67
Osteoprotegerin
in bone, effects of PTH/PTHrP, 496–497
discovery, 112

expression, 116–118
identification, 1084–1085
role in MAH, 1086–1087
Osteosarcomas
effects of IGFPB, 809–810
effects of insulin *in vitro,* 748–749
osteoblastic, culture models, 1504, 1506
Osteprogenitors, PTH regulation, 465–467
Ostoclasts
adhesion, inhibition of, 1372
lifespan, shorting of, 1372–1373
recruitment, inhibition of, 1372
Ovariectomy
effects of osteclastogenic cytokines, 699
effects on cancellous bone, 659
effects on turnover, 1614
induced bone loss, 699–702

P

Paget's disease
biochemical markers for, 1551, 1553–1554, 1556
biochemistry, 1251
calcitonin therapy, 1433
cellular biology, 1253–1255
characterization, 1249
complications, 1249
diagnosis, 1250
effects of bisphosphonates, 1376
etiology, 1255–1256
histopathology, 1250–1251
molecular biology, 1253–1255
occurrence, 1249
paramyxoviruses in, evidence, 1251–1253
therapy, 1251
Pancreas
effects of PTH/PTHrP, 525–527
MEN1 syndrome and, 1049, 1055
pathology, FBHH and, 1033
Paramyxoviruses, Paget's disease in, 1251–1253
Parathyroid cytosolic proteins, 414–415
Parathyroidectomy, 1050–1051
Parathyroid gland
abnormalities, MEN2 syndrome-associated, 1069
effects of
FBHH, 1036
Mg depletion, 345–346
vitamin D_3, 576
MEN1 syndrome and, 1053–1054
PTH gene expression, 408
tumors
clonality, 1018–1019
genetic derangements, 1019–1021
genetic pathogenesis, 1024–1025
molecular pathogenesis, 1025
Parathyroid hormone
anabolic properties
advantages, 1305
potential mechanisms, 1306–1307
biological action, 389
bone cell apoptosis regulation by, 161–162
cardiovascular activity, 531–532
cellular actions
cation/anion transport, 471–472
cytokine-mediation, 468–469
description, 465–469
hormone-mediation, 468–469
in collagen synthesis, 199
connexin regulation by, 293
ectopic secretion, 1025–1026
effects of
CaR therapy, 381, 1353
glucocorticoids, 725–726
Mg depletion, 347–348
NPS 2143, 1343–1344
NPS R-568, 1342, 1344
osteocytes, 98–99
protein kinases, 418
sex steroids, 417–418
vitamin D-3, 579
effects on
bone, 495–498
Ca homeostasis, 1339
cardiovascular system, 473–475
kidney
metabolism, 472–473
molecular mechanisms, 1118
phosphate transport, 469–471
OI, 1186
osteocytes, 498–499
pituitary function, 725
remodeling, 315
vasculature, 473–475
in endochondral ossification, 156
expression in bone, 493–494
genes
abnormalities, 1106–1109
characterization, 407
localization, 407
in hyperparathyroidism, 416–417
induced resorption, role of OPN, 244–245
levels in PHPT, 1557
mutations
characterization, 409
role in chondrodysplasias, 1127–1129
role in hypoparathyroidism
associated syndromes, 1109–1112
autosomal dominant type, 1107
autosomal recessive type, 1107–1108
isolated type, 1105
x-linked recessive type, 1109
role in PHP, 1112–1113
osteoblast regulation by, 465–467
osteoclast regulation by, 467–468
osteoporosis pathophysiology and, 1281–1283
peripheral metabolism, 463–465
phosphate transport regulation, 323–326
promoter sequences, 408
-PTHrP interactions
amino-terminal receptor domain, 393–395
characterization, 389–391
in CNS, 537–540
conformational changes, 397
conformation based design, 434–435

Parathyroid hormone (*continued*)
effects on
bone, 499–500
kidney, 473
mammary gland, 517–522
pancreas, 525–527
reproductive tissues, 522–525
skin, 515–517
integrated studies
advantages, 440–441
approaches, 441–442
contact sites, 442–445
molecular modeling, 445–450
photoreactive analogs, 442
interaction, 395–396
juxtamenbrane region, 395–396
ligand tethered, 445–450
mechanisms, 391–393
mid- and carboxy-terminal specific, 398–400
-PTH, interaction, 396–397
regulation, 397–398
signaling pathways
downstream activities, 428–429
dual intracellular, 427–428
selective ligands, 429–430
structure-activity relations, 424
subtype specificity switch, 426
target-specific ligands, 430
two-site dynamic model, 451–452
in vasculature, 522–525
regulation of
Ca excretion, 487–488
Ca in, 412–413
calcitriol in, 409–412
calreticulin in, 411–412
H excretion, 491–492
Mg excretion, 487–488
Na excretion, 491–492
Ph excretion, 489–491
phosphate in, 413–414
vitamin D metabolism, 492–493
role in
FBHH, 1035–036
humoral hypercalcemia of malignancy syndrome, 1094
osteoblast development, 71–73
secondary hyperparathyroidism, 416–417
type I osteoporosis, 1355
secretion, role of
CaR, 375
Mg, 344–345
signal transduction, 1137–1140
structure, primary, 424–426
therapy
adverse effects, 1318–1319
animal models, 1307–1308
bone metabolism markers, 1316
clinical studies, 1308–1309
delivery systems, 1318
effects on
BMD measurements, 1310, 1313
bone histology, 1315–1316
calcium balance, 1316–1317
fracture risk, 1309–1310
immunological response, 1318
with other treatments, 1313–1315
pharmacokinetics, 1317–1318
TIP39 receptor
characterization, 389–391
identification, 393, 426–427
tissue-specific expression, 407–408
Parathyroid hormone receptors
effects on kidney, 1119–1122
regulation of
Ca excretion, 488–489
H excretion, 491–492
Mg excretion, 488–489
Na excretion, 491–492
renal functions
cell signaling regulation, 484
signal transduction, 484
role in bone formation, 1120–1122
Parathyroid hormone-related peptides
amino-terminal receptor domain, 393–395
biological action, 389
bone, 493–494
characterization, 389–391
chimera, 437–439
cloning, 1080
conformational changes, 397
discovery, 1119
ectopic domain, cysteins characterization, 439–440
effects on
bone, 495–498
chondrocytes, 47–50
heart, 536–537
kidney, 483–485, 493
osteocytes, 498–499
identification, 393
juxtamenbrane region, 395–396
ligands, 1119–1122
mechanisms, 391–393
mid- and carboxy-terminal specific, 398–400
mutations
associated-genetic disorders, 440
role in Blomstand's lethal chondrodysplasia, 1127–1129
role in Jansen's metaphyseal chondrodysplasia, 1123–1125
as neuroprotective, 539–540
-PTH interactions
in CNS, 537–540
conformation based design, 434–435
description, 396–397
effects on
bone, 499–500
kidney, 473
mammary gland, 517–522
pancreas, 525–527
reproductive tissues, 522–525
skin, 499–500
integrated studies
advantages, 440–441
approaches, 441–442
contact sites, 442–445

molecular modeling, 445–450
photoreactive analogs, 442
ligand tethered, 450–451
signaling pathways
downstream activities, 428–429
dual intracellular, 427–428
selective ligands, 429–430
structure-activity relations, 424
subtype specificity switch, 426
target-specific ligands, 430
two-site dynamic model, 451–452
in vasculature, 522–525
purification, 1080
receptors, role in
bone formation, 1120–1122
epithelial-mesenchymal interactions, 1122
regulation, 397–398
role in
humoral hypercalcemia of malignancy syndrome, 1093–1094
malignancy-associated hypercalcemia
approaches to controlling, 1086–1087
mechanisms of action, 1083–1086
molecular aspects, 1080–1082
osteolysis, 1088–1089
osteolytic bone metastases, 1100
site-directed mutagenesis studies, 439
subtypes, 436–437
types, 435–436
in vasculature
effects on, 532–533
effects on anatomy, 532–533
expression, 533–534
mode of action, 533–534
Paraxis, 36
PDGF. *see* Platelet-derived growth factor
Peptide assay, as bone resorption marker, 219–220
Periodontal disease, BMP application, 921
Peroxisome proliferator-activated receptors, 982
Pfeiffer syndrome, 43
Pharmacogenetics, 1268–1269
Phenylalkylamines
NPS 2143, 1343–1344
NPS R-568
characterization, 1341–1342
effects on PTH, 1342
primary hyperparathyroidism, 1346–1348
secondary hyperparathyroidism
animal models, 1348–1350
clinical studies, 1351–1352
PHEX gene
rickets link, 328–329
tumor-induced osteomalacia link, 331–333
Phosphate
effects of calcitonin, 624–625
effects on PTH mRNA, 414–415
excretion, role of PTH/PTHR, 488–489
renal transport, effects of PTH, 469–471
role in PTH gene regulation, 412–414
therapy, for osteomalacia, 1212
Phosphoethanolamine, 1235, 1240
Phosphorus
deficiency, 321–332
function, 321
GI absorption, 322
impaired reabsorption, 326–334
renal excretion, 322–326
transport
effects of glucocorticoids, 725–726
mechanisms, 323–326
Photoreactive analogs, 442
Photosynthesis, previtamin Dd_3D
description, 587
in skin, 587–590
PHP. *see* Pseudohypoparathyroidism
PHPT. *see* Hyperparathyroidism, primary
Phytoestrogens, 667
PICP. *see* Procollagen type I propeptides
Pig models, 1643
PINP. *see* Procollagen type I aminoterminal propeptide
Pituitary adenylate cyclase-activating peptides, 784
Pituitary gland
function, effects of glucocorticoids, 725
tumors, MEN1 syndrome-associated
description, 1049–1050
pathology, 1055
treatment, 1052
P13 kinase, 146–147
Placenta, effects of PTH/PTHrP, 524
Plasminogen activator
in bone, 257–258
characterization, 257–258
gene expression in bone, 733
sequences, 1098
tissue-type, characterization, 257
Platelet conversion, 311–313
Platelet-derived growth factor
binding proteins, 820–821
clinical relevance, 821
effects on skeletal cells, 818–820
function, 821
osteoblastic bone metastases and, 1098
osteoporosis pathophysiology and, 1279
receptors, 820–821
synthesis by skeletal cells, 817–818
Polar moment of inertia, 27
Polymerase chain reaction
ligation mediated, 1514
reverse transcriptase
nascent transcript level analysis, 1508
transcription factor characterization, 1519–1520
PPARs. *see* Peroxisome proliferator-activated receptors
Pregnancy
effects of PTH/PTHrP, 524–525
effects on bone turnover, 1550
mammary epithelial cells and, 520–522
Preosteoclast cultures, current techniques, 1535–1536
Procollagen type I aminoterminal propeptide, 1547
Procollagen type I propeptides
circadian variability, 1551
description, 1547
levels in Paget's disease, 1553

Progesterone
effects onf VEGF-A expression, 892
receptors, effects on bone, 668–669
replacement therapy, 667–668
VDRdnucD response, 553–554
Progestins
coregulators, 657
disruption, rodent models, 661
effects on bone mass, 658
mechanism of action, 655–656
PRO-α genes
cis-acting elements binding, 200–201
first intron elements, 203–204
posttranscitptional regulation, 204
transcription factors binding, 200–201
upstream elements, organization, 201–203
Prostaglandins. *see also specific types*
connexin regulation by, 293
effects of
estrogen, 984
FGF, 838
glucocorticoids, 733
effects on
bone loss, 987–988
HHM, 1095
osteoblasts, 982–984
osteoclasts, 983–986
resorption, 778
VEGF-A expression, 892
metabolic pathways, 979–980
osteoporosis pathophysiology and, 1279
production, inhibition of, 983–984
receptors
characterization, 981–982
effects on
bone formation, 986–987
mechanical loading, 987
resorption, 986
Prostate cancer, role of TNFfl, 1098
Proteases of bone, 498
Proteinases, aspartic, 258
Protein-DNA incubation, 1518–1519
Protein isoprenylation, 1369–1371
Protein kinase A, 418
Protein kinase C
calcitonin and, 1431
CaR and, 375
PTH and, 418
Proteins. *see also specific proteins*
-DNA, interactions at PTH mRNA, 414–417
-DNA, interactions *in vitro* and *in vivo,* 1512–1515
leucine-rich, 228
Protein tyrosine phosphatases, 1366
Proteoglycans, 225–228
Proteolysis, bone cells, 809–810
Protooncogenes, expression in bone, 731
Proximal tubular
Na/H excretion, regulation by PTH, 492
Ph reabsorption, regulation by PTH, 487–488
Pseudohypoparathyroidism
definition, 1137
diagnosis, 1152–1154
genetics, 1152
identification, 1143
molecular classification, 1142–1143
pathophysiology, 1140–1142
patients with, 1112–1113
treatment, 1154–1156
type 2, 1150
type 1a
identification, 1143
multiple hormone resistance in, 1145–1149
neurological abnormalities, 1148
sensory abnormalities, 1146–1148
type 1b, 1149–1150
type 1c, 1150
Psoriasis, 595–597, 1412
PTPs. *see* Protein tyrosine phosphatases
Pyk2
-c-Src, role in osteoclast adhesion, 144–147
effects of calcitonin, 1431
Pyridinium
as bone resorption marker, 219
cross-links, 212
levels in heart transplants, 1561
levels in metastatic bone disease, 1563
Pyridinoline
levels in PHPT, 1548
as resorption marker, 1548
Pyridoxal 5′-phosphate, 1235, 1240–1242
Pyrophosphate, inorganic, 1235, 1242
Pyrroles cross-links, 212–213

Q

QCT. *see* Quantitative computed tomography
Quantitative computed tomography
general description, 1574–1576
and other techniques, comparisons, 1580
peripheral, 1577–1578
serial measurements, 1593–1596
Quantitative ultrasonometry
advantages, 1292–1293
description, 1578–1580
identifying patients for, 1583
QUS. *see* Quantitative ultrasonometry

R

Rabbit models, 1642–1643
Radiography
absorptiometry
description, 1577–1578
and other techniques, comparisons, 1580
description, 1600–1604
Raloxifene
effects on
bone, 682–683
breast, 684
cardiovascular system, 683–684
uterus, 684–685

studies, 681
thrombeoembolism association, 685
RANK. *see* Receptor activator of nuclear factor κβ
RANKL. *see* Receptor activator of nuclear factor κβ ligand
Rat models, 1641–1642
Receptor activator of nuclear factor κβ
-RANKL interaction, discovery, 112–114
regulation, 1085
role in
MAH, 1086–1087
osteoclast apoptosis, 157–159
osteoclast formation, 693–695
role in MAH, 1086–1087
signal transduction mechanism, 118–121
-TGF-β, cross-communication between, 121
Receptor activator of nuclear factor κβ ligand
in bone, effects of PTH/PTHrP, 496–497
deficient mice, 115–116
description, 109
effects on osteoclasts, 114–115
identification, 1084–1085
mutations, activating, 116
-RANK interaction, discovery, 112–114
role in postmenopausal osteoporosis, 945–946
Remodeling, bone
animal models, 1638
at BMU level
activation, 307–309
formation, 309–310
local factors, 311
mineralization, 310–311
origination, 304–307
direct activation, 315–316
effects of
calcitonin, 1431
IDDM, 743–744
IGF, 810–811
IL-4, 965
insulin, 743–746
NIDDM, 744
pharmacological agents, 314–315
pseudohypoparathyroidism, 1151
thyroid hormones, 712
effects on bone structure, 311–313
marrow spaces and, 315
microcallus formation and, 315–316
in osteoporosis, 313–314
physiology, 304
role of osteoclast apoptosis, 157
woven bone *vs.,* 315
Reporter genes, types, 1511–1512
Reproductive system
effects of
calcitonin, 627
raloxifene, 684–685
SERMs, 682
tamoxifen, 685–686
VDR in, 594–595
Resorption, bone
associated cell-matrix interactions, 266
biopsy, 1628–1629
coupling to formation, 1277
critical functions rule, 12
cycle, osteoclast role
cytoskeletal changes, 132–133
degradation product removal, 135–136
life span, 128–129
membrane domains, 130–132
polarization, 130–132
sealing zone formation, 129–130
surface attachment, 129–130
effects of
androgens, 764–766
FGF, 838–839
glucocorticoids, 727–729
IL-4, 965
IL-7, 966
INF-γ, 964
OPN, 243–244
PTH/PTHrP, 499–500
TGFβ, 905
thyroid hormones, 711–712
TNF, 963–964
effects on prostaglandin receptors, 986
factors effecting, 1550–1551
IL-1 stimulation, 961–962
inhibition by bisphosphonates
mechanisms of action level, 1366–1367
models, 1362–1365
relative potency, 1365–1366
markers, 218–220, 1547–1549
in osteoporosis, 1276–1277
phases, 307–309
PTH-induced, role of OPN, 244–245
role of
brandykinin receptors, 778
cathepsin K, 216
integrins, 270–271
kinin receptors, 778–779
septoclasts, 127–128
tumor-induced, effects of bisphosphonates, 1365
Respiratory system, effects of calcitonin, 626–627
RET protooncogene
characterization, 1024, 1067
mutations
role in MEN2 syndrome
correlations, 1070–1071
mechanism, 1071–1072
role in neural development, 1072
Rheumatoid arthritis, 156, 894–895
Rickets
clinical features, 1196–1197
diagnosis, 1204
hereditary hypophosphatemic, 329–331
therapy, 1204–1205, 1411
vitamin D-dependent
type I, description, 1197–1198
type II
biochemical features, 1198–1200
cellular defects, 1200–1204
clinical features, 1198–1200
mode of inheritance, 1199–1200
molecular defects, 1200–1204

Rickets (*continued*)
occurrence, 1197–1198
X-linked hypophosphatemic, 326–329
X-linked recessive hypophosphatemic, 333
RNA, messenger
PTH degradation, 414
splicing, CTR proteins, 607–609
stability, transcriptional control *vs.,* 1507–1508
Rod conversion, 311–313
ROS 17/2.8 cells, 1504
Ruffled border, 130–132

S

Saos-2 cells, 1506
Sclerotome differentiation, 37
SEAP. *see* Secreted alkaline phosphatase
Seasonal variability, effects on bone turnover, 1551
Secreted alkaline phosphatase, 1512
Selectins
characterization, 269
role in osteoblast functions, 278
Selective estrogen receptor modulators
function, 666–667
mechanism of action, 677–681
physiologic effects on
bone, 681
breast, 682
cardiovascular system, 681–682
reproductive tract, 682
Sepsis, calcitonin-associated, 630–631, 633
Septoclasts, 127–128
Serine kinase
activation, 932–933
characterization, 929–932
intracellular signaling
in cytoplasm, 935–936
in nucleus, 936
regulation, 937–938
structure, 933–935
target genes, 936–937
SERMs. *see* Selective estrogen receptor modulators
Serotonin
receptors, effects on bone, 789
transporter, effects on bone, 789
Serum proteins, description, 232–233
Sheep models, 1643–1644
SIBLING. *see* Small integrin-binding ligand with N-linked glycosylation
Signal transduction
adhesion-dependent, in osteoclasts, 143–144
androgen receptors, 758–760
BMP, SMAD
in cytoplasm, 935–936
in nucleus, 936
regulation, 937–938
structure, 933–935
target genes, 936–937
CSF-1, 949
$\alpha_v\beta_3$-mediated, 274
mechanical, electric fields in, 1483
microdomains, 539
NO, 966–968
protein binding, 889–890
PTH-PTHrP
downstream activities, 428–429
dual intracellular, 427–428
function, 1137–1140
selective ligands, 429–430
PTHR in
Ca excretion, 487–488
Mg excretion, 487–488
Na/H excretion, 491–492
Ph excretion, 490–491
renal cells, 484
RANK, 118–121
$1\alpha,25(OH)_2$ vitamin D_3, 549–551
Silicon-bone, interaction, 365
Skeleton
adaptation
belief in, 1477
cellular basis, 1482–1483
effects of age, 1483–1484
effects of exercise, 1480–1483
exercise constraints, 1484–1485
role of electric fields, 1483
androgen mode of action, 1462
biology, endogenous gene disruption, 1498–1499
development
cellular divisions, 5–6
effects of connexins, 293
embryonic
axial, 34–38
chondrogenesis, coupling, 52–53
intramembranous bone, 42–52
limb initiation, 38–40
mesenchymal condensation, 40–42
morphology, 33–34
osteogenesis, coupling, 52–53
patterning, 40–42
role of BMP, 922–923
formation, 8–11
genes affecting, analysis, 1494–1496
molecular regulation, 11–12
role of cadherins, 288–289
studying, 12–13
effects of estrogens, 655–656, 658
growth, effects of IDDM, 741–743
hypophosphatasia, 1236
intercellular Ca signaling, 297–298
male
interventional studies, 1474–1475
observational studies, 1472–1473
malignancy (*see specific diseases)*
osteoporosis treatment response to, 1637–1638
response
curvilinear nature of, 1481–1482
to HRT, animal models, 1637
role of androgens, 1456–1457
thyroid hormones
animal studies *in vivo,* 712–713
cell phenotypic responses, 711–712

gene products, 709–711
membrane actions, 709
nuclear receptors, 707–709
tissues, role of CaR, 1355
Skin
effects of PTH/PTHrP, 515–517
lipoma, *MEN1* gene and, 1059–1060
previtamin D-3 photosynthesis in, 587
Small integrin-binding ligand with N-linked glycosylation, 231
Sodium
effects of CaR, 381
excretion, role of PTH/PTHR, 491–492
-phosphate cotransporter, 413
Somatic cells therapy, 74–76
Somatostatin
characterization, 787
MEN1 syndrome treatment, 1051
Somitogenesis, 34–37
Southwestern blot, 1515–1516
SPARC, description, 228–229
SRC. *see* Steroid receptor coactivators
Stem cells. *see* Meschymal stem cells
Steroid receptor coactivators, 580–581
Sterol synthesis, blocking, role of FFP, 1369–1371
Strain
curve, 21–22
minimum effective, 1480
rate of, 1481
-stress, relationship, 18–20
Strength, tensile, 22
Stress
definition, 18
fractures
fluoride-induced, 1389–1390
occurence, 28
mediating, role of OPN, 246–248
patterns, 19
relaxation, 23
standard unit, 18
-strain
curve, 21–22
relationship, 18–20
Stroke-fracture association, 791–792
Stromal cells
-osteoblasts
M-CSF production by, 111–112
role in
OPG expression, 116–118
osteoclast differentiation, 109–111
osteoclast regulation, 111–112
RANKL regulation, 116–118
Stromelysin, 252
Strontium-bone interaction, 365–366
Substance P, 786
Sunscreens, 588
Surgery, orthopedic, 920
Syndecans
characterization, 269
expression in
cartilage, 281
syndecans, 279

T

Tamoxifen
effects on
bone, 685
breast, 685–686
cardiovascular system, 685–686
reproductive tract, 682, 685–686
thrombeoembolism association, 687
Tartrate-resistant acid phosphatase
assays, 1536
expression, 109–110, 1495
levels in Paget's disease, 1554
levels in PHPT, 1557
as resorption marker, 1549
T cells, role in RANKL regulation, 118
Telopeptides, cross-linked, 1548–1549
Tensile strength, 22
Tension, definition, 18–19
Testosterone. *see* Androgens
Tetracycline labeling, 1615–1616
Tetranectin, 229
Thiazide, effects on bone turnover, 1550
Threonine kinase
activation, 932–933
characterization, 929–932
intracellular signaling
in cytoplasm, 935–936
in nucleus, 936
regulation, 937–938
structure, 933–935
target genes, 936–937
Thrombospondin, description, 230–231
Thymus, PTH gene expression, 408
Thyroid gland
medullary cancer, 629–630
PTH gene expression, 408
Thyroid hormones
bone loss and, 714–715
effects on
chondrocytes, 46
skeleton
animal studies *in vivo,* 712–713
cell phenotypic responses, 711–712
gene products, 709–711
in humans, 713–714
membrane actions, 709
nuclear receptors, 707–709
osteoporosis pathophysiology and, 1280
phosphate transport regulation, 324–326
role in collagen synthesis, 199–200
Tissue-nonspecific alkaline phosphatase
biology, 1231–1232
circulating, 1242
deficiency, 1237
description, 1231
discovery, 1229
function, hypophosphatasia as model, 1243
history, 1229–1231
physiological role, 1229–1231
structural defects, 1238

Tissues
cultures
current techniques, 1530–1531
in vitro systems, 1529–1530
engineering, 74
TNALP. *see* Tissue-nonspecific alkaline phosphatase
TNF-related activation-induced cytokine, 113
Toremifene, 687
Torsion effects, 19–20
Trabecular bone
connectivity, 1631
strength of, 23
volume, 1631
TRAF. *see* Tumor necrosis factor receptor-associated factors
TRANCE. *see* TNF-related activation-induced cytokine
Transcription factors
collagen genes
mode of action, 203
proximal promoter binding, 200–201
function, 69–71
in osteoblast differentiation, 85–88
PTH, 495
regulatory complexes
analysis, 1507–1508
cloned or purified, characterization, 1518–1519
cloning, 1515–1518
DNA-protein interaction analysis, 1512–1515
initiation by nuclear run-on assays, 1508
multiprotein, purification/sequencing, 1516–1517
osteoblast cell culture models, 1504, 1506–1507
promoter analysis
assay selection, 1509–1510
reporter genes, 1511–1512
transfection methods, 1510–1511
Transcytosis, 274–275
Transfection
biolistic, description, 1511
methods, 1510–1511
stable, assay selection, 1509–1510
transient, assay selection, 1509
Transforming growth factor α
effects on osteoclasts, 121
osteoporosis pathophysiology and, 1279
phosphate transport regulation, 323–326
Transforming growth factor β
binding protein, 911–912
in bone
effects of FGF, 837
effects of PTH/PTHrP, 495
characterization, 904–905
connexin regulation by, 293
effects on
bone cells *in vitro,* 905–906
chondrocytes, 47
osteoclast formation, 948–949
VEGF-A expression, 891–892
gene expression in bone
effects of glucocorticoids, 732–733
targeted disruption of, 904
isoforms, 903–904
latency, 909–911
-RANKL, cross-communication between, 121
receptors, 906–907
role in
BMD, 1266–1267
bone cancer metastasis, 911–912
collagen synthesis, 194–196
osteoblast apoptosis, 160
osteoblast development, 71–73, 72
signaling pathways, 908–909
superfamily, 903
Transgenes, tissue-directed, 1494–1496
Transilial bone biopsy
in clinical research, 1633
complications, 1617
histology, 1628–1629
morphometric methods, 1629, 1631
in patient care, 1632
practical considerations, 1633
procedure, 1615–1616
reference data, 1631–1632
regions evaluated, 1629, 1631
specimen processing, 1617
timing, 1633
Transmission disequilibrium test, 1262
Transplantation
heart, associated bone loss, 1561–1562
kidney, associated bone loss, 1562
as osteopetrosis therapy, 1224–1225
TRAP. *see* Tartrate-resistant acid phosphatase
T scores. *see* Desitometry
Tumor necrosis factors
effects on bone, 963–964
expression, 109
α
effects on VEGF-A expression, 891
osteoporosis pathophysiology and, 1278–1279
role in collagen synthesis, 197–198
in osteoblasts, effects of estrogen, 662–663
receptor-associated, 118–119
role in
HHM, 1094–1095
MAH, 1087–1088
osteoclast
apoptosis, 158–159
formation, 693–695
ovariectomy-induced bone loss, 699–702
β, in cancer cells, 1098
Tumors
bone disease, 1376–1377
extrathyroidal neuroendocrine, 630
nonneuroendocrine, 630
suppressor genes, 1022, 1024
Turnover, bone
effects of
GH, 1443–1446
IGF-I, 1447–1448
Mg, 350
raloxifene, 682–683
as fracture risk marker, 1296–1298
postmenopausal rate, animal model, 1637
Tyrosine kinase, 889

U

Ultrasound, BMD, 1292–1293
Urokinase-type plasminogen activator, 257
Uterus, effects of PTH/PTHrP, 523–524

V

Vascular endothelial growth factor
  in bone formation, effects of FGF, 837
  effects on
    bone, 893–896
    cartilage, 893–896
    osteoclast formation, 948–949
  ligand family
    alternative splicing, 884–885
    postranslation processing, 885
    structure, 885–886
    viral, 886
  receptors
    activation, 889–890
    expression, 891–892
    structure, 886
    types, 887–888
  type A
    activities, 889–891
    expression, 891–892
    mechanisms, 889–891
Vasculature
  cells, uniquness, 883
  PTH in, 473–475
  PTHrP in
    effects on
      anatomy, 532–533
      contractile mechanisms, 532–533
      smooth muscles, 534
    expression, 533–534
    mode of action, 535–536
  role in remodeling, 311
Vasoactive intestinal peptide
  characterization, 781–783
  clinical observations, 791–792
Vasodilation, VEGF-A-induced, 891
VEGF. *see* Vascular endothelial growth factor
Versican (PG-100), 226
Viral vectors, description, 1510–1511
Viscoelasticity, 23–24
Vitamin D
  absorption, genetic determinants, 1269
  binding proteins, receptor interactions, 1413–1415
  compounds
    activating enzymes, 1412–1413
    analogs, 1409, 1411, 1416
    binding proteins, 1413
    clinical applications, 1411–1412
    genomic mechanisms, unraveling, 1417–1418
    hepatic clearance, 1416
    modeling, 1418
    molecular mechanisms of action, 1416–1417
    prodrugs, 1408
    target cell catabolic enzymes, 1415–1416
  deficiency, animal models, 1205
  effects of osteocytes, 98–99
  effects on chondrocytes, 47
  endocrine system, 548–549
  enhancer domain, 178
  function, 1195–1196
  hereditary deficiencies (*see* Osteomalacia; Rickets)
  metabolism
    effects of
      glucocorticoids, 725–726
      Mg depletion, 348–349
      PTH, 492–493
      sunlight, 573–574, 590–591
    in type II rickets, 1199
  osteoporosis pathophysiology and, 1280–1281
  production, hereditary deficiency, 1197–1198
  pseudohypoparathyroidism therapy, 1154–1156
  related genes
    response elements, 577–579
    transcriptional regulation, 579–581
  role in
    collagen synthesis, 199
    FBHH, 1036
    MAH, 1087
Vitamin D_3
  analogs, 561–565
  as arthritis treatment, 598
  conformational flexibility, 545–548
  effects on
    bone, 574–575
    calcitriol levels, 1204
    differentiation, 576–577
    hormone secretion, 577
    immune system, 577
    intestine, 575
    kidney, 575–576
    parathyroid glands, 576
    proliferation, 576–577
  genomic mechanism of action, 574
  nuclear receptor
    characterization, 551–552
    dimerization, 552–553
    evidence for, 558–559
    hormone response element, 553–554
    knockout model, 556–558
    ligand binding, 554–555
    ligand shape, 559–561
    mutations, 556
    receptor structure, 555–556
  photosynthesis, 587–590
  posttranscritpional effects *in vitro,* 1202–1203
  role in
    malignancy-associated hypercalcemia, 1081
    PTH gene regulation, 409–412
    PTHrP repression, 1086–1087
  signal transduction pathways, 549–551
  as skin disease treatment, 595–597
  structure, 545
  transcriptional regulation by, 577–579
  transcriptional effects *in vitro,* 1202–1203

Vitamin D receptors
in cancer cells, 591–592
characterization, 551–552
compound interactions, 1413–1415
deficient
DNA binding, 1201
hormone binding, 1201
nuclear uptake, 1201
dimerization, 552–553
evidence for, 558–559
function, 1195
genes, role in BMD, 1264–1265
heterodimerization, 580
homodimerization, 580
hormone response element, 553–554
in immune system, 592–594
interacting proteins complex, 581
knockout model, 556–558
ligand binding, 554–555
ligand shape for, 559–561
mutations, 556
phamacologic actions, 595
phosphorylation, 579–580
physiolgic actions, 595
receptor structure, 555–556
regulation, 579
reproductive system, 594–595
-transcription machinery interaction, 580–581
Vitamin K, role in osteoclast apoptosis, 159
Vitronectin, 231

W

Water metabolism, 379–381
WHO. *see* World Health Organization
Wolff's law, 17, 99, 1477
World Health Organization
osteopenia definition, 1582
osteoporosis classification, 1573–1574
Wounding healing, 821
Woven bone, 315

X

X-linked hypophosphatemic rickets, 326–329
X-linked recessive hypophosphatemic rickets, 333
X-ray energy, 1612–1613

Y

Yield point, 21–22
Young's modulus, 21

Z

Zone of polar activity, 38, 40
ZPA. *see* Zone of polar activity

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SECOND EDITION

Volume 2

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Contents

VOLUME 2

Contributors xi
Preface to the Second Edition xix
Preface to the First Edition xxi

CHAPTER 48
Vascular Endothelial Growth Factors **883**
Shun-ichi Harada and Kenneth A. Thomas

CHAPTER 49
Transforming Growth Factor-β **903**
L. F. Boneward

CHAPTER 50
Bone Morphogenetic Proteins **919**
Vicki Rosen and John M. Wozney

CHAPTER 51
Bone Morphogenetic Protein Receptors and Actions **929**
Kohei Miyazono

CHAPTER 52
Colony-Stimulating Factors **943**
Willy Hofstetter and Matthew T. Gillespie

CHAPTER 53
Local Regulators of Bone: IL-1, TNF, Lymphotoxin, Interferon-γ, IL-8, IL-10, IL-4, the LIF/IL-6 Family, and Additional Cytokines **961**
Mark C. Horowitz and Joseph A. Lorenzo

CHAPTER 54
Prostaglandins and Bone Metabolism **979**
Carol C. Pilbeam, John R. Harrison, and Lawrence G. Raisz

CHAPTER 55
Nitric Oxide and Other Vasoactive Agents **995**
Lee D. K. Buttery, Lucia Mancini, Niloufar Moradi-Bidhendi, Meg C. O'Shaughnessy, Julia M. Polak, and Iain MacIntyre

PART II
MOLECULAR MECHANISIMS OF METABOLIC BONE DISEASES

CHAPTER 56
Molecular Basis of PTH Overexpression **1017**
Geoffrey N. Hendy and Andrew Arnold

CHAPTER 57
Familial Benign Hypocalciuric Hypercalcemia and Neonatal Primary Hyperparathyroidism **1031**
Ghada El-Hajj Fuleihan, Edward M. Brown, and Hunter Heath III

CHAPTER 58
Multiple Endocrine Neoplasia Type 1 1047
Maria Luisa Brandi, Cesare Bordi, Francesco Tonelli, Alberto Falchetti, and Stephen J. Marx

CHAPTER 59
The Role of the *RET* Protooncogene in Multiple Endocrine Neoplasia Type 2 1067
Robert F. Gagel and Gilbert J. Cote

CHAPTER 60
Systemic Factors in Skeletal Manifestations of Malignancy 1079
Janet E. Henderson, Richard Kremer, and David Goltzman

CHAPTER 61
Local Factors in Skeletal Malignancy 1093
Gregory R. Mundy, Toshiyuki Yoneda, Theresa A. Guise, and Babatunde Oyajobi

CHAPTER 62
Molecular Basis of PTH Underexpression 1105
R. V. Thakker

CHAPTER 63
Jansen's Metaphysical Chondrodysplasia and Blomstrand's Lethal Chondrodysplasia: Two Genetic Disorders Caused by PTH/PTHrP Receptor Mutations 1117
Harald Jüppner, Ernestina Schipani, and Caroline Silve

CHAPTER 64
Pseudohypoparathyroidism 1137
Michael A. Levine

CHAPTER 65
Other Skeletal Diseases Resulting from G Protein Defects: Fibrous Dysplasia of Bone and McCune–Albright Syndrome 1165
Lee S. Weinstein

CHAPTER 66
Osteogenesis Imperfecta 1177
David W. Rowe

CHAPTER 67
Hereditary Deficiencies in Vitamin D Action 1195
Uri A. Liberman

CHAPTER 68
Oncogenic Osteomalacia 1209
Kenneth W. Lyles

CHAPTER 69
Osteopetrosis 1217
L. Lyndon Key, Jr., and William L. Ries

CHAPTER 70
Hypophosphatasia: Nature's Window on Alkaline Phosphatase Function in Man 1229
Michael P. Whyte

CHAPTER 71
Paget's Disease of Bone 1249
Frederick R. Singer and G. David Roodman

CHAPTER 72
Genetic Determinants of Bone Mass and Osteoporotic Fracture 1259
Stuart H. Ralston

CHAPTER 73
Pathophysiology of Osteoporosis 1275
Gideon A. Rodan, Lawrence G. Raisz, and John P. Bilezikian

CHAPTER 74
Evaluation of Risk for Osteoporosis Fractures 1291
Patrick Garnero and Pierre D. Delmas

PART III
PHARMACOLOGICAL MECHANISMS OF THERAPEUTICS

CHAPTER 75
Parathyroid Hormone 1305
A. B. Hodsman, D. A. Hanley, P. H. Watson, and L. J. Fraher

CHAPTER 76
Calcium 1325
Robert P. Heaney

CHAPTER 77
Calcium Receptors as Novel Drug Targets 1339
Edward F. Nemeth

CHAPTER 78
Bisphosphonates: Mechanisms of Action 1361
Herbert Fleisch, Alfred Reszka, Gideon A. Rodan, and Michael Rogers

CHAPTER 79
Fluoride in Osteoporosis 1387
Johann D. Ringe

CHAPTER 80
The Pharmacology of Estrogens in Osteoporosis 1401
Robert Lindsay and Felicia Cosman

CHAPTER 81
Vitamin D and Analogs 1407
Glenville Jones

CHAPTER 82
Molecular and Clinical Pharmacology of Calcitonin 1423
Mone Zaidi, Angela M. Inzerillo, Bruce Troen, Baljit S. Moonga, Etsuko Abe, and Peter Burckhardt

CHAPTER 83
Growth Hormone and Insulin-like Growth Factor-I Treatment for Metabolic Bone Diseases 1441
Clifford J. Rosen

CHAPTER 84
Anabolic Steroid Effects on Bone in Women 1455
Azriel Schmidt, Shun-ichi Harada, and Gideon A. Rodan

CHAPTER 85
Estrogen Effects on Bone in the Male Skeleton 1467
John P. Bilezikian, Sundeep Khosla, and B. Lawrence Riggs

CHAPTER 86
Mechanisms of Exercise Effects on Bone 1477
Robert Marcus

PART IV
METHODS IN BONE RESEARCH

CHAPTER 87
Application of Transgenic Mice to Problems of Skeletal Biology 1491
Stephen Clark and David Rowe

CHAPTER 88
Use of Cultured Osteoblastic Cells to Identify and Characterize Transcriptional Regulatory Complexes 1503
Dwight A. Towler and Rene St. Arnaud

CHAPTER 89
Current Methodologic Issues in Cell and Tissue Culture 1529
Robert J. Majeska and Gloria A. Gronowicz

CHAPTER 90
Biochemical Markers of Bone Metabolism 1543
Markus J. Seibel, Richard Eastell, Caren M. Gundberg, Rosemary Hannon, and Huibert A. P. Pols

CHAPTER 91
Methods and Clinical Issues in Bone Densitometry and Quantitative Ultrasonometry 1573
Glen M. Blake and Ignac Fogelman

CHAPTER 92
Controversial Issues in Bone Densitometry 1587
Paul D. Miller

CHAPTER 93
Macro- and Microimaging of Bone Architecture 1599
Yebin Jiang, Jenny Zhao, and Harry K. Genant

CHAPTER 94
Transilial Bone Biopsy **1625**
Robert R. Recker and
M. Janet Barger-Lux

CHAPTER 95
Animal Models in Osteoporosis Research **1635**
Donald B. Kimmel

CHAPTER 96
Defining the Genetics of Osteoporosis: Using the Mouse to Understand Man **1657**
C. J. Rosen, L. R. Donahue, and W. G. Beamer

Index for Volumes 1 and 2 1667

VOLUME 1

Contributors xiii
Preface to the Second Edition xxi
Preface to the First Edition xxiii

PART I
BASIC PRINCIPLES

A. Cell Biology

CHAPTER 1
Structure and Development of the Skeleton **3**
Sandy C. Marks, Jr., and Paul R. Odgren

CHAPTER 2
Biomechanics of Bone **17**
Dennis M. Cullinane and Thomas A. Einhorn

CHAPTER 3
Embryonic Development of Bone and the Molecular Regulation of Intramembranous and Endochondral Bone Formation **33**
Andrew C. Karaplis

CHAPTER 4
Mesenchymal Stem Cells and Osteoblast Differentiation **59**
Jane E. Aubin and James T. Triffitt

CHAPTER 5
Transcriptional Control of Osteoblast Differentiation and Function **83**
Thorsten Schinke and Gerard Karsenty

CHAPTER 6
The Osteocyte **93**
P. J. Nijweide, E. H. Burger, and J. Klein-Nulend

CHAPTER 7
Cells of Bone: Osteoclast Generation **109**
Naoyuki Takahashi, Nobuyuki Udagawa,
Masamichi Takami, and Tatsuo Suda

CHAPTER 8
Osteoclast Function: Biology and Mechanisms **127**
Kalervo Väänänen and Haibo Zhao

CHAPTER 9
Integrin and Calcitonin Receptor Signaling in the Regulation of the Cytoskeleton and Function of Osteoclasts **141**
Le T. Duong, Archana Sanjay, William Horne,
Roland Baron, and Gideon A. Rodan

CHAPTER 10
Apoptosis in Bone Cells **151**
Brendan F. Boyce, Lianping Xing, Robert J. Jilka,
Teresita Bellido, Robert S. Weinstein,
A. Michael Parfitt, and Stavros C. Manolagas

CHAPTER 11
Involvement of Nuclear Architecture in Regulating Gene Expression in Bone Cells **169**
Gary S. Stein, Jane B. Lian, Martin Montecino,
André J. van Wijnen, Janet L. Stein,
Amjad Javed, and Kaleem Zaidi

B. Biochemistry

CHAPTER 12
Type I Collagen: Structure, Synthesis, and Regulation **189**
Jerome Rossert and Benoit de Crombrugghe

CHAPTER 13
Collagen Cross-Linking and Metabolism 211
Simon P. Robins and Jeffrey D. Brady

CHAPTER 14
Bone Matrix Proteoglycans and Glycoproteins 225
Pamela Gehron Robey

CHAPTER 15
Osteopontin 239
Masaki Noda and David T. Denhardt

CHAPTER 16
Bone Proteinases 251
Richard C. D'Alonzo, Nagarajan Selvamurugan, Stephen M. Krane, and Nicola C. Partridge

CHAPTER 17
Integrins and Other Cell Surface Attachment Molecules of Bone Cells 265
Michael A. Horton, Stephen A. Nesbitt, Jon H. Bennett, and Gudrun Stenbeck

CHAPTER 18
Intercellular Junctions and Cell–Cell Communication in Bone 287
Roberto Civitelli, Fernando Lecanda, Niklas R. Jørgensen, and Thomas H. Steinberg

C. Bone Remodeling and Mineral Homeostasis

CHAPTER 19
Histomorphometric Analysis of Bone Remodeling 303
Susan M. Ott

CHAPTER 20
Phosphorus Homeostasis and Related Disorders 321
Marc K. Drezner

CHAPTER 21
Magnesium Homeostasis 339
Robert K. Rude

CHAPTER 22
Metals in Bone: Aluminum, Boron, Cadmium, Chromium, Lead, Silicon, and Strontium 359
Felix Bronner

CHAPTER 23
Biology of the Extracellular Ca^{2+}-Sensing Receptor (CaR) 371
Edward M. Brown

D. The Hormones of Bone

CHAPTER 24
Receptors for Parathyroid Hormone (PTH) and PTH-Related Peptide 389
Thomas J. Gardella, Harald Jüppner, F. Richard Bringhurst, and John T. Potts, Jr.

CHAPTER 25
Parathyroid Hormone: Molecular Biology 407
Justin Silver, Tally Naveh-Many, and Henry M. Kronenberg

CHAPTER 26
Parathyroid Hormone–Receptor Interactions 423
Michael Chorev and Michael Rosenblatt

CHAPTER 27
Actions of Parathyroid Hormone 463
Janet M. Hock, Lorraine A. Fitzpatrick, and John Bilezikian

CHAPTER 28
Renal and Skeletal Actions of Parathyroid Hormone (PTH) and PTH-Related Protein 483
F. Richard Bringhurst and Gordon J. Strewler

CHAPTER 29
Physiological Actions of Parathyroid Hormone (PTH) and PTH-Related Protein: Epidermal, Mammary, Reproductive, and Pancreatic Tissues 515
John J. Wysolmerski, Andrew F. Stewart, and T. John Martin

CHAPTER 30
Vascular, Cardiovascular, and Neurological Actions of Parathyroid-Related Protein 531
Thomas L. Clemens and Arthur E. Broadus

CHAPTER 31
1α,25(OH)$_2$Vitamin D$_3$: Nuclear Receptor Structure (VDR) and Ligand Specificities for Genomic and Rapid Biological Responses 545
Anthony W. Norman

CHAPTER 32
Vitamin D Gene Regulation 573
Sylvia Christakos

CHAPTER 33
Photobiology and Noncalcemic Actions of Vitamin D 587
Michael F. Holick

CHAPTER 34
Structure and Molecular Biology of the Calcitonin Receptor 603
Deborah L. Galson and Steven R. Goldring

CHAPTER 35
Calcitonin Gene Family of Peptides: Structure, Molecular Biology, and Effects 619
Kenneth L. Becker, Beat Müller, Eric S. Nylén, Régis Cohen, Jon C. White, and Richard H. Snider, Jr.

CHAPTER 36
Amylin and Calcitonin Gene-Related Peptide 641
Ian R. Reid and Jill Cornish

E. Other Systemic Hormones That Influence Bone Metabolism

CHAPTER 37
Estrogens and Progestins 655
David Rickard, Steven A. Harris, Russell Turner, Sundeep Khosla, and Thomas C. Spelsburg

CHAPTER 38
Selective Estrogen Receptor Modulators 677
Douglas B. Muchmore and Geoffrey Greene

CHAPTER 39
Mechanisms of Estrogen Action in Bone 693
Roberto Pacifici

CHAPTER 40
Thyroid Hormone and Bone 707
Paula H. Stern

CHAPTER 41
Clinical and Basic Aspects of Glucocorticoid Action in Bone 723
Barbara E. Kream and Barbara P. Lukert

CHAPTER 42
Effects of Diabetes and Insulin on Bone Physiology 741
Johan Verhaeghe and Roger Bouillon

CHAPTER 43
Androgens: Receptor Expression and Steroid Action in Bone 757
Kristine M. Wiren and Eric S. Orwoll

CHAPTER 44
Kinins and Neuro-osteogenic Factors 773
Ulf H. Lerner and Pernilla Lundberg

F. Local Regulators

CHAPTER 45
The Role of Insulin-like Growth Factors and Binding Proteins in Bone Cell Biology 801
Cheryl A. Conover and Clifford Rosen

CHAPTER 46
Platelet-Derived Growth Factor and the Skeleton 817
Ernesto Canalis and Sheila Rydziel

CHAPTER 47
Fibroblast Growth Factor (FGF) and FGF Receptor Families in Bone 825
Marja M. Hurley, Pierre J. Marie, and Robert Z. Florkiewicz

Index for Volumes 1 and 2 853

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Preface to the Second Edition

The success of the first edition of *Principles of Bone Biology* clearly indicated that this text met an important need in our field. Well-worn copies (often with a cracked spine!) can be found on the shelves of bone biology research laboratories and offices throughout the world. We knew from the outset that undertaking the first edition would include a commitment to producing a second one. Advances in bone biology over the past five years have moved forward at a dizzying pace, clearly justifying the need for a second edition at this time. The elucidation of the molecular interactions between osteoblasts and osteoclasts is one of many examples documenting this point. Studies of animals in which critical genes have been deleted or over-expressed have produced some surprises and added still further complexity to what we have already recognized as an extremely complex regulatory system controlling the development and maintenance of skeletal structures. These and many other advances have provided the background for further development of effective therapeutic approaches to metabolic bone diseases.

In preparing the second edition, we have asked all authors to provide extensive revisions of their chapters. Additionally, the second edition features new authors who have written 10 new chapters. Some chapters from the first edition have been consolidated or otherwise reconfigured to keep the total number of chapters essentially the same as in the first edition. Although the number of chapters and their organizational structure has been retained, the extraordinary amount of new information has led to an increase in size of many of the chapters along with more extensive referencing. As a result, the substantially larger second edition is being published in two volumes. Each volume contains a full table of contents and full indexing to help the reader find specific information. The somewhat smaller individual volumes should be easier to handle and hold up better to the extensive use we expect from readers.

As was the case in the first edition, we asked our authors to meet a tight schedule so that the text would be as up-to-date as possible. We are indebted to our many authors who successfully met this challenge. The updated chapters as well as the new ones have, therefore, been written in such a way that the newest and most exciting breakthroughs in our field are still fresh. This task could not have been completed without the help of the staff at Academic Press. We acknowledge, in particular, Jasna Markovac and Mica Haley. They have been enormously helpful in all phases of this effort.

We have enjoyed very much the task of bringing this second edition to you. We trust that this second edition will be even more useful to you than the first. Enjoy the book!

John P. Bilezikian
Lawrence G. Raisz
Gideon A. Rodan

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Preface to the First Edition

The world of modern science is undergoing a number of spectacular events that are redefining our understanding of ourselves. As with any revolution, we should take stock of where we have been, where we are, and where we are going. Our special world of bone biology is participating in and taking advantage of the larger global revolution in modern science. Often with shocking but delightful suddenness, we are gaining new insights into difficult issues, discovering new concepts to explain old observations, developing new approaches to perennial mysteries, and applying novel technological advances from other fields to our own. The pace with which the bone world is advancing is impressive not only to the most ardent optimists, who did not expect so much so soon, but also to the more sober minded who, only several years ago, would have brushed off the notion that progress could come with such lightening speed.

The rationale for this book is rooted in the recognition of the revolution in bone biology. We need a new repository of knowledge, bringing us both to the core and to the edge of our universe. Our goal is to provide complete, truly up-to-date, and detailed coverage of this exciting and rapidly developing field. To achieve this, we assembled experts from all over the world and asked them to focus on the current state of knowledge and the prospects for new knowledge in their area of expertise. To this end, *Principles of Bone Biology* was conceived. It is designed to be useful to students who are becoming interested in the field and to young investigators at the graduate or postgraduate level who are beginning their research careers. It is also designed for more established scientists who want to keep up with the changing nature of our field, who want to mine this lode to enrich their own research programs, or who are changing their career direction. Finally, this book is written for anyone who simply strives for greater understanding of bone biology.

This book is intended to be comprehensive but readable. Each chapter is relatively brief. The charge to each author has been to limit size while giving the reader information so complete that it can be appreciated on its own, without necessary recourse to the entire volume. Nevertheless, the book is also designed with a logic that might compel someone to read on, and on, and on!

The framework of organization is fourfold. The first 53 chapters, in a section titled "Basic Principles," cover the cells themselves: the osteoblast, the osteoclast, and the osteocyte; how they are generated; how they act and interact; what turns them on; what turns them off; and how they die. In this section, also, the biochemistry of collagenous and noncollagenous bone proteins is covered. Newer understandings of calcium, phosphorus, and magnesium metabolism and the hormones that help to control them, namely, parathyroid hormone, vitamin D metabolites, calcitonin, and related molecules, are presented. A discussion of other systemic and local regulators of bone metabolism completes this section.

The second section of this book, "Molecular Mechanisms of Metabolic Bone Diseases," is specifically devoted to basic mechanisms of a variety of important bone diseases. The intention of these 17 chapters is not to describe the diseases in clinical, diagnostic, or therapeutic terms but rather to illustrate our current understanding of underlying mechanisms. The application of the new knowledge summarized in Part I to pathophysiological, pathogenetic, and molecular mechanisms of disease has relevance to the major metabolic bone disorders such as osteoporosis, primary hyperparathyroidism, and hypercalcemia of malignancy as well as to the more uncommon disorders such as familial benign hypocalciuric hypercalcemia, pseudohypoparathyroidism, and osteopetrosis.

The third section of this book, "Pharmacological Mechanisms of Therapeutics," addresses the great advances that have been made in elucidating how old and new drugs act to improve abnormalities in bone metabolism. Some of these drugs are indeed endogenous hormones that under

specified circumstances are useful therapies: estrogens, vitamin D, calcitonin, and parathyroid hormone are representative examples. Others agents such as the bisphosphonates, fluoride, and calcium are reviewed. Finally, agents with therapeutic potential but still in development such as calcimimetics, insulin-like growth factors, transforming growth factor, bone morphogenetic protein, and fibroblast growth factor are presented with a view to the future. The intent of this 12-chapter section is not to provide step-by-step "how-to" instructions for the clinical uses of these agents. Such prescribing information for established therapies is readily found in other texts. Rather, the underlying mechanisms by which these agents are currently believed to work is the central point of this section.

The fourth and final section of this book, "Methods in Bone Research," recognizes the revolution in investigative methodologies in our field. Those who want to know about the latest methods to clone genes, to knock genes out, to target genes, and to modify gene function by transfection and by transcriptional control will find relevant information in this section. In addition, the selection and characteristics of growth conditions for osteoblastic, osteoclastic, and stem cells; animal models of bone diseases; assay methodologies for bone formation and bone resorption and surrogate bone markers; and signal transduction pathways are all covered. Finally, the basic principles of bone densitometry and bone biopsies have both investigative and clinical relevance. This 15-chapter section is intended to be a useful reference for those who need access to basic information about these new research technologies.

The task of assembling a large number of international experts who would agree to work together to complete this ambitious project was formidable. Even more daunting was the notion that we would successfully coax, cajole, and otherwise persuade authors of 97 chapters to complete their tasks within a six-month period. For a book to be timely and still fresh, such a short time leash was necessary. We are indebted to all the authors for delivering their chapters on time.

Finally, such a monumental undertaking succeeds only with the aid of others who helped conceive the idea and to implement it. In particular, we are grateful to Jasna Markovac of Academic Press, who worked tirelessly with us to bring this exciting volume to you. We also want to thank Tari Paschall of Academic Press, who, with Jasna, helped to keep us on time and on the right course. We trust our work will be useful to you whoever you are and for whatever reason you have become attracted to this book and our field. Enjoy the book. We enjoyed editing it for you.

John B. Bilezikian
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CHAPTER 48

Vascular Endothelial Growth Factors

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Introduction

The evolution of complex animal life has depended on the development of many adaptations. One of the most apparent of these is a vascular system that allows cells within an organism to have continuous access to an adequate supply of oxygen and nutrients and a means by which potentially toxic by-products of metabolism can be removed. In addition, the vascular network provides an efficient means for the transport of hormones that coordinate rapid physiologic responses and of immune cells that help combat invading pathogens and remove cellular debris. Virtually all organs, including bone, contain a vascular system that develops during early embryogenesis. Even the few adult tissues that have either only a marginal or no vascular supply, such as hyaline cartilage and the cornea, were vascularized during their development, later becoming avascular by a controlled regression of many or all of the original vessels.

The vascular system is lined from the interior of the heart to the smallest capillaries by a unique cell type denoted the vascular endothelial cell. The stability of the vascular system is reflected by very low vascular endothelial cell proliferation rates corresponding to intervals of years between rounds of division for each cell. Despite this general mitotic quiescence, endothelial cells can respond rapidly and robustly to appropriate mitogenic stimuli. The growth of new blood vessels, or angiogenesis, is typically initiated by the migration of vascular endothelial cells from existing capillaries and smaller venules and is sustained by mitosis of these cells. In healthy adults, however, little spontaneous angiogenesis appears to occur, except during the female estrus cycle and to support tissue repair.

The molecular mechanisms by which growth, maintenance, and remodeling of the vascular system are controlled have been elucidated in increasing detail over the past two decades. Protein mitogens, including several members of the fibroblast growth factor family, have been described that can induce mitosis and migration of a variety of cells in culture, including vascular endothelial cells, and can promote blood vessel growth *in vivo.* However, in the late 1980s a protein growth factor was discovered that is a uniquely selective mitogen for vascular endothelial cells. It was identified and purified by several groups on the basis of two substantially different activities—the induction of vascular permeability *in vivo* and mitosis of cultured vascular endothelial cells—thereby giving rise to the names vascular permeability factor (VPF) and vascular endothelial growth factor (VEGF). This protein is not only a selective mitogen for vascular endothelial cells, but also is able to promote angiogenesis *in vivo.* An additional intriguing feature of VEGF is the induction of its expression by hypoxia, perhaps one of the earliest recognized inducers of physiologically appropriate angiogenesis.

Because the focus of this chapter and most current work on this protein centers on its control of vascular growth, the VEGF nomenclature will be used. We will provide an overview of this angiogenic protein and of several homologous family members and will describe recent progress in elucidating the biology of this group of protein growth factors, including their relevance to bone biology. Several other reviews on this topic have been published (Thomas, 1996; Ferrara and Davis-Smyth, 1997; Neufeld *et al.,* 1999; Larrivee and Karsan, 2000).

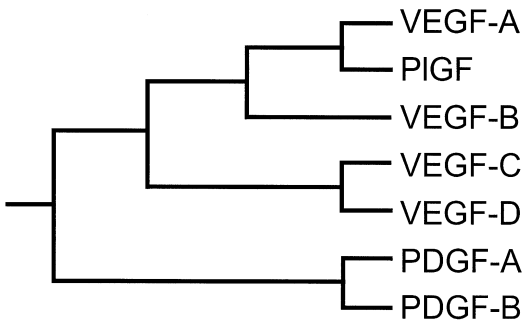


Figure 1 VEGF/PDGF dendritic tree. The degree of relatedness among PDGF and VEGF family members, inferred by their amino acid sequence homology, is shown schematically. VEGF family members are more closely related to each other than to either of the more distantly related PDGFs (Yamada *et al.,* 1997).

VEGF Ligand Family

Soon after the amino acid sequence of VEGF was established, it was recognized to be distantly related to platelet-derived growth factor (PDGF). Subsequently, four additional homologous VEGF family members, denoted placental growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D were identified. To distinguish the originally discovered VEGF from several other VEGF family members that were subsequently identified, it is now also denoted VEGF-A. On the basis of amino acid sequence homologies, the evolutionary relatedness of these proteins has been inferred (Yamada *et al.,* 1997), as illustrated in Fig. 1. Mapping of the VEGF genes (Vincenti *et al.,* 1996; Mattei *et al.,* 1996; Paavonen *et al.,* 1996; Yamada *et al.,* 1997) has shown them to be dispersed throughout the human genome with no two family members located on the same chromosome. Nevertheless, genomic cloning of each of the VEGF family members has revealed a generally similar exon/intron arrangement (Fig. 2).

Alternative Splicing

Alternative splicing of exons that encode polypeptides within the C-terminal regions of several of these proteins generates distinct isoforms. For example, the VEGF-A gene is composed of eight coding exons (Tischer *et al.,* 1991) with exons 3 and 4 encoding the core PDGF homologous receptor-binding region. Alternative mRNA splicing gives rise to three major and several minor polypeptide isoforms. Inclusion of all eight coding exons ($VEGF_{189}$), removal of exon 6 ($VEGF_{165}$), or deletion of exons 6 and 7 ($VEGF_{121}$) generate the principal isoforms. A less common form is generated by deletion of exon 7 ($VEGF_{145}$). Exons 6 and 7 encode polypeptide regions rich in positively charged basic amino acid residues that promote ionic binding to negatively charged heparan-containing proteoglycans. These

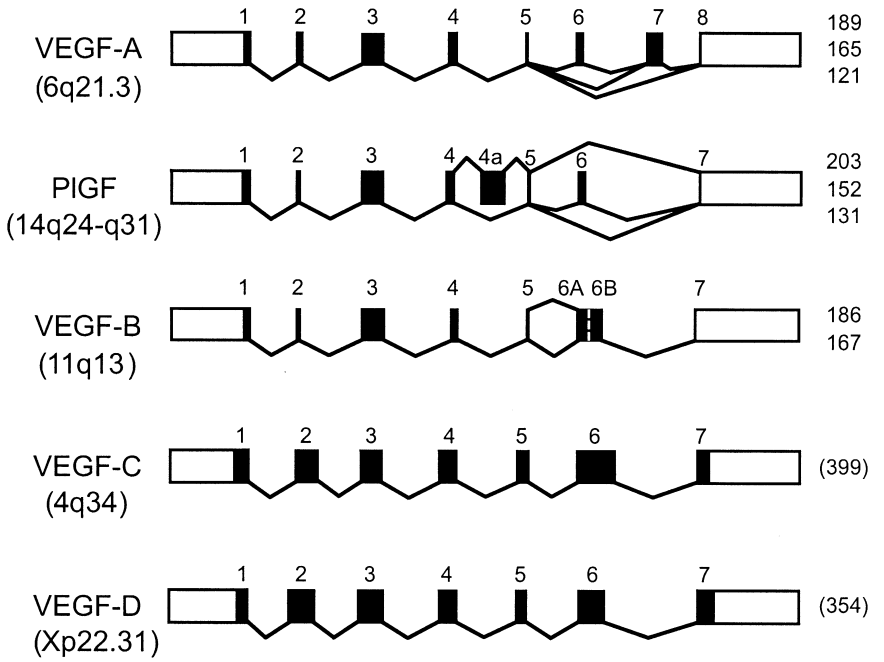


Figure 2 VEGF gene locations and structures. The human chromosomal location is listed in parentheses below the VEGF name. The exon/intron structure of the VEGF family members is shown. Exons are numbered sequentially and connected to represent alternative splicing. The mature amino acid lengths of the isoforms are listed on the right. VEGF-C and -D lengths listed in parentheses are full-length translation products because the secretory signal peptide lengths are not experimentally established and extensive and potentially heterogeneous posttranslation proteolytic modifications occur.

heparan proteoglycans are found on cell surfaces and within extracellular matrices, including basement membranes, upon which many types of cells reside, including vascular endothelial cells. $VEGF_{121}$, devoid of both cationic polypeptides inserts, does not bind to heparan proteoglycans. In contrast, $VEGF_{165}$ binds in a readily reversible manner to these sulfated oligosaccharides and $VEGF_{189}$ binds notably tighter. The heparan sulfate-binding forms can be released from matrix either by cleavage of the heparan oligosaccharide chains with heparanases or by proteolysis of the VEGF carboxy-terminal heparan-binding region by plasmin and perhaps other proteases (Houck *et al.,* 1992).

Somewhat different splicing events also generate three PlGF isoforms that, after removal of the 18 residue secretory leader sequence, contain 131, 152, and 203 amino acid residues (Fig. 2). Again, the shortest form is devoid of C-terminal heparin-binding sequences, whereas the 152 amino acid form contains a 21 residue cationic C-terminal region that promotes heparin binding (Maglione *et al.,* 1993). $PlGF_{203}$ is generated by inclusion of an exon encoding 72 amino acid residues but is devoid of the cationic polypeptide sequence that promotes binding of $PlGF_{152}$ to heparin (Cao *et al.,* 1997).

VEGF-B transcripts are also subject to alternative splicing (Fig. 2). Exon 6 can utilize two different splice acceptor sites (Grimmond *et al.,* 1996; Olofsson *et al.,* 1996). If the internal splice site is used then the 5′ end of this exon (exon 6A) is skipped, resulting in the translation of exons 6B and 7 to encode VEGF-B_{167}. Alternatively, if the splice acceptor at the 5′ end of exon 6 is used then translation proceeds through exon 6A and, in a different reading frame, into exon 6B. Translation is terminated by an in-frame stop codon within the alternative reading frame of exon 6B to generate VEGF-B_{186}. Although exon 7 is still transcribed, it is not translated. The C terminus of VEGF-B_{186} is not homologous to other VEGF sequences. The VEGF-C and VEGF-D genes each contain seven coding exons (Chilov *et al.,* 1997; Rocchigiani *et al.,* 1998). To date, no functional alternative splice forms have been described.

Posttranslational Processing

The primary translation products of all of the VEGF family members contain amino-terminal hydrophobic secretory leader sequences that are proteolytically removed upon secretion. The exact lengths of these leader sequences have not been determined for VEGF-C or -D. All of the VEGFs contain N-glycosylation sites and are secreted as glycoproteins. In addition to alternative splicing, posttranslational limited proteolysis can further modify some of the VEGF isoforms. For example, removal of the cationic C-terminal region from VEGF-A_{189} by urokinase can promote its binding to high-affinity receptors (Plouet *et al.,* 1997). The processing pathway of VEGF-C, shown in Fig. 3, involves the removal of N- and long C-terminal sequences (Joukov *et al.,* 1997) encoded by exons 5–7. Exon 6 generates a silk-like motif, and exons 5 and 7 encode Cys-rich sequences; the functions of these regions are unknown. As described later, receptor binding can be modulated by these proteolytic modifications. Similar proteolytic processing of VEGF-D (Stacker *et al.,* 1999) removes N- and Cys-rich C-terminal regions (Rocchigiani *et al.,* 1998).

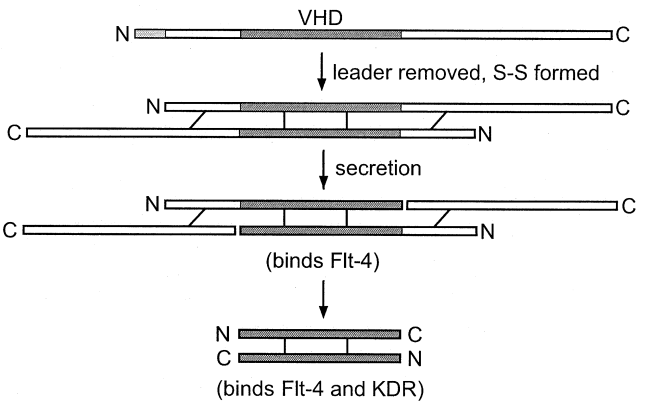


Figure 3 VEGF-C posttranslational processing. The N-terminal secretory leader region is shown in light gray and the VEGF homology domain (VHD) is denoted in dark gray. After secretion, the leader is removed proteolytically and interchain disulfides are made. The chains are cleaved near the C terminus of the VHD domains but the C-terminal extensions are still retained through intersubunit disulfide bonds. These dimers can bind Flt-4 but not KDR. A second set of proteolytic cleavages near the N termini of the two chains generates VEGF-C dimers consisting of the VHD receptor-binding domains that can bind Flt-4 and KDR (Enholm *et al.,* 1998).

VEGF Structure

All of the VEGF family members appear to function as dimers. The crystal structure of the core region of VEGF-A common to all of the receptor-binding isoforms has confirmed the similarity of its tertiary structure (Muller *et al.,* 1997a,b) with that determined previously for PDGF-B. As shown in Fig. 4 (see also color plate), each dimeric subunit is composed of an N-terminal helix that interacts principally with the adjacent subunit and is essential for dimerization (Siemeister *et al.,* 1998) and a central four-stranded β sheet. The subunits are arranged in a side-by-side antiparallel manner. The eight Cys residues within each subunit that are conserved among all VEGF and PDGF homologues participate in three intrasubunit and two intersubunit disulfide bonds. Disulfide bonds between subunits have also been identified in PlGF and VEGF-B (Olofsson *et al.,* 1996), although, surprisingly, nondisulfide-linked homodimers have been proposed to predominant in the mature fully processed forms of VEGF-C (Joukov *et al.,* 1997) and VEGF-D (Stacker *et al.,* 1999). In addition to homodimers, VEGF-A has been shown to form stable disulfide cross-linked heterodimers with PlGF (DiSalvo *et al.,* 1995), VEGF-B (Olofsson *et al.,* 1996), and VEGF-C (Joukov *et al.,* 1997). VEGF/PlGF heterodimers, identified in normal rhesus bronchial epithelial cells (Cao *et al.,* 1996), promote endothelial cell mitosis and migration and are angiogenic *in*

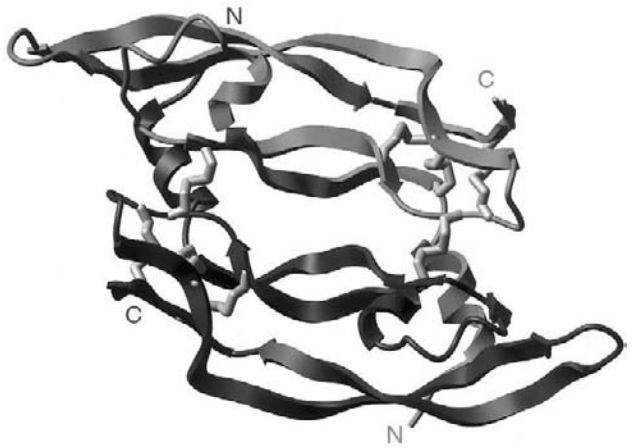


Figure 4 VEGF-A structure. The high-resolution crystal structure of the VEGF-A dimer, corresponding to the 121 isoform, shows the two subunits to be related by a twofold rotation axis perpendicular to the plane of the illustration. Each subunit is composed of four β strands and a single N-terminal α helix that interacts primarily with the adjacent subunit. Each subunit also has eight conserved Cys residues that form three intrachain disulfide bonds near one end of the subunit fold and two interchain disulfide bonds at opposite ends of the fold (Muller *et al.,* 1997a,b). (See also color plate.)

vivo (Cao *et al.,* 1996), albeit with lower specific activities than VEGF-A homodimers.

Viral VEGFs

Two virus-derived proteins have been identified that bind and activate VEGF receptors. The first, denoted VEGF-E, is a dimeric VEGF homologue encoded by an orf virus that normally infects sheep and, with much lower frequency, humans (Lyttle *et al.,* 1994). Infection is characterized by the appearance of highly vascularized edematous dermal lesions. The second is the HIV-1 tat protein (Albini *et al.,* 1996; Mitola *et al.,* 1997), also known as a transactivator of HIV and host genes. The selective advantage provided by the viral acquisition of these genes and retention of their ability to activate VEGF receptors has not been established experimentally.

VEGF Receptor Family

Three homologous transmembrane receptors, denoted Flt-1 (VEGFR-1), KDR/Flk-1 (VEGFR-2), and Flt-4 (VEGFR-3), have been identified that bind with high affinity (K_d ~10–400 p*M*) to different subsets of the known VEGFs (Fig. 5). The cellular responsiveness to different VEGFs is determined principally by the restricted expression of these receptors. Flt-1 is present on many types of endothelial cells, monocytes (Barleon *et al.,* 1996), and dendritic cell progenitors (Wu *et al.,* 1996). The expression of KDR is almost exclusively limited to vascular endothelial cells and their progenitors (Asahara *et al.,* 1997). Flt-4 is expressed primarily by lymphatic endothelial cells. However, during development it is expressed by a subset of

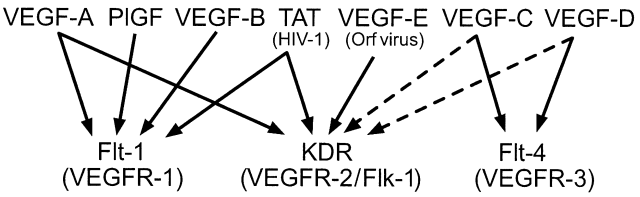
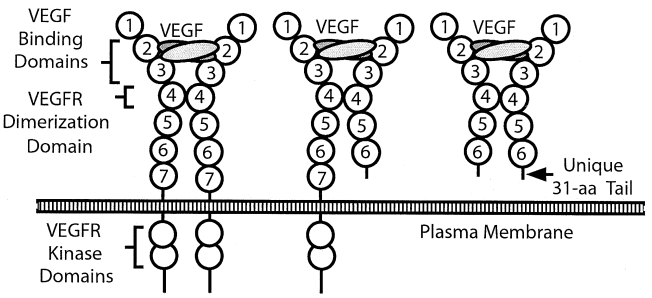


Figure 5 Ligand–receptor-binding selectivity. The seven VEGF ligand family members are listed along the top with arrows pointing to which of the three high-affinity tyrosine kinase receptors they each bind. Dotted arrows from VEGF-C and -D to KDR denote high-affinity binding only following full proteolytic processing. Tat and VEGF-E are HIV-1 and orf vial homologues.

venule vascular endothelial cells that differentiate into lymphatic endothelial cells (Kaipainen *et al.,* 1995).

Receptor Structure and Ligand Interaction

Flt-1, KDR, and Flt-4 have been mapped to human chromosomes 13q12 (Imbert *et al.,* 1994), 4q12 (Spritz *et al.,* 1994; Terman *et al.,* 1992), and 5q33-q35 (Armstrong *et al.,* 1993), respectively. The intron/exon structures of mouse Flt-1 (Kondo *et al.,* 1998) and human KDR have been determined to contain 30 and 28 exons (Yin *et al.,* 1998). Each approximately 1300 amino acid long 200-kDa receptor is composed of seven extracellular immunoglobulin (Ig)-like domains, a single short transmembrane-spanning polypeptide, and an intracellular tyrosine kinase (Fig. 6). The VEGF-binding regions of Flt-1 (Cunningham



	Chromosome	Length (aa)
Flt-1/VEGFR-1	13q12	1338
sFlt-1/sVEGFR-1	13q12	687
KDR/VEGFR-2	4q11-q13	1355
Flt-4/VEGFR-3	5q33-q35	1298

Figure 6 VEGF receptor–ligand complex schematic. As shown in the left most complex, the membrane-spanning tyrosine kinase receptors (Flt-1, KDR, and Flt-4) are composed of seven extracellular immunoglobulin (Ig)-like domains, a single transmembrane-spanning polypeptide, and an intracellular split kinase domain. VEGF binding has been mapped to Ig-like domains 2 and 3. The fourth Ig-like domains appear to interact directly with each other in a ligand-induced receptor dimer. A complex between the truncated soluble Flt-1 receptor and a full-length receptor is shown in the center. The sFlt-1 receptor can form stable homodimers and heterodimers with the corresponding extracellular region of KDR so are thought to be able to form dimers with full-length Flt-1 and KDR. Finally, a VEGF-stabilized dimer of sFlt-1 is shown on the right.

et al., 1997) and KDR (Fuh *et al.,* 1998) have been localized to extracellular Ig-like domains 2 and 3. Deletion of domain 2 in Flt-1 abolishes VEGF-A binding, which can be restored by insertion of domain 2 of KDR. The chimeric receptor exhibits the ligand-binding specificity of KDR rather than Flt-1. The Flt-4 receptor binds VEGF-C and -D with high affinity but not the other VEGF family members, including VEGF-A. Replacement either of the first three domains or of just domain 2 in Flt-4 with the corresponding Flt-1 domains generates chimeric receptors that can bind VEGF-A (Davis-Smyth *et al.,* 1996).

Ligand–Receptor Complex Structure

A crystal structure of VEGF-A bound to domain 2 of Flt-1 (Weismann *et al.,* 1997) has revealed their relative orientations, as illustrated in Fig. 7 (see also color plate), and details of the interaction surface. Both subunits within the VEGF-A homodimer interact with each Flt-1 domain 2 molecule through an interaction surface partitioned approximately 65 and 35% between the two ligand subunits. The interface between VEGF-A and receptor is predominately hydrophobic, as is typical of other protein–protein interactions. It also contains a single buried ion pair between a Flt-1 Arg and a VEGF-A Asp residue and 14 buried water molecules, 7 of which bridge the ligand and receptor. VEGF-A appears to undergo no major conformational change on complex formation. Because the approximately cylindrical VEGF-A dimer can bind a receptor at each end, it promotes Flt-1 receptor dimerization, which is also stabilized by direct contacts between Ig-like domain 4 (Barleon *et al.,* 1997). Similar interactions could occur within KDR and Flt-4 homodimers and Flt-1/KDR heterodimers (Kendall *et al.,* 1996).

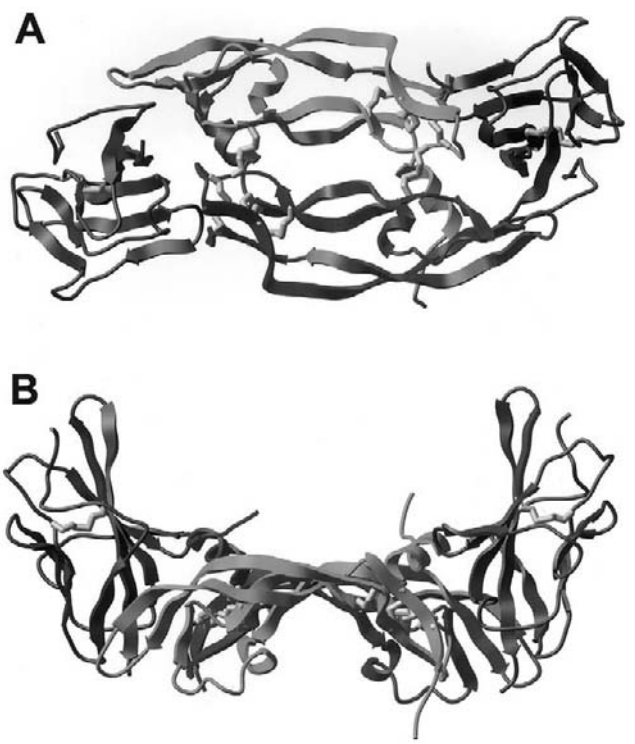


Figure 7 Structure of a complex between VEGF-A and domain 2 of Flt-1. (A) The backbone conformation determined by crystal structure analysis shows the interaction between the ends of the VEGF-A dimer subunits (red and blue) with domain 2 of Flt-1 (green). Both VEGF-A subunits interact with each receptor domain. Additional interactions are thought to occur with domain 3. (B) The same complex rotated 90° (Weismann *et al.,* 1997). (See also color plate.)

Soluble Receptor

A naturally occurring truncated soluble form of the Flt-1 receptor, sFlt-1, contains the N-terminal six Ig-like domains but is devoid of the membrane proximal Ig-like domain, transmembrane polypeptide, and intracellular kinase, all of which are replaced by a unique 31 amino acid sequence (Fig. 6). The soluble receptor is generated by alternative splicing of the Flt-1 primary transcript in which 31 codons of a retained intron are translated through to the first in-frame stop codon (Kendall and Thomas, 1993). It binds VEGF-A, PlGF (Kendall *et al.,* 1994), and presumably VEGF-B and HIV-1 tat with high-affinity characteristic of full-length Flt-1 but, because it is not able to initiate signal transduction, functions as an inhibitor. The sFlt-1 receptor forms VEGF-stabilized homodimers in a manner presumably equivalent to the interactions that stabilize dimerization of the extracellular regions of the full-length Flt-1 receptor. In addition, sFlt-1 can form VEGF-stabilized heterodimers with the corresponding extracellular region of KDR (Kendall *et al.,* 1996). The ability of these extracellular regions to dimerize infers that (1) dimerization of sFlt-1 with either full-length Flt-1 or KDR can abrogate receptor activation in a dominant-negative manner by preventing receptor kinase transphosphorylation as described in the following section on receptor activation and (2) heterodimers can also form between full-length Flt-1 and KDR in a manner analogous to that established to exist between PDGF receptor α and β. Stable expression of sFlt-1 by tumor cells can inhibit their angiogenesis-dependent growth as xenografts *in vivo* (Goldman *et al.,* 1998) consistent with its inhibitory activity *in vitro.*

Additional Receptors

In addition to high-affinity receptors, heparin-binding VEGF isoforms can bind to at least two additional types of lower affinity receptors. Neuropilin-1 (NP-1) is a 130-kDa membrane-bound protein containing a short intracellular tail that can mediate cell adhesion and bind the chemorepellant semaphorin-3A in neuronal cells. NP-1 is also expressed on vascular endothelial cells and has been found to bind the heparin-binding isoforms VEGF-A_{165}, $PlGF_{152}$, VEGF-B_{167}, and VEGF-E, but not the nonheparin-binding isoforms VEGF-A_{121}, $PlGF_{131}$, and $PlGF_{203}$ (Soker *et al.,* 1998; Makinen *et al.,* 1999; Migdal *et al.,* 1998; Wise *et al.,* 1999). The NP-1 receptor enhances the binding of

VEGF-A_{165} to KDR so appears to facilitate its function (Soker *et al.,* 1998). A natural 90-kDa soluble NP-1, generated like sFlt-1 by translation of a retained intron through to the first in-frame stop codon within an alternatively spliced mRNA, is expressed and presumably antagonizes the function of NP-1 (Gagnon *et al.,* 2000). The homologous NP-2, which binds semaphorins-C and -3F, has also been shown to bind VEGF-A_{165} and, in contrast to NP-1, VEGF-A_{145} (Gluzman-Poltorak *et al.,* 2000).

The identity of the heparan sulfate proteoglycans that can bind to the polycationic regions of VEGFs are not yet well characterized. However, one such heparan proteoglycan has been identified as the glycosylphosphatidylinositol-anchored glypican-1 (Gengrinovitch *et al.,* 1999), the only glypican known to be expressed in the vascular system. As expected, it binds VEGF-A_{165} but not VEGF-A_{121}.

The biologic relevance of the differential binding of VEGF isoforms to neuropilins and heparan proteoglycans is not entirely clear. They could increase the apparent potency of isoforms of VEGF to which they bind by partitioning them to the cell surface, thereby increasing their local concentration in the vicinity of the high-affinity receptor. In addition, they could participate in ternary complexes with VEGF ligands and high-affinity receptors. The affinity of VEGF-A_{165} and, to an even greater degree, VEGF-A_{189} for heparan proteoglycans in extracellular matrices, including basement membranes, could contribute to their localization within the vicinity where they are expressed. Another possible function of the binding of the longer forms of VEGF to heparan proteoglycans might be to sequester them to extracellular matrices in a form that can later be mobilized rapidly by lysis with either proteoglycanases or proteases (Houck *et al.,* 1992). The more readily diffusible VEGF-A_{121} might contribute to establishing chemotactic gradients for endothelial cells and, if cleared more rapidly, might provide transient stimulation.

Receptor Activation

The binding of VEGF to its high-affinity receptors can increase receptor tyrosine kinase catalytic activity and trigger the activation of signal transduction proteins by binding to receptor phosphotyrosine residues.

Tyrosine Kinase Activity

Ligand-mediated dimerization of high-affinity transmembrane growth factor receptors brings the intracellular tyrosine kinase domains into close proximity where they can effectively transphosphorylate each other on specific tyrosine residues. The consequence of this receptor transphosphorylation has been shown to increase the effective activity of the kinase and to trigger the binding of downstream signal transduction proteins. The details of VEGF receptor kinase structure and function are becoming available. A crystal structure of a modified form of the KDR kinase, in which 50 of 68 amino acid residues in the long "kinase insert loop" were removed to facilitate crystallization, has been determined (McTigue *et al.,* 1999). Like other receptor tyrosine kinases, it is formed of two domains with the active site in between them as shown in Fig. 8 (see also color plate). The amino-terminal domain consists of a twisted five-strand antiparallel β sheet and one α helix, whereas the larger C-terminal domain contains two antiparallel β strands and seven helices. Several functionally important loops between elements of secondary structure are present near the catalytic site. The flexible activation loop contains two tyrosine residues at positions 1054 and 1059 that, in the unphosphorylated state, are thought to interfere with substrate binding. Once phosphorylated, the analogous loops of related tyrosine kinases change conformation, perhaps mediated by the interaction of the phosphotyrosine side chains with other surface residues, thereby facilitating the access of ATP and peptide substrates. Phosphorylation of the two tyrosines in this KDR loop increase substrate binding as reflected by lower K_M values, resulting in greater potential catalytic turnover. In contrast, K_{cat}, the intrinsic catalytic activity at saturating substrate concentrations, does not appear to change (Kendall *et al.,* 1999).

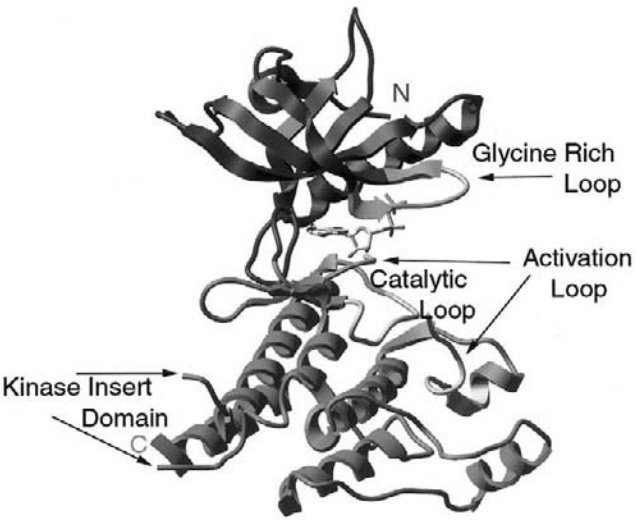


Figure 8 Structure of KDR tyrosine kinase domains. The backbone conformation of the split intracellular tyrosine kinase domain, determined by X-ray crystal structure analysis, is shown with the amino- and carboxy-terminal halves in blue and red, respectively. The amino-terminal half is largely composed of a β sheet with a single long α helix, whereas the C-terminal half consists of mainly α helical structure. ATP- and polypeptide-binding sites are near the interface of these N- and C-terminal folding domains. The glycine-rich, activation and kinase insert loops are shown in yellow, black, and green, respectively. The activation loop contains tyrosine residues 1054 and 1059 that upon autophosphorylation are thought to increase access of substrates to their binding sites. Most of the kinase insert loop was removed to facilitate crystallization so its conformation is largely unknown (McTigue *et al.,* 1999). (See also color plate.)

Signal Transduction Protein Binding

In related kinases, phosphorylated tyrosine residues have been identified not only in activation loops, but also in juxtamembrane regions, kinase insert loops, and sequences C-terminal of the kinase domains. These phosphorylated tyrosine residues can contribute to signal transduction by serving as binding sites for other signal transduction proteins that can themselves become phosphorylated and mediate one or more downstream signaling pathways. In addition to the two phosphorylated tyrosine residues in the activation loop of KDR, two more at positions 951 and 996 (Dougher-Vermazen *et al.,* 1994) in the kinase insert loop and one at position 1175 in the C-terminal region (Igarashi *et al.,* 1998) have been identified and several others likely exist. VEGF-induced phosphorylation of KDR results in the association of signaling adapter proteins, kinases, and phosphatases at several phosphorylated KDR sites. The downstream sequence of steps in signaling has not been fully elucidated and, in fact, several pathway branches corresponding to different biological activities, such as mitosis, survival, migration, vasodilation, permeability, and induction of extracellular matrix degradation, might exist (reviewed in Petrova *et al.,* 1999; Larrivee and Karsan, 2000).

VEGF-A Activities and Mechanisms

The angiogenic activity of VEGF-A has been the focus of much of the effort to understand its biologic activity *in vivo.* VEGF-A is active in animal models of angiogenesis and in peripheral (Baumgartner *et al.,* 1998) and cardiac ischemia (Pearlman *et al.,* 1995). In addition, VEGF has been shown to drive pathologic angiogenesis in neovascular ocular diseases, some types of inflammation, and a wide range of cancers.

The genetics of VEGF-A and its receptors demonstrate their importance in normal development. Not only homozygous but also heterozygous gene knockouts are embryonically lethal in mice (Carmeliet *et al.,* 1996; Ferrara *et al.,* 1996). The phenotypes show a noticeable deficit in vascularization in heterozygous mice that is amplified proportionally in homozygotes. This very unusual lethal heterozygous phenotype demonstrates the importance of appropriate VEGF levels for viable embryonic development. Conversely, transgenic expression of VEGF-A under the control of either the keratin K6 or rhodopsin gene regulatory regions results in hypervascular development in the skin (Larcher *et al.,* 1998) and retina (Okamoto *et al.,* 1997), respectively.

Homozygous, but not heterozygous, gene knockouts of the VEGF-A receptors KDR (Shalaby *et al.,* 1995) and Flt-1 (Fong *et al.,* 1995) are each embryonically lethal but with substantially different phenotypes. KDR mouse knockouts exhibit a virtual absence of vascular endothelial cells. In contrast, endothelial cell-lined vessels are present in Flt-1 knockout mice but appear disorganized. These mice have an increased number of endothelial cell progenitors, thought to be the consequence of an increased commitment to hemangioblast generation that leads to altered vessel pattern formation (Fong *et al.,* 1999). Surprisingly, mice with a homozygous knockout of just the Flt-1 kinase region develop normal vessels and survive. However, they do exhibit strongly suppressed macrophage migration (Hiratsuka *et al.,* 1998) consistent with the known chemotaxic response to VEGF-A and PlGF mediated by Flt-1 (Barleon *et al.,* 1996).

VEGF-A can directly induce the principal activities in the cascade of events culminating in vessel growth, including endothelial cell mitogenesis, survival, adhesion, and migration. In addition, VEGF-A can induce vasodilation and vascular permeability that might contribute to vascular tone and barrier functions. Although the details of how VEGF-A induces these responses are not completely defined, several aspects of the signaling pathways have been identified.

Mitogenesis

Vascular endothelial cell mitogenic activity is mediated predominantly, if not solely, through the KDR receptor, which is almost exclusively expressed on vascular endothelial cells. VEGF induces maximal tyrosine phosphorylation of KDR after 5 min of exposure followed by receptor downregulation, reflecting internalization (Esser *et al.,* 1998). Several growth factors induce mitogenesis by a receptor-mediated cytoplasmic signal transduction cascade that starts with receptor autophosphorylation, which promotes binding of the adapter protein Shc through its SH2 phosphotyrosine-binding domain. Shc then binds a second adapter Grb2 that complexes with SOS, a protein that promotes the release of GDP from inactive Ras and its subsequent binding and activation by GTP. Activated Ras can bind and activate the Ser/Thr kinase Raf that then phosphorylates and activates MEK, a dual kinase that phosphorylates MAPK/ERK on both Thr and Tyr residues. Upon activation, MAPK translocates into the nucleus where it modulates transcriptional events required for the initiation of cell division.

Shc can bind to phosphotyrosine 1175 on activated KDR (Warner *et al.,* 2000), become phosphorylated on Tyr-317, and bind the Grb2-SOS complex (Kroll and Waltenberger, 1997). The Shc-like kinase Sck can also bind to KDR phosphotyrosine 1175 (Igarashi *et al.,* 1998) and might function in a similar manner to that proposed for Shc. Although the level of GTP-bound Ras is increased and the Raf-MEK-MAPK pathway is activated in endothelial cells in response to VEGF-A, surprisingly a dominant-negative Ras has been reported not to block VEGF-induced phosphorylation of MAPK. However, inhibitors of protein kinase C (PKC) do significantly reduce the MEK activation of MAPK and subsequent DNA synthesis (Takahashi *et al.,* 1999).

The Raf-MEK-MAPK downstream pathway could be activated by an alternate upstream pathway starting with the activation of the c-Src tyrosine kinase upon binding to an unidentified phosphotyrosine on KDR and the binding of phospholipase Cγ (PLCγ) through the VEGF-receptor-associated protein (VRAP), an adapter protein that binds through its central SH2 domain to KDR phosphotyrosine 951 (Wu *et al.,* 2000b). PLCγ is then activated by tyrosine phosphorylation that occurs, at least in part, by KDR-activated c-Src (He *et al.,* 1999). Activated PLCγ hydrolyzes the membrane-anchored phosphatidylinositol 4,5-bisphosphate (PIP_2), generating water-soluble inositol 1,4,5-trisphosphate (IP_3) and the membrane product diacylglycerol (DAG). IP_3 binds and activates an IP_3-gated Ca^{2+} release channel in the endoplasmic reticulum, resulting in the release of Ca^{2+} that, either directly or through promotion of calmodulin binding to target proteins, can modulate multiple cellular processes. Elevated Ca^{2+} can also alter PKC so that it translocated to the membrane where it is activated by binding to DAG. Exactly how PKC activates Raf is not yet established.

Ca^{2+} released by PLCγ-generated IP_3 can also activate endothelial cell nitric oxide synthase (eNOS) (He *et al.,* 1999) at least, in part, by promoting calmodulin binding to it. Nitric oxide (NO) can activate guanylate cyclase, thereby generating cGMP that can activate protein kinase G (PKG) by binding to four sites on its regulatory subunit. The Ser/Thr PKG can in turn bind and activate Raf that, as described earlier, stimulates the MEK-MAPK mitogenic pathway (Hood and Granger, 1998; Parenti *et al.,* 1998). In this manner, VEGF-stimulated growth of endothelial cells is also dependent on NO generated by eNOS.

KDR might also be able to initiate negative feedback control of its activation. In response to VEGF-A stimulation, GAP, a protein that decreases Ras activity by accelerating the hydrolysis of Ras-bound GTP to GDP, also becomes phosphorylated and complexes either directly or indirectly to KDR (Guo *et al.,* 1995). In addition, the tyrosine phosphatases SHP-1 and SHP-2 associate with phosphorylated KDR presumably through their SH2 domains. Tumor necrosis factor (TNF), which diminishes VEGF-stimulated KDR activity, appears to do so by promoting the association of SHP-1 with KDR (Guo *et al.,* 2000).

Cell Survival

VEGF-A promotes the survival of vascular endothelial cells under a variety of stresses, such as serum starvation in culture (Gerber *et al.,* 1998). This survival activity is also mediated through KDR. The regulatory subunit of phosphatidylinositol 3-kinase (PI3K), an enzyme that phosphorylates phosphatidylinositols at the D3 position of the inositol ring, has two SH2 domains that can bind directly to receptor phosphotyrosine residues and becomes phosphorylated in response to VEGF, which allows it to recruit the PI3K catalytic subunit (Thakker *et al.,* 1999). In addition, the KDR adapter protein VRAP can bind constitutively to PI3K (Wu *et al.,* 2000). The 3-phosphoinositol product of VEGF-activated PI3K can bind and activate PKB/Akt, a Ser/Thr kinase that inhibits the activity of caspases, proteases that mediate programmed cell death or apoptosis. Akt is also activated by PI3K-dependent phosphorylation possibly mediated through the 3-phosphoinositol-mediated activation of another kinase. Therefore, VEGF binding to KDR induces both proliferation and survival. Inhibition of survival signals can initiate the apoptosis of vascular endothelial cells, leading to vascular regression.

Adhesion and Migration

VEGF-A promotes adhesion of endothelial cells to extracellular matrix components by several integrins, including the vitronectin-binding $\alpha v\beta 5$ and $\alpha v\beta 3$ integrins (Byzova *et al.,* 2000; Friedlander *et al.,* 1995; Watanabe and Dvorak, 1997). Phosphorylated KDR forms complexes either directly or indirectly with the $\beta 3$ integrin subunit (Soldi *et al.,* 1999) and activates $\alpha v\beta 3$ ligand binding by an activated KDR-dependent mechanism involving PI3K and Akt (Byzova *et al.,* 2000). VEGF induces increased endothelial cell expression of the $\alpha 1\beta 1$ and $\alpha 2\beta 1$ collagen (Senger *et al.,* 1997) and $\alpha v\beta 3$ vitronectin (Senger *et al.,* 1996)-binding integrins and the intracellular adhesion molecule ICAM-1 (Radisavljevic *et al.,* 2000). VEGF also induces the phosphorylation of focal matrix adhesion kinase and the focal adhesion-associated component paxillin (Abedi and Zachary, 1997; Wu *et al.,* 2000a) and promotes actin reorganization (Morales-Ruiz *et al.,* 2000).

The growth of new capillaries requires vascular endothelial cell migration, which depends on the modulation of matrix attachment, intercellular adhesion, and cytoskeletal proteins. VEGF increases the migration of vascular endothelial cells probably initiated through its activation of KDR, as the KDR-specific ligand VEGF-E promotes endothelial cell migration (Meyer *et al.,* 1999). Within 15 to 60 min of VEGF-induced KDR phosphorylation, the cellular adherens junction components VE-cadherin, β-catenin, plakoglobulin, and p120 become tyrosine phosphorylated, perhaps loosening cell–cell contacts to facilitate not only mitosis but also migration and possibly permeability (Esser *et al.,* 1998). VEGF also promotes endothelial cell migration by activating $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha 5\beta 1$, and the collagen-binding $\alpha 2\beta 1$ integrins (Byzova *et al.,* 2000). Although the details of the migration signaling pathway are not yet clear, it appears to require active PI3K (Byzova *et al.,* 2000), Akt, and eNOS (Morales-Ruiz *et al.,* 2000; Radisavljevic *et al.,* 2000). Activated Akt phosphorylates eNOS Ser-1177, which appears to be necessary and, in fact, sufficient to induce endothelial cell migration (Dimmeler *et al.,* 2000). NO has been shown to promote spontaneous oscillations in matrix adhesion that, in the presence of a gradient of a chemotactic factor such as VEGF-A, could

facilitate directed migration (Eisei *et al.,* 1998). VEGF-A, PlGF, and HIV-1 Tat can also promote the migration of monocytes. Although these cells are devoid of KDR, they do have Flt-1 (Barleon *et al.,* 1996) to which these ligands bind and can induce phosphorylation (Gruber *et al.,* 1995; Mitola *et al.,* 1997).

Vasodilation

VEGF-A induces NO-dependent vasodilation *in vivo* that contributes to the control of vascular tone. The receptor responsible for this response might not be KDR. An antibody that specifically activates KDR but not Flt-1 was reported to elicit an angiogenic but not a vasodilatory response (Malavaud *et al.,* 1997). The NO activation pathway appears to be similar, at least in part, to that contributing to mitogenesis (Papapetropoulos *et al.,* 1997). The activation of Akt also leads to the production and release of the potent vasodilator nitric oxide. Endothelial NO formation activates smooth muscle guanylate cyclase, causing cGMP production. VEGF also leads to the activation of phospholipase A_2, resulting in a three- to four-fold increase of the vasodilator prostacyclin (PGI_2) within 45 to 60 min (He *et al.,* 1999).

Vascular Permeability

As described previously, VEGF-A was originally identified in tumor ascites fluid as vascular permeability factor (VPF) on the basis of its activity in a guinea pig vascular dye extravasation assay (Senger *et al.,* 1983). Increased permeability promotes the leakage of fibrinogen and other plasma proteins that are proposed to provide a matrix that facilitates the migration of endothelial cells (Dvorak *et al.,* 1995). NO also appears to function in the signaling pathway mediating permeability by the activation of guanylyl cyclase to produce cGMP, which activates PKG (Wu *et al.,* 1996). NO donors and prostacyclin analogs administered together but not alone increase VEGF-induced permeability, whereas the cyclooxygenase (COX) inhibitor indomethacin inhibits VEGF-induced vascular permeability, thus implicating prostaglandin metabolites as contributors to this activity (Murohara *et al.,* 1998).

Expression of VEGFs and VEGFRs

The primary cellular source of VEGF-A appears to be cells other than those of the vascular endothelium. This paracrine expression of VEGF allows various organs and tissues to control their vascularization and is under the control of a variety of mediators, including other growth factors and cytokines, oncogenes, hormones, metabolites, and, notably, hypoxia. Induction of transcription and stabilization of mRNA can each contribute to increased VEGF-A protein expression.

Growth Factors and Cytokines

VEGF-A expression can be increased by the mitogenic growth factors PDGF (Brogi *et al.,* 1994; Mukhopadhyay *et al.,* 1995; Stavri *et al.,* 1995), bFGF (Koochekpour *et al.,* 1995), FGF-7/KGF (Frank *et al.,* 1995), EGF (Sone *et al.,* 1996), heregulin β1 (Yen *et al.,* 2000), and IGF-1 (Warren *et al.,* 1996) in a variety of normal and malignant cultured cells. It is also induced by TGF-β1 (Pertovaara *et al.,* 1994), TNFα (Frank *et al.,* 1995), IL-1β (Li *et al.,* 1995), and IL-6 (Cohen *et al.,* 1996). In the case of PDGF, induction of expression has been mapped to GC-rich Sp1 recognition sites within the VEGF-A promoter (Finkenzeller *et al.,* 1997). The ability of these factors to induce VEGF-A expression could explain reported angiogenic activities of some of them that do not directly stimulate endothelial cell mitosis.

Oncogenes

Several downstream signal transduction-related oncogenes have also been shown to induce VEGF-A expression. Mutant K-Ras (Okada *et al.,* 1998), H-Ras (Rak *et al.,* 1995), and v-Raf (Grugel *et al.,* 1995) each induce VEGF-A expression in different transformed tumor cells that presumably contribute to tumor angiogenesis. Wild-type p53, p73, the von Hippel-Lindau (VHL) suppressor gene, and Smad4 can each inhibit VEGF-A promoter activity. Inactivating p53 mutations increase VEGF-A expression (Mukhopadhyay *et al.,* 1995) and also diminish expression of the antiangiogenic protein thrombospondin (Volpert *et al.,* 1997). Similarly, silencing of the p53-like p73 gene by hypermethylation in some leukemias and lymphomas can eliminate its ability to repress VEGF-A promoter function (Salimath *et al.,* 2000). Loss of function of the VHL tumor suppressor protein that normally represses VEGF-A transcription by directly binding the promoter-binding Sp1 transcription factor results in increased VEGF expression (Mukhopadhyay *et al.,* 1997). Deletion of Smad4, a protein in the TGF-β1 signal transduction pathway that is lost at high frequency in pancreatic and gastrointestinal cancers, results in increased VEGF-A expression and diminished expression of thrombospondin (Schwarte-Waldhoff *et al.,* 2000).

Hormones and Metabolites

Hormones, including estrogen (Hyder *et al.,* 1996), testosterone (Joseph *et al.,* 1997; Ruohola *et al.,* 1999), and thyroid-stimulating hormone (Kanji *et al.,* 1995), can each stimulate VEGF-A expression. Progesterone and several synthetic progestins used in oral contraceptives also increase VEGF expression, whereas the antiprogestin RU-486 blocks induction by progesterone (Hyder *et al.,* 1998). VEGF-A transcription but not stabilization is also induced by the prostaglandin PGE_2 (Harada *et al.,*

1994). COX-2, an inducible cyclooxygenase that converts arachidonate to PGH_2, the substrate for a set of prostaglandin synthases, is overexpressed in benign polyps and colon cancers (Williams *et al.,* 1999). COX-2 inhibitors have been shown to retard tumor growth in mice. Fibroblasts from COX-2 homozygous knockout mice exhibit a greater than 90% reduction in their ability to produce VEGF-A, and decreased vascular density was observed in tumors grown in these mice (Williams *et al.,* 2000).

Advanced glycation end products (AGE) generated by the nonenzymatic covalent attachment of glucose to proteins, a diagnostic indicator of diabetes, also increase VEGF-A expression. AGE bovine serum albumin (BSA), but not nonglycated BSA, induces VEGF-A expression in a dose-dependent manner (Lu *et al.,* 1998; Yamagishi *et al.,* 1997). Glucose deprivation has also been shown to increase VEGF-A expression (Shosuke *et al.,* 1998; Shweiki *et al.,* 1995) and mRNA stabilization (Stein *et al.,* 1995). Therefore, lack of glucose control leading either to elevated or to reduced levels can result in the induction of VEGF-A expression. For example, AGEs are elevated in the ocular vitreous in proliferative diabetic retinopathy (Hirata *et al.,* 1997) and increase VEGF expression within the retina (Lu *et al.,* 1998). VEGF-A appears to be the primary angiogenic agent supporting retinal and choroidal neovascularization and diabetic retinopathy (Aiello *et al.,* 1994) and, perhaps, age-related macular degeneration (Kliffen *et al.,* 1997).

Hypoxia

Perhaps the most intriguing inducer of VEGF-A expression is hypoxia (Shweiki *et al.,* 1992). Decreased pO_2 has been long recognized to induce compensatory neovascular growth. Cultured cells can increase VEGF-A production from 10- to 50-fold by lowering the O_2 from 21 to 3% or less. Although detectable VEGF-A is not typically expressed by endothelial cells, it can be induced in hypoxia (Namiki *et al.,* 1995; Nomura *et al.,* 1995). Induction of VEGF expression is also seen in ischemic normal and cancerous tissues *in vivo.* Hypoxia is now known to increase VEGF-A production both by inducing transcription (Finkenzeller *et al.,* 1995; Levy *et al.,* 1995; Liu *et al.,* 1995) and by stabilizing its mRNA (Ikeda *et al.,* 1995; Shima *et al.,* 1995; White *et al.,* 1995) from a half-life of 30–45 min under normoxia to 6–8 hr under hypoxia (2% O_2). Induction of transcription presumably occurs through activation of 5′ DNA promoters and enhancers, whereas stability elements appear to reside in the 3′-untranslated region of VEGF-A mRNA. A functional 47-bp transcriptional, hypoxia-inducing factor-1 (HIF-1)-binding site is located nearly 1 kb 5′ of the VEGF-A transcriptional initiation site (Forsythe *et al.,* 1996). Hypoxia-stabilizing elements are located in the 3′-untranslated region (UTR) (Levy *et al.,* 1996b) within which are found three AU-rich sequences that form hypoxia-inducible RNA–protein complexes (Claffey *et al.,* 1998; Levy *et al.,* 1996a) containing the HuR protein (Levy *et al.,* 1998).

Other VEGF Family Members

Less is known of the functions of the more recently identified VEGF family members. Although none of these mammalian homologues appear to be as critical to the induction and maintenance of the vasculature as VEGF-A, they might be equally important for other crucial biologic activities.

PlGF and VEGF-B

The biologic function of the PlGF, which binds selectively to Flt-1, is not yet clear. PlGF is highly expressed not only in placenta (Cao *et al.,* 1997; Maglione *et al.,* 1993), but also in vascular endothelial cells (Yonekura *et al.,* 1999). PlGF typically exhibits little, if any, vascular endothelial cell mitogenic activity (Birkenhager *et al.,* 1996; DiSalvo *et al.,* 1995) consistent with the Flt-1 knockout phenotype. The function of the Flt-1 receptor is also somewhat enigmatic. Although it binds VEGF-A with even higher affinity than KDR, it does not appear to efficiently mediate a significant mitogenic response in most *in vitro* endothelial cell assays. The Flt-1-specific ligand PlGF has been observed to increase VEGF mitogenic activity *in vitro* so this receptor has been proposed to function as a decoy, sequestering low concentrations of VEGF that might otherwise stimulate KDR inappropriately (Park *et al.,* 1994). However, chimeric CSF-1 extracellular/VEGFR intracellular receptor transfectants provide a somewhat different interpretation. An intracellular KDR chimera mediates proliferation whereas a Flt-1 chimera does not. The Flt-1 chimera inhibited the activity of a cotransfected KDR chimera. Therefore, the cytoplasmic regions of Flt-1 appear to antagonize KDR responses (Rahimi *et al.,* 2000).

PlGF acting through Flt-1 can induce the migration of monocytes (Barleon *et al.,* 1996) and endothelial cell expression of procoagulant tissue factor (Clauss *et al.,* 1996). A chimeric Flt-1 in which the juxtamembrane region is replaced by the corresponding sequence from KDR confers endothelial cell migration but not proliferation activity (Gille *et al.,* 2000). VEGF-A/PlGF heterodimers, identified in tumor cells (DiSalvo *et al.,* 1995) and rhesus brochus epithelial cells (Cao *et al.,* 1996), can induce migratory and mitogenic activity in endothelial cells, albeit with lower specific activities than VEGF-A homodimers, and are angiogenic *in vivo* (Birkenhager *et al.,* 1996). Perhaps these heterodimers function by promoting the formation of KDR/Flt-1 receptor heterodimers. VEGF-B, also acting through Flt-1, can induce the expression of endothelial cell urokinase and plasminogen activator inhibitor-I, suggesting that it might have a role in the regulation of extracellular matrix degradation, cell adhesion, and migration (Olofsson *et al.,* 1998).

VEGF-C and VEGF-D

VEGF-C binds and activates lymphatic endothelial cell Flt-4 (Joukov *et al.,* 1996; Lee *et al.,* 1996) and drives lymphatic endothelial cell mitosis (Fitz *et al.,* 1997). The lymphatic system is involved primarily in the drainage of interstitial fluid, immune function, and inflammation. VEGF-C is present in T cells, lymphocytes, and platelets (Wartiovaara *et al.,* 1998). Although VEGF-C transcription is not induced by hypoxia, it can be increased by PDGF, EGF, and TGF-β (Enholm *et al.,* 1997) and by the proinflammatory cytokines IL-1α, IL-1β, and TNFα (Ristimak *et al.,* 1998). Overexpression of VEGF-C in the skin of transgenic mice causes lymphatic, but not vascular, endothelial cell proliferation and vessel enlargement (Jeltsch *et al.,* 1997). A homozygous mouse Flt-4 gene knockout is embryonically lethal with defective blood vessel development and fluid accumulation in the pericardial cavity (Dumont *et al.,* 1998). Primary human lymphoedema, a rare genetic disorder leading to swelling of the extremities, is correlated with mutations that inactivate the tyrosine kinase activity of Flt-4 (Karkkainen *et al.,* 2000). VEGF-C can acquire KDR-binding activity (Joukov *et al.,* 1996), along with corresponding functional increases in endothelial cell migration, proliferation, and vascular permeability (Joukov *et al.,* 1997) by the proteolytic removal of N- and C-terminal regions to generate a core VEGF receptor-binding homology domain similar to that illustrated in Fig. 4. The close homologue VEGF-D (Orlandini *et al.,* 1996), expressed in greatest abundance in lung, also binds to Flt-4 and can be activated proteolytically to bind KDR (Stacker *et al.,* 1999). Therefore, undefined proteolytic activation mechanisms can confer vascular angiogenic activity on these lymphangiogenic agents.

VEGF and Bone/Cartilage

Angiogenesis and Chondrogenesis/Osteogenesis

Both intramembranous and endochondral bone formations are associated with the formation of blood vessels, which leads to the recruitment of hematopoietic osteoclast–precursor cells as well as perivascular osteoblast–progenitor cells. Intramembraneous ossification is characterized by capillary ingrowth into a mesenchymal condensation of embryonic connective tissue cells, followed by differentiation of these mesenchymal cells into osteoblasts that secrete collagen and noncollagenous matrix proteins, resulting in mineralized bone matrix termed woven bone, which is then remodeled into lamellar bone.

Endochondral Osteogenesis

Although it has been assumed that angiogenesis is essential for osteogenesis, until recently the functional role of angiogenesis in osteogenesis, as well as molecular mechanisms involved in this process, was not well understood. In contrast to intramembranous ossification, which takes place mainly during development, endochondral bone formation occurs in development as well as in pathological osteogenesis such as fracture repair and ectopic ossification. Angiogenesis has been implicated in endochondral bone formation, where avascular cartilage is replaced by bone, thus becoming one of the most highly vascularized tissues. Endochondral osteogenesis is initiated by the condensation of mesenchymal cells that later differentiate into chondrocytes, which secrete cartilaginous matrix and form cartilage. As the cartilage area enlarges through cell proliferation and matrix deposition, chondrocytes become further differentiated. Compression and hypertrophy lead to calcified cartilage and apoptotic cell death. One of the earliest events that leads to replacement of calcified cartilage by mineralized bone matrix is the invasion of blood vessels from the metaphysis into calcified cartilage, resulting in the recruitment of cells involved in resorption of the calcified cartilage and deposition of bone matrix. Angiogenesis has also been implicated in pathological osteogenesis processes such as fracture repair (Ferguson *et al.,* 1999; Glowacki, 1998), ossification of articular cartilage (Fenwick *et al.,* 1999), distraction osteogenesis (Jazrawi *et al.,* 1998; Li *et al.,* 1999), and ectopic bone formation (Harada *et al.,* 1995).

A number of angiogenic factors are expressed in bone and cartilage, including FGFs (De Luca and Baron, 1999), members of the bone morphogenetic protein (BMP)/growth and differentiation factor (GDF) family (Bailon Plaza *et al.,* 1999; Carey and Liu, 1995; Sakou *et al.,* 1999), TGF-β (Serra *et al.,* 1999), IGF-I (Shinar *et al.,* 1993), EGF, PDGF, and CYR61 (Babic *et al.,* 1998). In addition, a number of antiangiogenic factors are also present in cartilage, such as thrombospondins, chondromodulin-I (Hiraki *et al.,* 1997), and developmental endothelial locus-1 (Hidai *et al.,* 1998), implicated in the avascular nature of cartilage. However, the role of these factors in angiogenesis in bone and cartilage is not clear.

Expression of VEGF-A in skeletal tissue and bone cells was first demonstrated *in vitro* in osteoblastic cells and *in vivo* in RNA isolated from bone tissue (Harada *et al.,* 1994). Abundant expression of VEGF-A mRNA and its protein were documented histologically in hypertrophic chondrocytes of adult rat tibiae, adult mouse tibiae (Gerber *et al.,* 1999), avian growth plate (Carlevaro *et al.,* 2000), and fetal human growth plate (Horner *et al.,* 1999). VEGF-A was also expressed in hypertrophic chondrocytes during fracture repair in mice (Ferguson *et al.,* 1999), as well as during ectopic osteogenesis in rats (Harada *et al.,* 1995), but was not observed in resting or proliferating chondrocytes. Expression of VEGF-A was also observed during chondrocyte differentiation *in vitro* in avian chondrocytes (Carlevaro *et al.,* 2000) and in rat chondrocytic cells. These expression patterns of VEGF-A, in combination with the well-documented role of VEGF and its receptors in angiogenesis, strongly implicate VEGF-A in angiogenesis and the blood vessel invasion, essential for endochondral ossification. A number of VEGF antagonists and anti-

bodies are being developed toward the suppression of tumor angiogenesis. One such protein, a chimera containing the three N-terminal extracellular Ig-like domains of soluble Flt-1 fused to an Fc fragment of IgG, was used in mice to address the role of VEGF in endochondral ossification (Gerber *et al.,* 1999; Harper and Klagsbrun, 1999). Treatment of 24-day-old mice with the soluble Flt-1 IgG chimera resulted in marked expansion of the hypertrophic chondrocyte zone and in disorganized blood vessels that failed to invade this region. Neutralization of VEGF action by soluble Flt-1 IgG also results in decreased trabecular bone formation and shortening of the femora, indicating the role of VEGF in endochondral ossification. However, the expression of genes associated with chondrocyte differentiation, i.e., type II and type X collagen, was not altered in these mice, suggesting that the expansion of the hypertrophic chondrocyte zone resulted from the increased life span of terminally differentiated chondrocytes rather than an alteration in the proliferation or maturation of chondrocytes (Gerber *et al.,* 1999; Harper and Klagsbrun, 1999).

The phenotype of the growth plate treated with soluble Flt-1 IgG resembles that of mice with a null mutation of MMP-9/gelatinase B, i.e., expansion due to delayed chondrocyte apoptosis and delayed vascularization and ossification (Vu *et al.,* 1998). MMP-9/gelatinase B is a matrix metalloproteinase highly expressed in TRAP-positive osteoclasts and chondroclasts. A similar growth plate phenotype has also been reported in mice with null mutation of MT1-MMP (Zhou *et al.,* 2000), supporting an essential role for these MMPs in the resorption of calcified cartilage, in neovascularization of calcified cartilage, and in endochondral ossification. In addition, conditional inactivation of VEGF-A alleles in chondrocytes was carried out using the Cre/loxP system in mice expressing Cre-recombinase under the control of the human type II collagen promoter (Haigh *et al.,* 2000). While most mice with deletion of a single VEGF-A allele in Cre-expressing cells died in uterus due to aberrant development of the dorsal aorta, few mice survived and developed an increased hypertrophic zone of the growth plate (Haigh *et al.,* 2000). A similar growth plate phenotype has also been reported in mice exclusively expressing VEGF-A_{120} (mouse isoforms are one amino acid residue shorter than the corresponding human forms) as a result of Cre/loxP-mediated deletion of exons 6 and 7, which encode VEGF-A_{164} and VEGF-A_{188} (Carmeliet *et al.,* 1999). Unlike VEGF-A (-/-) mice, these mice survive until the perinatal period and develop ischemic cardiomyopathy and a growth plate phenotype (Maes *et al.,* 2000).

Taken together, these findings indicate that VEGF-A, expressed in hypertrophic chondrocytes, plays an essential role in endochondral ossification, capillary invasion, chondrocyte apoptosis, and resorption of calcified cartilage by chondroclasts, which also require MMPs expression (Fig. 9).

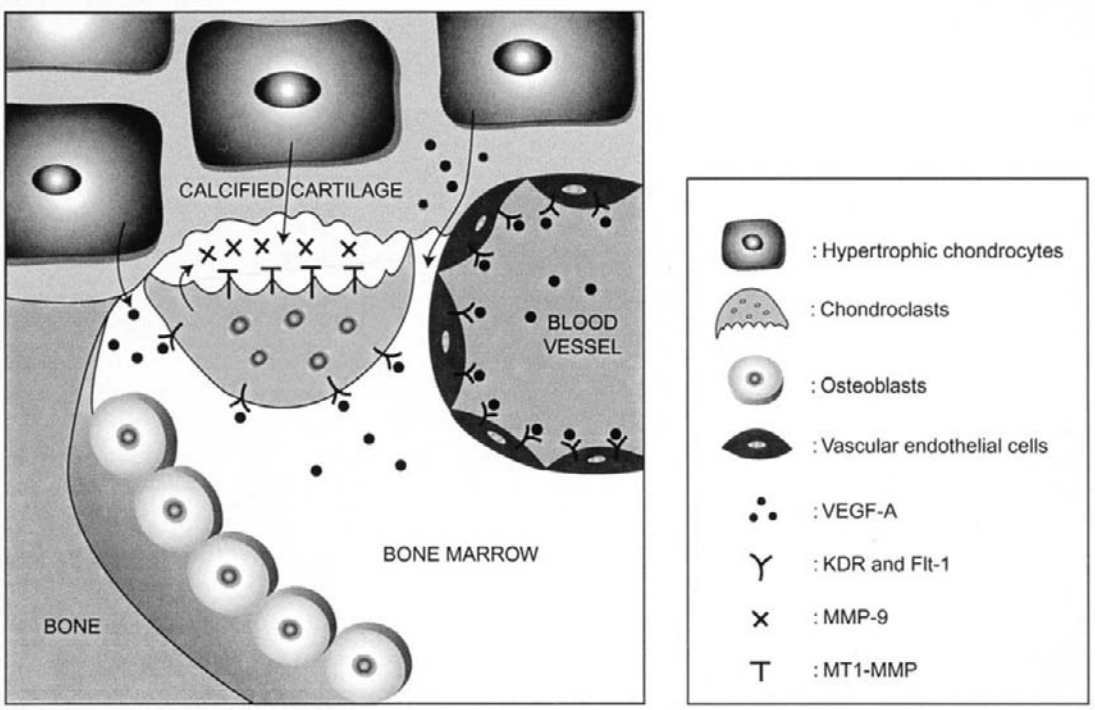


Figure 9 Role of VEGF-A in endochondral osteogenesis. VEGF-A released from hypertrophic chondrocytes induces capillary invasion to calcified cartilage, leading to the recruitment of chondroclasts that resorb calcified cartilage and osteoblasts that generate mineralized bone matrix. These actions of VEGF-A are also required for the death of hypertrophic chondrocytes. MMP-9 and MT1-MMP expressed in chondroclasts are essential for resorption of calcified cartilage as well as for capillary invasion and apoptosis of hypertrophic chondrocytes. VEGF-A may act directly on chondroclasts (and osteoclasts) to induce differentiation, survival, and migration.

Osteoblasts

In contrast to the well-documented role of VEGF-A expression in hypertrophic chondrocytes, a functional role for VEGF-A expression in osteoblasts has not yet been established. VEGF-A mRNA and protein are expressed *in vitro* in primary calvarial osteoblasts and in osteoblastic cell lines (Goad *et al.,* 1996; Harada *et al.,* 1994; Schlaeppi *et al.,* 1997; Steinbrech *et al.,* 1999; Wang *et al.,* 1996). Its expression is upregulated by prostaglandins E_2 and E_1, both inducers of osteogenesis, and is suppressed by dexamethasone, a negative regulator of osteogenesis (Harada *et al.,* 1994). In mouse and human osteoblastic cell lines, VEGF-A mRNA and its protein levels are also induced by other osteogenic factors, such as IGF-I (Goad *et al.,* 1996), TGF-β (Chua *et al.,* 2000; Saadeh *et al.,* 1999), 1,25(OH)$2D_3$ (Schlaeppi *et al.,* 1997; Wang *et al.,* 1996, 1997), endothelin-1 (Kozawa *et al.,* 2000), and bone morphogenetic proteins (Harada *et al.,* 1995; Yeh and Lee, 1999). Expression of VEGF-A is also upregulated by hypoxia via induction of hypoxia-inducible factor-1a, which could also mediate the upregulation of VEGF-A expression by IGF-I (Steinbrech *et al.,* 1999, 2000).

However, in contrast to these *in vitro* observations, analysis of VEGF expression *in vivo* in bone tissue failed to detect high levels of VEGF-A expression in osteoblasts. An experiment using a tail suspension hind limb unloading rat model showed that unloading results in reduction of VEGF expression and blood vessel number and area in tibiae (Barou *et al.,* 2000). Interestingly, mechanical loading rapidly induced angiogenesis (Jazrawi *et al.,* 1998) and the expression of VEGF-A mRNA in a distraction model in rats (unpublished information). Expression of VEGF-A mRNA in adult rat tibia diaphysis is induced rapidly by systemic treatment with PGE_2 (Machwate *et al.,* 1995), implicating the possible mediation of PGE_2 in this effect. Taken together, these observations suggest a possible role for VEGF-A and angiogenesis in bone metabolism changes induced by mechanical stress. Further detailed analyses of VEGF-A expression *in vivo* in osteoblasts, following application of these stimuli, as well as functional studies *in vivo* in animals, using tissue-selective gene targeting or neutralization of VEGF-A, are required to determine the role of VEGF-A and of angiogenesis in mechanical stress-induced bone metabolism and in bone remodeling (Parfitt, 2000).

Osteoclasts

There is increasing evidence for a role of VEGF-signaling pathways in hematopoietic cells. Targeted gene disruption of KDR indicates its potential role in hematopoiesis in addition to vasculogenesis (Shalaby *et al.,* 1997). Detailed *in vitro* analysis documented KDR as a marker for human postnatal hematopoietic cells (Ziegler *et al.,* 1999). In addition, monocyte/macrophage lineage cells are shown to express Flt-1, which could play a role in chemotactic responses (Barleon *et al.,* 1996; Clauss *et al.,* 1996). Expression of neurotropin-1, known to interact with $VEGF_{165}$ but not $VEGF_{121}$, was also reported in bone marrow stromal cells that support hematopoiesis (Tordjman *et al.,* 1999). A report in op/op osteopetrotic mice suggests a novel role for VEGF in osteoclasts derived from hematopoietic precursor cells (Niida *et al.,* 1999). op/op mice develop osteopetrosis due to the absence of the macrophage colony-stimulating factor (M-CSF) essential for osteoclastogenesis and for the generation of monocytes/macrophages (Yoshida *et al.,* 1990). The osteopetrotic phenotype of these mice is found only during their youth (rescuable by a single injection of M-CSF), suggesting the possible compensation by other growth factors or cytokines (Begg *et al.,* 1993). An injection of VEGF-A was found to result in osteoclast differentiation and osteopetrosis rescue in op/op mice (Niida *et al.,* 1999). In addition, neutralization of VEGF-A with either the soluble Flt-1–IgG chimera or the anti-VEGF-A antibody results in loss of osteoclasts in adult op/op mice or in young op/op mice after a single injection of M-CSF. Furthermore, in bone marrow cell cultures, VEGF-A, in combination with RANK ligand, induces the formation of osteoclast-like cells and their survival, indicating that VEGF-A can substitute for M-CSF to stimulate osteoclastogenesis and osteoclast survival (Niida *et al.,* 1999). A similar effect of VEGF-A on osteoclastogenesis has also been reported in a rabbit osteoclast culture system (Nakagawa *et al.,* 2000). Expression of both KDR and Flt-1 is found in osteoclasts isolated from bone tissue and *in vitro* in osteoclast-like cells (Gerber *et al.,* 1999; Nakagawa *et al.,* 2000; Niida *et al.,* 1999). Furthermore, PlGF, which interacts with Flt-1 but not with KDR, was as effective as VEGF-A in osteoclastogenesis in op/op mice, suggesting the involvement of Flt-1 in VEGF effects on osteoclasts (Niida *et al.,* 1999). Interestingly, in mice treated with the soluble Flt-1-IgG chimeric protein, there is a substantial reduction in the number of TRAP-positive chondroclasts at the cartilage–bone junction, suggesting the possible role of VEGF signaling in the recruitment and/or differentiation of chondroclasts essential for cartilage resorption (Gerber *et al.,* 1999) (Fig. 9).

Arthritis

Rheumatoid arthritis (RA) is characterized by chronic joint inflammation and infiltration by activated T cells and macrophages (Feldmann *et al.,* 1996; Feldmann and Maini, 1999). The overgrowth of synovial lesions results in the destruction of articular cartilage and bone. Angiogenesis is an indispensable process in the chronic proliferative synovitis and pannus formation in RA (Koch, 1998; Walsh, 1999). VEGF-A mRNA and protein are highly expressed in subsynovial macrophages and synovial-lining cells of inflammatory joints from RA patients (Ikeda *et al.,* 2000; Lu *et al.,* 2000; Nagashima *et al.,* 1995). Increased levels of VEGF-A protein were also found in synovial fluid, as well as in serum of RA patients, which may correlate with disease activity (Harada *et al.,* 1998; Maeno *et al.,* 1999; Pale-

olog *et al.,* 1998). A report on type II collagen-induced murine arthritis model showed that treatment with soluble Flt-1 linked to polyethylene glycol results in reduced joint inflammation and reduced bone and cartilage destruction (Miotla *et al.,* 2000), suggesting that blockage of VEGF activity could be beneficial in RA. Osteoarthritis (OA) is characterized by a progressive loss of articular cartilage, thickening of the trabeculare of the subchondral bone, and formation of new bone and cartilage, giving rise to osteophytes (McAlindon and Dieppe, 1989). Increased angiogenic activity was documented in articular cartilage from OA patients (Fenwick *et al.,* 1999), suggesting that vascularization could be part of the inflammation and ectopic endochondral ossification in avascular articular cartilage.

Summary

The discovery and initial characterization of the VEGF family of growth factors provide insight into the control of vascular and lymphatic angiogenesis. Vascular angiogenesis clearly contributes to the organization and remodeling of a variety of tissues, including bone. The importance of VEGFs to the growth, remodeling, and repair of bone could provide an important framework in which to appreciate the integrated control of vascular and bone biology.

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CHAPTER 49

Transforming Growth Factor-β

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TGFβ Superfamily

TGFβ1 is the prototype and the founding molecule for the TGFβ superfamily. This family has grown to include more than 40 members, including TGFβ isoforms, activins and inhibins, Mullerian inhibitory substance, growth differentiation factors (GDFs), and an ever increasing number of bone morphogenetic proteins (BMPs). Members of this superfamily appear to mediate many key events in growth and development maintained evolutionarily from fruit flies to mammals. The actions of these proteins appear to be mediated through structurally similar serine/threonine kinase transmembrane receptors.

TGFβ isoforms consist of four distinct proteins: TGFβ1–3 and 5. Mammalian TGFβ1 and chicken TGFβ4 are products of homologous rather than duplicated genes (Burt and Jakowlew, 1992). TGFβ1–3 are expressed differentially in mammalian tissues, and each binds with different affinities to TGFβ receptors and appears to have slightly different biological effects (for review, see Centrella *et al.,* 1994). Greater homology exists between the mature regions as compared with the precursor regions, suggesting that the precursor regions may also provide distinct functions (Graycar *et al.,* 1989) (or the pro-peptides are under less selective pressure). BMPs more closely resemble proteins involved in differentiation during embryogenesis such as *Drosophila decapentaplegic* and Vg-1 than TGFβ. TGFβ will only induce new bone formation when injected in close proximity to bone. Unlike BMPs, TGFβ will not produce bone when injected into ectopic sites (Ozkaynak *et al.,* 1990).

Isoforms of TGFβ

The isoforms of TGFβ have been "lumped" together and their effects on bone generalized: however, the isoforms can have very different effects depending on tissue and receptor expression. This generalization must be kept in mind when reading this chapter. An excellent example of different, opposing, effects of the isoforms can be found in the cranial suture of the rat calvaria where removal of TGFβ-3 results in obliteration of the suture, but is prevented by removing TGFβ-2 (Opperman *et al.,* 1999). In the assays in which the various isoforms have been tested, small variations in biological effects have been observed (for review, see Centrella *et al.,* 1994), however, the dramatic differences in tissue distribution suggest specific functions for each isoform. For example, 80–90% of TGFβ in bone is the TGFβ1 isoform, however, the major form produced in kidney (approximately 50%) is TGFβ2 (Marra *et al.,* 1996) and prostate produces 30 to 70 times more TGFβ2 than 1 (Zhao *et al.,* 1997).

TGFβ isoforms are regulated differently. The promoter regions for TGFβ1, TGFβ2, and TGFβ3 show little similarity (Kim *et al.,* 1989; Malipiero *et al.,* 1990; Lafyatis *et al.,* 1990). TGFβ genes also appear to be regulated post-transcriptionally, which may account for the lack of correlation between mRNA expression and protein secreted from some cells. A 5′-untranslated region of the TGFβ1 gene appears to regulate the translation of TGFβ1 mRNA into protein (Kim *et al.,* 1992).

The various isoforms bind with different affinities to TGFβ receptors. The type II TGFβ receptor has a 100-fold

higher affinity for TGFβ 1 and 3 compared to TGFβ 2 (Lin *et al.,* 1992), whereas the endoglin receptor has much higher affinity for TGFβ 2 than for TGFβ 1 or TGFβ 3 (Cheifetz *et al.,* 1992), and the type III receptor has equal affinity for all three isoforms (Lopez-Casillas *et al.,* 1993). The exchange of amino acids 92–95 of TGFβ 1 for the corresponding amino acids of TGFβ 2 abolishes TGFβ 1 high-affinity binding to the type II receptor (Qian *et al.,* 1996). In a human colon carcinoma cell line that responds to TGFβ 2 but not TGFβ 1, TGFβ 2 but not TGFβ 1 bound to type I receptors (Zhou *et al.,* 1995). Investigators are now involved in unraveling the specificity of each isoform for each receptor by determining receptor- and ligand-binding sites (Burmester *et al.,* 1998).

The three-dimensional structure of the TGFβ1 and β2 isoforms has been determined and has served to be the prototype to determine the structure of the other members of the TGFβ superfamily. The TGFβ2 tertiary structure was determined by X-ray crystallography (Daopin *et al.,* 1992) and the structure of TGFβ1 by nuclear magnetic resonance imaging (Hinck *et al.,* 1996). The shape of TGFβ isoforms has been compared with an outstretched hand with curled fingers. Hydrophobic patches are on the "heel" of the "hand" and on the "fingertips." This is unlike most soluble proteins, which are spherical and have a hydrophobic core. When the two TGF monomers bind together to form the homodimer, the hydrophobic "heel" of one molecule touches the hydrophobic "fingers" of the other molecule. The central core of the hydrophobic molecule contains four molecules of water of unknown function. The other members of the TGFβ superfamily will probably contain this central core. Variable regions between the members of this superfamily appear to be at the tips of the "heel" of the molecule. The TGFβ receptors may bind through the back of the "hand." Several investigators are currently determining the tertiary structure of the receptors alone and bound to ligand. Analysis of the three-dimensional structure of the ligand and its receptors should lead to the production of agonists and antagonists for pharmaceutical use (Hoffman, 1992).

Results of Targeted Disruption of the TGFβ Gene or "KnockOut" Mice

Mice that have the targeted deletion of the genes for each TGFβ isoform have different phenotypes, again supporting the hypothesis that each isoform has specific functions. Interestingly, the deletion of a particular isoform of TGFβ in knockout animals does not appear to be compensated for by the other nondeleted isoforms. Targeted disruption of the TGFβ1 gene in mice results in death due to inflammatory disease (Boivin *et al.,* 1995; Kulkarni *et al.,* 1993; Shull *et al.,* 1992). These mice appear perfectly normal until after weaning when massive infiltration of lymphocytes and macrophages into several organs occurs. This leads to organ failure and death. However, the puzzling component of these studies was that the animals appeared normal until after weaning. The puzzle to the normal appearance and perinatal survival of TGFβ1 null mice was solved by Letterio *et al.* (1994). These investigators determined that TGFβ was being passed from the mother to pups through the placenta and also through breast milk. This resulted in maternal rescue of the TGFβ1 null embryos and newborn. These mice were the first example of a gene knockout experiment that was not equivalent to a protein knockout. To eliminate the effects of multifocal inflammation, TGFβ null deletions were created in severe combined immunodeficiency (SCID) mice (Diebold *et al.,* 1995). The longevity in these mice is increased fivefold, and inflammation is eliminated. T-cell marker expression, elevated during inflammation, such as major histocompatability complex class II and intercellular adhesion molecule I, is normal and cardiac cell proliferation is normal. Although inflammation is absent in immunodeficient TGFβ1 null mice and although they do survive into adulthood, they are 50–80% the size of their TGFβ1-expressing SCID littermates and show a lack of vigor and do not thrive. Therefore, it has been difficult to study the effects of a lack of TGFβ 1 on several developmental systems such as bone. However, these mice do have mineralization defects in their bones and teeth and are smaller than their normal siblings, indicating that TGFβ is important for normal bone development (D'Souza *et al.,* 1998; Geiser *et al.,* 1998).

Mice that lack the TGFβ 2 isoform have a wide range of developmental defects, including cranial defects, cardiac, lung, and urogenital defects (Sanford *et al.,* 1997). Mice lacking TGFβ3 show failure of the palatal shelves to fuse, which leads to cleft palate. No craniofacial abnormalities were observed, suggesting that TGFβ3 affects palatal shelf fusion by an intrinsic, primary mechanism (Proetzel *et al.,* 1995). These studies corroborate those of Brunet *et al.* (1995), who found that antibody and antisense oligonucleotides to TGFβ3 but not TGFβ1 or TGFβ2 prevented normal embryonic palate fusion in the mouse. Clearly, the three TGFβ isoforms play different roles in various tissues. Mice that lack the TGFβ type II receptor die *in utero* of a yolk sac hematopoiesis and vasculogenesis defect (Oshima *et al.,* 1996). These studies, utilizing targeted disruption of genes for TGFβ and receptor, emphasize the importance of these molecules during development.

TGFβs

TGFβ is a potent multifunctional cytokine whose major effects in the body appear to be as a regulator of cell growth, a stimulator of matrix production, and an inhibitor of the immune system. Early attempts to purify bone-derived growth factors resulted in the purification of TGFβ1 and TGFβ2 (for review, see Bonewald and Mundy, 1990). The discovery of large amounts of TGFβ in bone led to the discovery in numerous laboratories that TGFβ is a major player in bone remodeling. Agents important for new

bone formation or maintenance of bone mass appear to increase the amount of TGFβ in bone. For example, bones from ovariectomized rats have less TGFβ than normal rats (Finkelman *et al.,* 1991), vitamin D-deficient rats have less TGFβ than vitamin-repleted animals (Finkelman *et al.,* 1992), and studies using intermittent treatment with parathyroid hormone showing increased bone mass also show increased TGF β (Pfeilschifter *et al.,* 1990). The only other factor that appears to share this characteristic with TGFβ is insulin-like growth factor.

In Vitro Effects of TGFβ on Bone Cells

The bone remodeling process can be broken down arbitrarily into several stages. In the recruitment and chemotactic stage, the precursors for bone-forming cells are recruited to the site for future bone formation. After arriving, these cells will proliferate and then begin to differentiate, at which time the bone-forming cells begin to produce osteoid. The osteoid then becomes mineralized. TGFβ has dramatic effects on each of these stages of the bone remodeling process. TGFβ is a potent chemotactic factor that recruits a number of different cell types to sites of repair and inflammation but also recruits osteoblast precursors to sites of bone formation (Pfeilschifter *et al.,* 1990). The chemotactic epitope of TGFβ has been identified as residues 368 to 374 (Postlethwaite and Seyer, 1995). This synthetic peptide was shown to induce the chemotactic migration of neutrophils, monocytes, and fibroblasts. However, this peptide remains to be tested on osteoblast precursors.

In some osteoblast cultures, TGFβ will stimulate proliferation, whereas in others it will inhibit proliferation (for review, see Bonewald and Mundy, 1990). This is probably due to the stage of differentiation of the osteoblast population and the factors influencing experimental conditions. The major effect of TGFβ on the osteoblast is to cause this cell to differentiate and to become a matrix-producing cell. The two major functions of TGFβ are its effects on cell proliferation and matrix formation. Table I gives a list of proteins that are stimulated by TGFβ and a list of those that are inhibited. As can be seen from the list, proteins that are stimulated mainly appear to be matrix proteins and those that are inhibited appear to be proteases. When isolated calvaria are incubated with TGFβ *in vitro,* an increase in bone matrix formation and the number of osteoblasts is observed (Hock *et al.,* 1990).

Although TGFβ stimulates the formation of osteoid, it actually suppresses the formation of mineral in osteoid (for review, see Bonewald and Dallas, 1994). TGFβ will suppress the mineralization of rabbit chondrocytes and fetal rat calvarial cells. TGFβ also suppresses markers of the calcified matrix such as osteocalcin (see Table I). TGFβ inhibits osteocalcin expression in both fetal rat calvarial cells and ROS 17/2.8 cells and in $1,25D_3$-induced osteocalcin production by MG-63 cells. In *in vivo* models examining the effects of injections of TGFβ over murine calvaria (see below), it was only after the injections of TGFβ had ceased that the osteoid became mineralized. Therefore, once TGFβ has initiated new bone formation, it must be removed before mineralization can occur.

Table I TGFβ Is a Potent Stimulator of Matrix Formation[a]

Stimulates	Inhibits
Fibronectin	Procollagenase
Fibronectin receptor	Plasminogen activator
Collagen	Osteocalcin
Osteonectin	
Osteopontin	
Integrins (vitronectin receptor)	
Proteoglycans	
Decorin	
Tissue inhibitor metalloproteinases	
Plasminogen activator inhibitor	
Progelatinase (72 kDa)	
TGFβ	
LTBP	

[a] TGFβ stimulates the synthesis of matrix proteins and their receptors. TGFβ inhibits matrix degradation by increasing the production of protease inhibitors and decreasing the production of proteases (Roberts *et al.,* 1988). Not consistent with the inhibition of several proteases by TGFβ is the stimulation of progelatinase. As an inhibitor of mineralization, TGFβ also inhibits its marker, osteocalcin. TGFβ has autocrine effects and is a potent inducer of its very important regulatory molecule, the latent TGFβ binding protein (LTBP).

TGFβ generally has inhibitory effects on osteoclastic bone resorption. TGFβ appears to inhibit bone resorption by inhibiting the formation and activation of osteoclasts (for review, see Bonewald and Mundy, 1990). In bone systems where TGFβ stimulates resorption, this effect was shown to be mediated by the generation of prostaglandin by TGFβ (Tashjian *et al.,* 1985), possibly by the induction of prostaglandin synthase II in osteoblast cells (Pilbeam *et al.,* 1993). Therefore, TGFβ may act on osteoclast precursors to inhibit their proliferation and their formation and may inhibit the resorption process of mature cells by inducing cell death.

It has been shown that TGFβ may induce the apoptosis of mature osteoclasts (Hughes *et al.,* 1994). TGFβ has been shown to be responsible for apoptosis or programmed cell death in a number of tissues, such as liver, uterine epithelial cells, and hemopoietic cell lines, and will cause cell death of various tumor cell lines, such as prostate, liver, and kidney tumors (for review, see Bursch *et al.,* 1992). In the hemopoietic system, cytokines such as interleukin-6 act as positive regulators of cell growth, whereas TGFβ provides negative regulation in the form of programmed cell death (Sachs and Lotem, 1994). This may also be true as far as osteoclasts are concerned, as interleukin-6 also appears to be a positive regulator of osteoclasts (Ohsaki *et al.,* 1992).

Therefore, the function of TGFβ as an inducer of programmed cell death can be extended to bone with the specific target being the osteoclast.

However, to further justify calling TGFβ a multifunctional cytokine, TGFβ has also been shown to enhance osteoclast formation. TGFβ has been shown to enhance osteoclast formation in cultures of spleen cells and T lymphocytes (Horwood *et al.,* 1999), in marrow cultures cotreated with RANKL and M-CSF (Sells *et al.,* 1999), and to stimulate the formation of osteoclast-like cells in cultures of a human leukemia cell line, FLG 29.1(Fiorelli *et al.,* 1994).

In Vivo Effects of TGFβ on Bone

TGFβ will induce new bone formation if injected in close proximity to bone. Unlike bone morphogenetic proteins, TGFβ will not produce bone when injected into ectopic sites. The earliest *in vivo* experiments were performed using injection of TGFβ over the calvaria of mice (Mackie and Trechsel, 1990; Marcelli *et al.,* 1990; Noda and Camilliere, 1989). These studies showed conclusively that TGFβ stimulates new mineralized bone formation; however, whether these effects of TGFβ are mediated through prostaglandin are controversial. One study found no effect of indomethacin on TGFβ-induced new bone formation (Mackie and Trechsel, 1990), whereas one study saw a partial inhibition of new bone formation by indomethacin (Marcelli *et al.,* 1990). Soon after these studies were performed, it was shown that a single injection of TGFβ1 can induce bone closure of a nonhealing skull defect (Beck *et al.,* 1991). When TGFβ is combined with demineralized bone matrix, an acceleration of osteoinduction is observed (Kibblewhite *et al.,* 1993). The demineralized bone matrix used in this study has little ability to induce the synthesis of new bone by itself. However, in combination with TGFβ, an increase in the formation of trabecular bone is observed in conjunction with greater resorption of the demineralized bone matrix carrier. TGFβ has also been shown to initiate chondrogenesis and osteogenesis when applied to the rat femur (Joyce *et al.,* 1990b).

Systemic administration of TGFβ appears to stimulate cancellous bone formation in both juvenile and adult rats (Rosen *et al.,* 1994), in the nonload bearing rat (Machwate *et al.,* 1995), and in the aging mouse model (Gazit *et al.,* 1995). However, Kalu and co-workers (1993) did not find significant effects of TGFβ2 on the loss of cancellous bone after ovariectomy. Even though no significant effect was observed on bone loss, TGFβ2 did prevent the increase in TRAP(+)-multinucleated cells and caused a decrease in the number of trabecular osteoclasts. These studies suggest that bone loss in these different systems is mediated through different mechanisms and that TGFβ treatment may be more efficacious in some conditions of bone loss as compared to others. Perhaps in the ovariectomized rat model, resorption is accelerated greatly beyond the levels of formation so that TGFβ has little effect on bone loss.

Transgenic mice overexpressing active TGFβ 2 driven by the osteocalcin promoter have been described as osteoporotic (Erlebacher and Derynck, 1996), and mice overexpressing the dominant-negative TGFβ type II receptor driven by the same promoter have been described as osteopetrotic (Filvaroff *et al.,* 1997). These observations appear to contradict many of the observations concerning the function of TGFβ outlined in the previous paragraphs. However, one must take into consideration the following points when interpreting the role of TGFβ in bone based on the phenotype of these mice. (1) Transgenic animals do not represent a physiological, but a potentially pathological effect of TGFβ due to overexpression of active TGFβ. (2) Clearly, the expression of TGFβ must be tightly regulated. It must be readily available for on-site activation and then must be removed or inactivated. (3) Excess amounts of TGFβ actually inhibit mineralization. (4) TGFβ is thought to act as a coupling factor in bone to couple resorption with formation. If excess amounts of TGFβ are present, then bone remodeling is uncoupled. (5) The major isoform in bone is TGFβ1 not 2 and, as described earlier, the isoforms can have distinctly different affects on bone. (6) The osteocalcin promoter is expressed to a greater extent in mineralizing osteoblasts and osteocytes as compared to early osteoblasts (Mikuni-Takagaki *et al.,* 1995; Sims *et al.,* 1997). Therefore, overexpression of active TGFβ or the dominant- negative type II receptor in these animals is only occurring very late in osteoblast differention. TGFβ probably has its bone stimulatory effects in early phases of osteoblast differentiation. (7) The expression of osteocalcin, the TGFβ isoforms, and TGFβ type II receptor are clearly very different during bone formation and the mineralization process (Horner *et al.,* 1998). The type II receptor is not normally highly expressed during late stages of osteoblast differentiation. Together, these observations show that the regulation of TGFβ is critical in remodeling and disease. Bone disease can potentially result from an imbalance in TGFβ isoforms and their bioavailability.

TGFβ Receptors

Essentially all cells have receptors that specifically bind TGFβ (for reviews, see Heldin *et al.,* 1997; Hu *et al.,* 1998). Early studies using cross-linking techniques identified these receptors as type I, type II, and type III using band migration on SDS–PAGE (Cheifetz *et al.,* 1986; Segarini *et al.,* 1987; Wakefield *et al.,* 1987). The type I receptor gave a band ~65–70 kDa, the type II a band of 85–110 kDa, and the type III migrated as a diffuse band −200 kDa. These three major classes of receptors have been cloned (Franzen *et al.,* 1993; Lin *et al.,* 1992; Wang *et al.,* 1991). The type I and II receptors appear to be responsible for transducing intracellular signaling upon binding with TGFβ, whereas the type III receptor, now

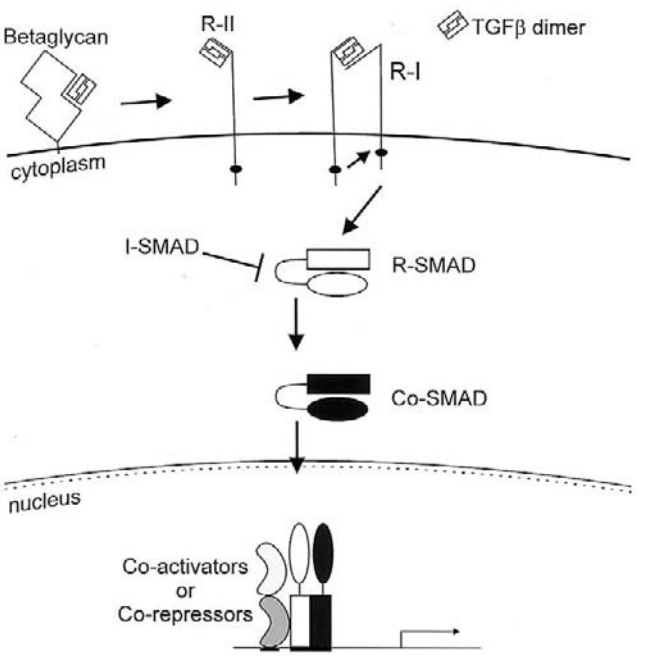

Figure 1 TGFβ receptor function and signaling mechanisms. Free TGFβ dimer is proposed to bind to the type II receptor or alternatively, β-glycan, also known as the type III receptor, presents cell surface-bound TGFβ to type II receptors. The β-glycan is displaced by the type I receptor for cell signaling to occur. The type II receptor is constitutively active. Following ligand binding, the type II receptor complexes with and activates the type I receptor. Instead of the simplified interactions as shown, the TGFβ receptors probably form large complexes. Data suggest that the type II receptor exists as a stable homodimeric complex. It is likely that the signaling TGFβ complex is a tetramer consisting of two type II chains and two type I chains. The type I receptor phosphorylates regulatory Smads, such as Smad-2 and -5. Inhibitory Smads (such as Smad-7) can block the interaction of the regulatory Smads with Smad-4, the major Co-Smad, which moves the complex to the nucleus. The specificity of the effects of TGFβ on the cell type is probably due to the composition of coactivators and/or corepressors present in the nucleus.

known as β-glycan, possesses only a short intracellular domain that does not appear to be involved in TGFβ signaling (Wang *et al.,* 1991). Although additional cell surface proteins that bind to TGFβ have been identified through cross-linking experiments (Centrella *et al.,* 1994; Massagueà, 1992), most attention has now been focused on type I–III receptors (see Fig. 1). The type III receptor is an integral membrane protein that is modified heavily by glycosaminoglycan groups, which probably accounts for its diffuse migration on gel electrophoresis. The nonglycosylated form of this receptor is still able to bind ligand. The type III receptor binds with equal affinity to the three TGFβ isoforms. It has been suggested that this receptor may function as a concentrating ligand before presentation to signal-transducing receptors type I and type II (Lopez-Casilles *et al.,* 1993; Massaguè *et al.,* 1990).

A considerable amount of time lapsed between the first cross-linking studies showing the presence of TGFβ receptors in 1985 and 1986 and determination of the actual signaling mechanism via serine/threonine kinase activity. Insight into the mechanism of signaling was provided when the type II activin receptor was cloned (Mathews and Vale, 1991). This receptor was identified as a transmembrane serine/threonine kinase. Since that time the cDNA cloning of various type I and type II receptors for TGFβ, activins, and BMPs has shown that all appear to be transmembrane serine/threonine kinases. It appears that the receptor signaling process for TGFβ is likely to serve as a model for the rest of the TGFβ superfamily. Both type I and type II receptors have short extracellular domains with a long cytoplasmic region that mainly consists of the kinase domain. The type I receptor also contains a conserved SGSGSGLP motif between the transmembrane domain and the kinase domain. These TGFβ receptors interact in both homomeric and heteromeric fashion.

Two systems have been proposed for the interactions of type I and type II receptors to promote TGFβ signaling. Wrana and colleagues (1994) proposed that TGFβ binds directly to the type II receptor, which is a constitutively active kinase. The bound TGFβ is then recognized by the type I receptor. These become a complex in which the type II receptor phosphorylates the type I receptor. Phosphorylation of the type I receptor is proposed to propagate signaling to downstream substrates. This signaling pathway is probably responsible for mediating the antiproliferative effects of TGFβ as well as effects on gene responses.

A second model proposes two distinct receptor-associated signaling pathways (Chen *et al.,* 1993). In this model, the type II receptor is required for signaling of growth inhibition. However, the second pathway is mediated through the type I receptor without signaling through the type II receptor as long as the extracellular domain of the type II receptor is present to provide ligand interaction with the type I receptor. This study was performed by producing a truncated type II receptor that could act as a dominant-negative mutant. This signaling through the type I receptor is responsible for the effects of TGFβ on extracellular matrix.

All three TGFβ receptor types appear to be expressed on osteoblasts, and the ratio and expression of these receptors can be modified by osteotropic factors. Studies on bone cells have been performed using cross-linking of radiolabeled TGFβ to the cell surface as initially performed by Segarini and co-workers (1989) where they described for the first time the presence of the three receptors on fetal rat osteoblasts. Subsequently, it was shown that parathyroid hormone (PTH) enhances the binding of TGFβ to all three receptors in fetal rat calvarial osteoblast-enriched cultures (Centrella *et al.,* 1988). This was in contrast to the effect of PTH on fibroblast-enriched cultures where PTH had no effect on TGF binding. Bone morphogenetic protein 2 (BMP 2) also appears to modulate the expression of TGFβ receptors (Centrella *et al.,* 1995b). In fetal rat calvarial osteoblast-like cells, the expression of type III and II receptors declined compared to the type I receptor in parallel with an increase in osteoblast-like activity. BMP 2 appears to accelerate this process in both osteoblast cultures and ROS 17/2.8 cells. Therefore, BMP 2 alters the TGFβ-binding profile in ways that are consistent with a differentiation

toward the osteoblast phenotype. This is in contrast to the effects of glucocorticoid on the TGFβ receptor profile where an increase in binding at type III sites is observed with a corresponding decrease in binding to type I and type II receptors (Centrella *et al.,* 1991). BMP 2 appears to override the effects of glucocorticoid on binding of TGFβ to receptors (Centrella *et al.,* 1995b). These studies, using BMP 2 and glucocorticoid on fetal rat calvarial cells, are in contrast to studies using the mouse mesenchymal stem cell line C3H-10T1/2 treated with retinoic acid, which predisposes these cells toward the osteoblast phenotype (Gazit *et al.,* 1993). In these studies, treatment with retinoic acid consistently decreased type III, type II, and type I receptor binding to TGFβ. The same effects were also observed using the rat cell line ROBC26. The authors suggested that downregulation of the TGFβ receptor might be an essential component of the osteoblast differentiation process.

Two separate TGFβ receptor signaling pathways are present in osteoblasts (Takeuchi *et al.,* 1995). Four clonal osteoblastic cell lines were examined that include MC3T3-E1 cells, MG-63, SaOS2, and UMR-106. Very different receptor profiles were observed with these cell lines. Both MC3T3 and MG-63 expressed all three receptor types. However, SaOS cells only expressed type I and type III and UMR-106 cells only expressed the type III receptor. These cells were examined for the effects of TGFβ on growth inhibition, proteoglycan synthesis, and fibronectin synthesis. Even though the SaOS cells lacked the type II receptor by cross-linking studies, they still responded to exogenous TGFβ with an increase in fibronectin synthesis. Even though the type II receptor did not bind to TGFβ, it was detectable by immunoprecipitation. Their data suggest that cells lacking signaling type II receptor escape the effects of TGFβ on proliferation and that the effect of TGFβ on fibronectin synthesis is under control of the type I receptor. Therefore, it is the combination of cell surface receptors for TGFβ that determines the response of osteoblasts to this cytokine.

TGFβ Signaling Pathways

An impressive amount of information concerning the intracellular events that follow the interactions between type I and type II receptors has been gained since 1996 (see Fig. 1). Direct substrates for the type I receptor appear to be Smad-2 and Smad-3, also known as receptor-activated Smads (R-Smads), whereas negative regulators of this interaction include Smad-6 and Smad-7, the inhibitory Smads (I-Smads) (Hayashi *et al.,* 1997; Imamura *et al.,* Hu *et al.,* 1998; de Castecker *et al.,* 2000). The discovery and naming of the Smads originated from studies of the dpp signaling pathway in *Drosophila* (Massague *et al.,* 1997). The Smads share structural homology through two domains called MH-1 and MH-2. These two regions are highly conserved evolutionarily. The MH-1 domain represses the MH-2 domain, which possesses constitutive transcription activity. It is proposed that the Smads associate as trimers through the MH-2 domain. Smad-2 or Smad-3 heterozygous mutant mice are viable, but the compound heterozygous Smad-2/Smad-3 mutant is lethal, suggesting a gene dosage effect and that the relative level of Smad-2 and -3 influences the nature of the TGFβ response (Weinstein *et al.,* 2000). Smad-2 and Smad-3 have been shown to interact with beta-tubulin and chemical disruption of tubular networks interferes with Smad2 binding to tubulin and activation of TGFβ signals. This suggests that the cytoskeleton is also involved in TGFβ signaling.

Smad-4, also called a common mediator (Co-Smad), appears to bring cytoplasmic Smad-2 and Smad-3 into the nucleus where together they can regulate the transcription of target genes (de Winter *et al.,* 1997) (see Fig. 1). Smad-4 was found to be homologous to a gene deleted in pancreatic carcinomas called "deleted in pancreatic cancer-4" or DPC-4 (Hahn *et al.,* 1996). A number of Smad-4-independent TGFβ responses have been identified (for review, see de Castecker *et al.,* 2000). These include Jun N-terminal kinase (JNK) and extracellular signal-related kinases (ERK) 1/2 mitogen-activated protein (MAP) kinase pathways. The potential exists for other co-Smads to be identified.

Other substrates for the type I receptor have been reported, which include FKBP12, WD40, and farnesyl transferase (see Hu *et al.,* 1998). However, the signaling function of these pathways is less clear than the Smad pathway. TGFβ has also been shown to activate a MAP-kinase pathway specified by the serine-threonine kinase TAK-1 (Yamaguchi *et al.,* 1995), and TGFβ has been implicated as well in G protein-mediated and ras-signaling pathways (for review, see de Castecker *et al.,* 2000). Because inhibitors of MAP kinases and p38 can clearly affect TGFβ signaling, Smads are not responsible for all TGFβ signaling.

Several transcription regulators and accessory proteins modulate TGFβ signaling. Negative modulators and inhibitors of the TGFβ signaling pathway include, in addition to the inhibitory Smads, the 5′TG 3′ interacting factor (TGIF), histone deacetylases (HDAC), Smad ubiquitination regulatory factor 1 (Smurf 1), calmodulin, a primary modulator of calcium signaling, and others (for more information, see Zimmerman and Padgett, 2000). Positive regulators include SARA, the Smad anchor for receptor activation (Tsukazaki *et al.,* 1998), that appears to function by bringing Smad-2 into close proximity with a cluster of residues in the kinase region of the type I receptor. Other SARA-like proteins may exist that bind to other Smads. Additional transcription activators include FAST proteins, AP-1 members, TFE3, a basic helix-loop-helix transcription factor that has been shown to cooperate with Smad-3 and -4, Polyoma virus enhancer-binding protein 2/core-binding factor factor (PEBP2/CBF), Olf-1/EBF associated zinc finger (OAZ), MSG1, CREB-binding protein (CBP)/p300, and others (for review, see Zimmerman and Padgett, 2000). Interestingly, the vitamin D nuclear receptor, VDR, interacts specifically with Smad-3. Activation of TGFβ signaling results in a Smad-3-mediated

enhancement of VDR-dependent transcription through the MH1 domain instead of the MH2 domain as is observed with other coactivators (Yanagi *et al.,* 1999; Yanagisawa *et al.,* 1999). This may have implications for bone growth and remodeling as TGFβ and $1,25(OH)_2D_3$ have been shown to synergistically increase alkaline phosphatase in primary human bone cells and the MG-63 osteoblast cell line (Wergedahl *et al.,* 1992; Bonewald *et al.,* 1992) This shows that TGFβ through Smad-3 is cooperatively enhancing VDR-regulated pathways.

Latency of TGFβ

TGFβ is secreted by cells in culture in biologically latent forms. Latent TGFβ can be activated *in vitro* by transient acidification or alkalinization or by the action of chaotropic agents (Gehron-Robey *et al.,* 1987; Lawrence *et al.,* 1985; Pircher *et al.,* 1986). Release of the mature TGFβ 25-kDa homodimer from the latent complex is necessary for TGFβ to exert its effects on target cells. TGFβ is the only growth factor known to be produced in a latent or inactive form (see Fig. 2).

The precursor or latency-associated peptide (LAP) is all that is necessary to confer latency. This latent complex, containing only LAP and the TGFβ homodimer, has been referred to as the "small latent complex" of 100 kDa. Approximately 50% of latent TGFβ made by bone cells is this form (Bonewald *et al.,* 1991), whereas other cell types produce little or none of this form. The small latent complex may have a unique function in bone by acting as a circulating, more readily activatable form.

Bone cells also produce the "large latent complex" as described in other cell types (Dallas *et al.,* 1995; Miyazono *et al.,* 1988; Tsuji *et al.,* 1990). The large latent complex contains a protein called the latent TGFβ-binding protein or

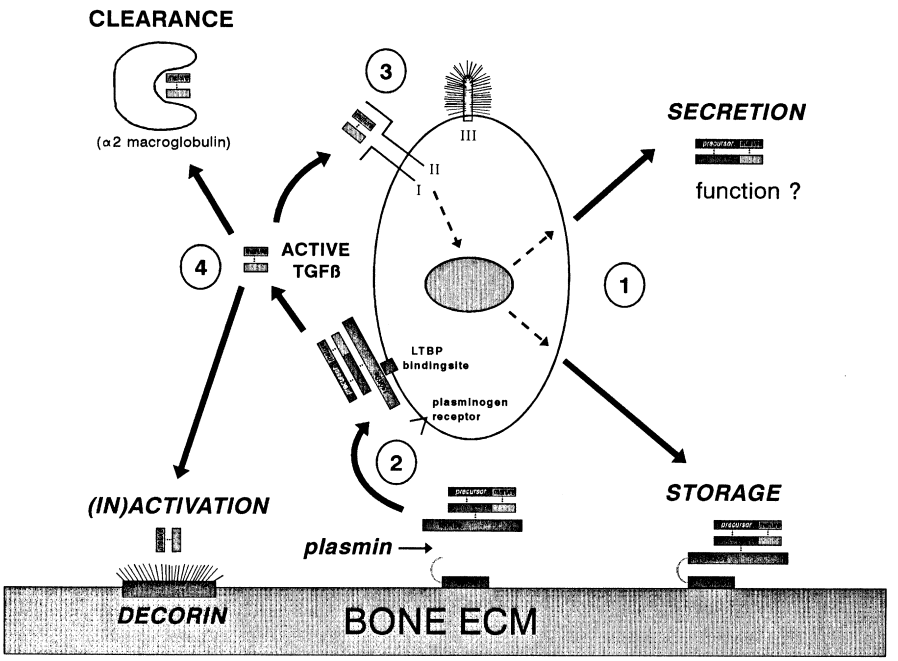

Figure 2 The osteoblast and TGFβ. The osteoblast produces latent TGFβ, can activate latent TGFβ upon proper stimulation, and can respond to TGFβ through specific receptors. Bone cells are unique in producing at least two latent forms of TGFβ (1): one that contains the TGFβ binding protein or LTBP and one that does not. The function of the complex lacking LTBP is unknown but perhaps it functions as a circulating form of latent TGFβ. In the complex containing LTBP, LTBP appears to direct the latent complex to bone for storage. Plasmin, a known matrix remodeling agent, will cause the release of latent TGFβ from the matrix by cleavage of the LTBP at a protease-sensitive hinge region (2). This plasmin-released complex appears similar to the platelet latent complex. It is proposed that binding sites exist on the cell surface for the cleaved LTBP, which can allow the tethered latent complex to be activated by cell surface-associated plasmin. Active TGFβ binds to the type II receptor, which in turn causes the association with the type I receptor (3). The type II receptor is a kinase that will activate the type I receptor, which in turn sends a signal to the nucleus. The type III receptor, known as β-glycan, is a proteoglycan that will bind TGFβ but does not appear to signal. TGFβ has an autocrine effect in that TGFβ will induce the production of its own mRNA in the form of latent TGFβ. Active TGFβ is removed from the circulation by association with α_2-macroglobulin (4) (O'Conner-McCourt and Wakefield, 1987). Other molecules are known to either activate or inactivate TGFβ. Decorin in the kidney inhibits TGFβ (Border *et al.,* 1992), but decorin in bone appears to maintain the activity of TGFβ (Takeuchi *et al.,* 1994). Thrombospondin appears to activate and maintain activity of TGFβ (Schultz-Cherry *et al.,* 1995).

LTBP-1 (see Fig. 3). This protein is truncated at the amino terminus in the latent complex produced by platelets (Wakefield *et al.,* 1987; Kanzaki *et al.,* 1990). We and others have shown that the nontruncated LTBP protein targets the small latent TGFβ to the matrix for storage (Dallas *et al.,* 1995; Taipale *et al.,* 1994). This protein does not confer latency to the complex.

Activation of Latent TGFβ

The earliest experiments examining the activation of latent TGFβ used dissociating agents such as acid, chaotropic agents, or heat treatment of conditioned media containing inactive TGFβ (Brown *et al.,* 1990). These were followed by experiments using recombinant small latent TGFβ to examine the activation process by proteases such as plasmin (Lyons *et al.,* 1990) or cells such as osteoclasts (Oreffo *et al.,* 1989). These experiments, using recombinant latent TGFβ, are more representative of the processing that occurs of the "small" latent complex. More attention has focused on the release and subsequent activation of the matrix-bound "large" latent TGFβ complex. Release and activation of matrix-bound latent TGFβ occur during matrix remodeling through the activity of proteases such as plasmin (Nunes *et al.,* 1997) and the cellular activity of cells such as bone-resorbing osteoclasts (Dallas *et al.,* 1997).

Several proteases have been implicated in the activation of latent TGFβ. The activation of LTGFβ in cocultures of endothelial cells and smooth muscle cells was shown to be due to the generation of plasmin (Sato *et al.,* 1990). Plasmin activates LTGFβ by proteolytically cleaving the precursor or LAP at multiple sites (Lyons *et al.,* 1990). Retinoids were found to increase the activation of LTGFβ in endothelial cells/smooth muscle cell cocultures by increasing membrane-associated plasminogen activator/plasmin levels (Kojima and Rifkin, 1993).

Several stimulated cell types have been shown to activate latent TGFβ, such as macrophages treated with interferon-γ (Twardzik *et al.,* 1990), with IgG or lipopolysaccharide (Schalch *et al.,* 1991), with mesenchymal cells treated with glucocorticoids (Rowley, 1992), with endothelial cells treated with fibroblast growth factor (Flaumenhaft *et al.,* 1992), and with cocultures of endothelial cells with pericytes or smooth muscle cells (Sato and Rifkin, 1989). Vascular smooth muscle cells treated with angiotensin II activate latent TGFβ (Gibbons *et al.,* 1992), and fetal fibroblasts treated with antiestrogens also produce active TGFβ (Colletta *et al.,* 1990). Keratinocytes treated with retinoic acid secrete active TGFβ2 (Glick *et al.,* 1989). Therefore, many cell types activate latent TGFβ, depending on whether other factors are present in the cellular environment.

Bone cells activate latent TGFβ, but through different mechanisms (see Fig. 3). Human osteoblast-like cells treated with glucocorticoids activate latent TGFβ (Oursler *et al.,* 1993). Parathyroid hormone-treated osteoblast-like cells, UMR-106-01 cells and neonatal mouse calvarial cells, appear to activate latent TGFβ using the plasminogen activator system (Yee *et al.,* 1993). Isolated avian osteoclasts upon treatment with retinol will activate latent TGFβ (Oreffo *et al.,* 1989), but this activation was shown not to be due to plasmin (Bonewald *et al.,* 1996). Oursler (1994) showed that avian osteoclast-like cells isolated using an antibody that recognizes the 121F antigen, a protein related to superoxide dismutase, will produce and activate latent TGFβ. No significant inhibition of latent TGFβ activation was observed using a series of protease inhibitors, including those that block plasmin, although a combination of five protease inhibitors significantly blocked activation. It has been shown that one of the mechanisms whereby osteoclasts can activate latent TGFβ is through matrix metalloproteinases (MMPs)(Dallas *et al.,* 1997). Both MMP-9 and MMP-2 have been shown to activate latent TGFβ (Yu and Stamenkovic, 2000).

A noncellular mechanism has been described in which latent TGFβ is activated by extracellular organelles called matrix vesicles (Boyan *et al.,* 1994). Matrix vesicles found in osteoid are associated with matrix calcification. Alone, these organelles have no effect on latent TGFβ; however, upon pretreatment of these organelles with $1,25(OH)_2D_3$, both recombinant latent TGFβ1 and recombinant latent TGFβ2 are activated. Matrix vesicles have been shown to contain proteases, such as plasminogen activator, and therefore one of the nongenomic effects of $1,25D_3$ on the membranes of these organelles may be the release of TGFβ-activating proteases.

Thrombospondin, a glycoprotein involved in cell growth, adhesion, migration, and angiogenesis, will activate latent TGFβ (Schultz-Cherry *et al.,* 1995). In cell-free systems, thrombospondin activates both small and large latent TGFβ (Murphy-Ullrich *et al.,* 1992; Schultz-Cherry *et al.,* 1994). Activation was shown to be a two-step process. The first process involves the actual binding of latent TGFβ to thrombospondin, and the second step involves the release of active TGFβ. The sequences of thrombospondin responsible for these two activities were identified and synthetic peptides made that would either block the binding of TGFβ to thrombospondin or prevent activation of the molecule. Peptides interfering with the first function (GGWSHW) or those interfering with the second function (KRFK) may have potential therapeutic uses where TGFβ activity requires modulation, such as in wound healing or fibrotic scar formation.

It has been suggested that TGFβ-1 null mice and thrombospondin-1 null mice share similar abnormalities (Crawford *et al.,* 1998). When thrombospondin-1 null mice were treated with a peptide that mimics the thrombospondin activation of TGFβ, several organ abnormalities reverted back to wild type. Synthetic peptide, KRFK, made to a repeat in thrombospondin, can activate latent TGFβ by cleaving LAP (Schultz-Cherry *et al.,* 1995), and an antagonist peptide, LSKL, can prevent this activation. Investigators suggest that thrombospondin may be the best physiological activator of latent TGFβ, even more so than the plasmin/plasminogen-activator system described by Flaumenhaft and co-workers (1992, 1993; Kojima and Rifkin,

1993). It remains to be determined if this is true for all organ systems, especially bone.

Proteases other than plasmin will activate latent TGFβ. Elastase will cleave matrix-bound latent TGFβ, but will not activate the small latent complex. Whereas both MMP-9 and MMP-2 activate latent TGFβ, only MMP-9, but not MMP-2, will cleave LTBP-1. Prostate-specific antigen will specifically activate latent TGFβ-2 but not latent TGFβ-1, nor will it release matrix-bound latent TGFβ (Zhao *et al.,* 1997). This is the first example of isoform-specific activation. Release of latent TGFβ from the matrix through the cleavage of LTBP-1 does not necessarily result in the activation of latent TGFβ (Dallas *et al.,* 1994, 1995). Cleavage of LTBP may be a means of releasing latent TGFβ that can then be available for activation at a site distant from the storage site. Plasmin will activate small latent TGFβ, but much lower concentrations (10- to 100-fold less) will release matrix-bound latent TGFβ, suggesting that a more physiological function of plasmin is to release the latent complex from the matrix rather than to activate the soluble forms.

Latent TGFβ-Binding Protein (LTBP)

TGFβ may be unique among growth factors because of its association with the protein LTBP (Figs. 2 and 3). The function of LTBP may not only be to target or deliver latent TGFβ to the matrix, but also to provide a vehicle for its release from the matrix and to target the modified released latent complex to the cell surface for activation. Additionally, and independent from its association with TGFβ, LTBP appears to function as a structural extracellular matrix protein required for new bone formation (Dallas *et al.,* 1995). The relationship between TGFβ and LTBP could represent an intricate system for regulating the actions of this multifunctional growth factor.

LTBP appears to be a structural extracellular matrix protein involved in the formation of large fibrillar structures in the extracellular matrix of bone cells (Dallas *et al.,* 1995). Both antibodies and antisense oligonucleotides to LTBP will inhibit the formation of bone-like nodules by fetal rat calvarial cells. The fibrillar structures found in these cultures contain LTBP complexed with the small latent TGFβ and free LTBP not complexed to TGFβ. These fibrillar structures also contain fibrillin-1. In long bones *in vivo,* LTBP-1 and fibrillin colocalized to the surface of newly forming osteoid and bone (Dallas *et al.,* 2000). The presence or composition of other proteins within these fibrils is not known. Plasmin cleaves LTBP at a protease-sensitive hinge region, which leaves the 60-kDa amino terminus of the LTBP in the matrix and releases the latent complex containing a 130-kDa cleaved fragment of LTBP similar in composition to that described for platelets. This latent complex appears to be targeted to the cell surface for activation by plasmin (see Fig. 3.)

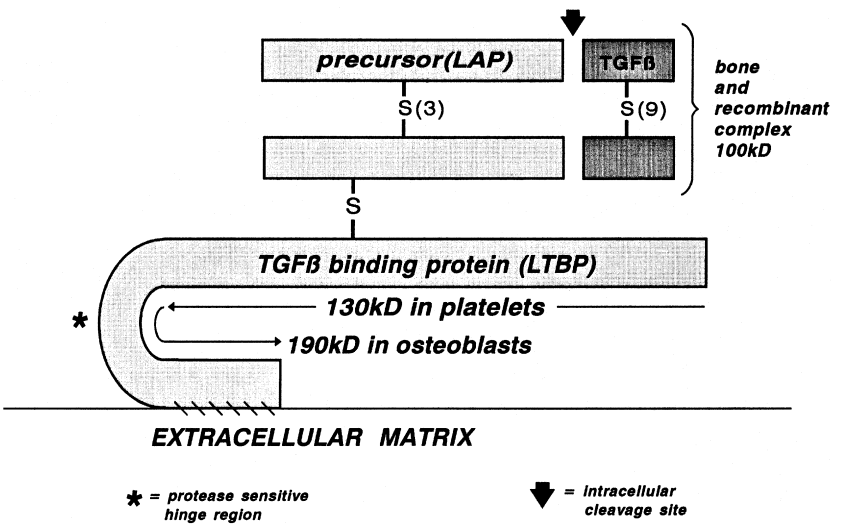

Figure 3 Various forms of latent TGFβ. Various forms of latent TGFβ are produced by different cell types. Bone cells produce the 100-kDa latent complex, which lacks the TGFβ binding protein or LTBP. This 100-kDa complex appears to be identical to the recombinant material secreted by transfected Chinese hamster ovary cells. Platelets produce the 100-kDa complex covalently associated through disulfide bonds with a truncated form of LTBP of 130 kDa. Bone cells also make the latent complex in which the 100-kDa latent TGFβ is covalently attached to a 190-kDa LTBP. In all latent complexes, the mature TGFβ homodimer must be dissociated from the precursor to become biologically active. LTBP is not necessary for processing or secretion of latent complex in bone cells and it does not confer latency to the complex. LTBP appears to direct the latent complex to the matrix for storage. LTBP itself forms fibrillar structures in the matrix. Latent TGFβ can be released from the matrix through the action of plasmin on the matrix-bound latent complex. LTBP contains a protease-sensitive hinge region through which this occurs.

Mutations in LTBP may result in connective tissue disorders. Sequence analysis of LTBP reveals a number of features characteristic of matrix or adhesion molecules, such as 16 epidermal growth factor-like repeats, an RGD sequence, putative calcium-binding domains, and an 8 amino acid motif identical to the cell-binding domain of β2 laminin (Kanzaki *et al.,* 1990; Tsuji *et al.,* 1990). Several of these features are shared with the fibrillin family of extracellular matrix proteins in addition to a novel cysteine repeat motif that is unique to the fibrillin family (Maddox *et al.,* 1989; Rosenbloom *et al.,* 1993; Sakai *et al.,* 1986; Zhang *et al.,* 1994). Fibrillins are major components of connective tissue microfibrils. Marfan syndrome and related disorders of the cardiovascular and skeletal system have been linked to mutations in genes for the fibrillin family of extracellular matrix proteins of which LTBP appears to be a new member (Dietz *et al.,* 1991; Lee *et al.,* 1991; Lonngvist *et al.,* 1994; Maslen *et al.,* 1991; Milewicz *et al.,* 1992). Women with Marfan's syndrome have increased osteopenia, which may predispose them to hip fracture (Kohlmeier *et al.,* 1995). Marfan-like connective tissue and skeletal disorders for which no current explanation is available or of unknown etiology may be due to mutations in members of the LTBP family.

To date, four LTBP genes have been isolated containing 15–19 EGF-like repeats: three novel eight cysteine (8-Cys) repeats and a "hybrid domain" that shares homology with the EGF-like and eight cysteine repeats (for review, see Taipale *et al.,* 1988). Many extracellular matrix proteins contain EGF-like repeats that mediate noncovalent protein–protein interactions, but only LTBPs and fibrillins contain the 8-Cys domains that mediate covalent interactions. The third 8-Cys repeat in LTBP-1 and -3 efficiently binds all three TGFβ isoforms through the latency-associated peptide: LTBP-4 has much weaker binding and LTBP-2 and the fibrillins 1 and 2 do not bind at all (Saharinen and Keski-Oja, 2000). LTBP-2 shares 41% homology with LTBP-1 and has very similar overall structural homology (Moren *et al.,* 1994). LTBP-3 shares 38% homology with LTBP-1, is produced by MC3T3-E1 osteoblast cells, and forms a complex with the TGFβ1 precursor (Yin *et al.,* 1995). It was suggested that LTBP-3 may be an early marker of osteoblast differentiation. This supports the observation of Dallas *et al.* (1995, 2000) showing that LTBP-1 is expressed very early in fetal rat calvarial cell culture and in the periosteal layer of developing bone. This new family of LTBP-like molecules may have important functions in bone formation.

Deletion of LTBP-2 appears to be a neonatal lethal (Shipley *et al.,* 2000). One other LTBP other than LTBP-1, LTBP-3, has been shown to be made by bone cells, specifically by MC-3T3 E1 cells (Yin *et al.,* 1995). The expression of LTBP-3 in *Xenopus* is found in the skull and notochord (Quarto *et al.,* 1999). Rifkin and co-workers (1999) have successfully deleted the gene for LTBP-3 in mice. These mice have a short snout, bossy forehead, closed parietal suture, short long bones, and overgrown incisors, probably because the snout is shortened. The gene deletions for LTBP 1 and 4 have not yet been accomplished.

Role of TGFβ in Bone Cancer Metastasis

TGFβ normally functions as a tumor suppressor. Mice that have a haploinsufficiency for TGFβ are more susceptible to tumorigenicity (Tang *et al.,* 1998). Acquisition of resistance to TGFβ growth inhibition obviously plays a major role in the progression to malignancy, as cancer cells are less susceptible to growth inhibition by TGFβ than normal cells (Fynan and Reiss, 1993). This resistance can be due to mutations and/or deletions in any component of the TGFβ receptor signaling pathway, as described by Sherr and colleagues (1996). In addition to mutations in receptors and signaling molecules, resistance to TGFβ can be due to the low expression of receptors and induction of TGFβ resistance by growth factors or viral transforming proteins and can occur at levels beyond the TGFβ signaling pathway, such as mutations in cell cycle regulators such as Rb, p15, p21, and p300/CBP, all targets of TGFβ (for review, see Taipale *et al.,* 1998).

TGFβ can support cancer growth by suppressing the immune system while simultaneously stimulating normal support cells to secrete growth factors for cancer cells. In the case of bone metastatic breast cancer, TGFβ can induce the production of the bone-resorbing factor parathyroid hormone-related peptide (PTHrp), which in turn stimulates osteoclastic bone resorption, thereby releasing growth factors, including TGFβ from the bone matrix (Guise *et al.,* 1996; see also Chapter 61). This creates a "vicious" cycle of events that sustains and supports growth of the breast cancer. Neutralizing PTHrp decreases osteoclastic bone resorption and tumor burden in bone. Guise and co-workers (1996) showed that a neutralizing monoclonal antibody to PTHrp(1-34) inhibits the development of breast cancer metastases, MDA-MB-231 cells, to bone in the nude mouse model. Similar results have been obtained with lung cancer cells (Iguchi *et al.,* 1996). MCF-7 breast cancer cells do not express PTHrp and do not metastasize to bone: however, when stably transfected with a vector carrying the gene for PTHrp, these cells do metastasize to bone (Thomas *et al.,* 1999), indicating that PTHrp is a bone metastatic factor.

TGFβ stimulates the production of PTHrp in several tumor types (for review, see Chirgwin and Guise, 2000). Because bone contains other growth factors (such as the IGFs, FGFs, PDGFs, and BMPs), these were tested for stimulation of PTHrp production by MDA-MB-231 cells. Only TGFβ stimulated PTHrp production (Yin *et al.,* 1999). These investigators also stably transfected the TGFβ type II dominant receptor in MDA-MB-231 cells and found considerably less bone destruction and less tumor burden. Expression of a constitutively active TGFβ type II receptor reversed the dominant-negative blockade, resulting in increased PTHrp production, enhancement of osteolytic bone metastasis, and decreased survival (Yin *et al.,* 1999). Introduction of cDNA

for PTHrp constitutively driven by the CMV promoter increased PTHrp production and accelerated the development of osteolytic bone metastases greatly. In these cells, TGFβ appears to be signaling through both the Smad pathway as described earlier and the p38 MAPkinase pathway downstream of TAK1 to regulate PTHrp production (Kakonen *et al.,* 2000). Thus, TGFβ receptor activation is important for the development and progression of osteolytic bone metastases.

Activation of latent TGFβ may also play a role in tumor invasion and angiogenesis. Yu and Stamenkovic (2000) have shown that cell surface-localized matrix metalloproteinase-9 activates TGFβ, thereby promoting tumor growth and invasion. Because many cancers express the matrix metalloproteinses, it will be important to determine if these play a role in bone metastasis.

In addition to the mechanisms described earlier, the loss of LTBP-1 expression or regulation may also be associated with malignancy. LTBP-1 expression is lost in malignant prostatic cells (Eklov *et al.,* 1993), gastrointestinal carcinomas (Mizoi *et al.,* 1993), and SV40-transformed fibroblasts (Taipale and Keski-Oja, 1996). Loss of LTBP-1 and thereby the loss of targeting of latent TGFβ to the extracellular matrix may be another mechanism whereby transformed cells escape regulation by TGFβ. It is not known if this is true for tumors that metastasize to bone.

Role of TGFβ in Osteoporosis

TGFβ may play a role in osteoporosis in humans. Low bone mass has been shown to be the most important risk factor for the development of osteoporotic fractures. Women in Denmark with a variant form of TGFβ-1 have low bone density and an increased susceptibility to osteoporosis (Langhdahl *et al.,* 1997). This variant is caused by a one base deletion in the intron sequence 8 bases prior to exon 5 in the TGFβ1 gene, which theoretically would result in less TGFβ1. This variant was also associated with increased bone turnover in both osteoporotic and normal women. However, this association with increased susceptibility to osteoporosis was not associated with peak bone mass, bone growth, early postmenopausal bone loss, or response the hormone replacement therapy (HRT) (Langhdahl *et al.,* 1997). This variant of TGFβ may be influencing susceptibility to osteoporotic fractures through other mechanisms than regulation of peak bone mass. Yamada and co-workers (1988) have found a polymorphism of the TGFβ1 gene that is a determinant of bone mass and is a susceptibility factor for osteoporosis. A substitution of thymidine at position 29 to a cytosine resulted in a proline for leucine substitution at amino acid 10. This polymorphism was found to be associated with higher peak bone mass in both males and females, and the thymidine-containing allele was an independent risk factor for osteoporosis in Japanese women. Of course in both of the studies, the target of the TGFβ is unknown.

Animal studies support a role for TGFβ in osteoporosis. Decreased TGFβ mRNA expression and decreased deposition of TGFβ occur in bones from ovariectomized rats (Finkelman *et al.,* 1992). Osteopenia in old male mice was due to reduced TGFβ (Gazit *et al.,* 1988). These investigators found less TGFβ stored in the matrix of long bones from these aged mice compared to young mice and less TGFβ produced by marrow osteogenic cells. The osteoprogenitors from aging mice are more responsive to TGFβ, and bone formation was stimulated by injections of TGFβ into these animals. This study supports previous studies showing that TGFβ is an important bone growth factor. Systemic administration of TGFβ 2 has been shown to prevent bone loss due to immobilization in rats (Machwate *et al.,* 1995).

It has been speculated that estrogen may partially maintain bone mass through TGFβ and that raloxifene maintains bone mass through the induction of TGFβ3 expression (Yang and Termine, 1994). The TGFβ3 promoter appears to contain a region that is positively regulated by raloxifene but that is unrelated to ERE-like sequences. Apparently this region can be activated by both estrogen and raloxifene, despite the fact that it does not have the conventional characteristics of an estrogen receptor.

Summary

The drive and determination to discover osteoinductive factors were responsible for the discovery of the TGFβs and other members of this superfamily in bone. However, with their discovery has come the challenge of unraveling their function in bone and the challenge to control the use and delivery of these factors. Nature has developed a surprisingly complex system of regulation—from latency and activation of the TGFβ molecule to an intricate system of receptors and a vast array of regulatory molecules that either enhance or inhibit the activity of this factor. Greater understanding of TGFβ, its effects, and its regulation is necessary to apply this information for potential clinical use. The challenge continues.

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CHAPTER 50

Bone Morphogenetic Proteins

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Introduction

The idea that the remarkable regenerative capacity of bone resided within bone itself came from the pioneering work of Urist (1965), who described the isolation of a bone inductive extract from adult bone and demonstrated the ability of this extract to induce new endochondral bone formation at ectopic sites in rodents. Much effort went into the characterization of this extract, which Urist called bone morphogenetic protein (BMP), but it was not until the genes responsible for the bone inductive activity were cloned using recombinant DNA methods that our understanding of the exact nature of the signals involved in bone formation began to take shape (Celeste *et al.,* 1990; Kingsley, 1994a; Luyten *et al.,* 1989; Ozkaynak *et al.,* 1990, 1992; Sampath and Reddi, 1981; Sampath *et al.,* 1987, 1991; Urist *et al.,* 1973; Wang *et al.,* 1988, 1990; Wozney *et al.,* 1988). We now recognize that BMPs are part of a large multigene family, the transforming growth factor β (TGF-β) superfamily, whose members are related to each other by relative degrees of sequence similarity but that possess a wide-ranging number of biological functions (Kingsley, 1994b; Rosen and Thies 1992; Fig. 1).

Members of the BMP subfamily show an identifying pattern of seven conserved cysteine residues in the mature, carboxy-terminal portion of the protein, which is where BMP activity resides. Mature BMP proteins are synthesized as larger precursor molecules that are processed to approximately 30,000 molecular weight dimers before their secretion from the cell.

Members of the BMP family initiate their cellular action by binding to transmembrane serine/theronine kinases known as type I and type II receptors. These closely related proteins are composed of a short cysteine-rich extracellular domain, a single transmembrane-spanning domain, and an intracellular domain with serine and theronine kinase regions (Franzen *et al.,* 1993; Lin *et al.,* 1992). BMPs can bind to both type I and type II receptors alone with low affinity (Liu *et al.,* 1995). High-affinity binding of BMPs is only achieved when both receptors are present. The same BMP can bind more than one type II receptor, which in turn can interact with different type I receptors. The nature of the signal seems to be dependent on the composition of the receptor complex and on the specificity of the type I receptor kinase (see Chapter 48).

Once secreted, the availability of BMPs for receptor interactions is mediated by the presence of extracellular antagonists that appear to bind to BMP proteins and prevent their subsequent interaction with BMP receptors. These include noggin for BMP-2, -4, -7, and GDF5 and 6, chordin for BMP-2, -4, and -7, follistatin for BMP-2, -4, -7, and BMP-11/GDF11, and gremlin for BMP-2, -4, and GDF5 (Dionne *et al.,* 2001; Gamer *et al.,* 1999; Gazzero *et al.,* 1998; Pereira *et al.,* 2000; Piccolo *et al.,* 1996). While the role for extracellular BMP antagonists in development is clear, how these same antagonists affect BMP signaling in the adult skeleton is only beginning to be investigated.

The osteogenic capacity of many BMPs has been proven by implanting individual pure recombinant BMPs in the rat ectopic assay, a measure of *de novo* osteoinduction, and using this stringent criteria, BMP-2, BPMs-4 through 7, and BMP-9 have been shown to be bone inductive molecules (Celeste *et al.,* 1992, 1994; D'Alessandro *et al.,* 1991; Gitelman *et al.,* 1994; Wozney *et al.,* 1988). In addition, heterodimeric forms of BMPs exist and several exhibit an enhanced bone inductive ability (Aono *et al.,* 1995; Israel *et al.,* 1996). In each case, the BMP protein acts to initiate the cascade of biological events known as endochondral bone formation, in which mesenchymal stem cells lay down a cartilaginous structure that is then resorbed and replaced by bone (Reddi, 1981) .

The following sections describe current thinking on the utility of BMPs as therapeutic agents and discuss how these

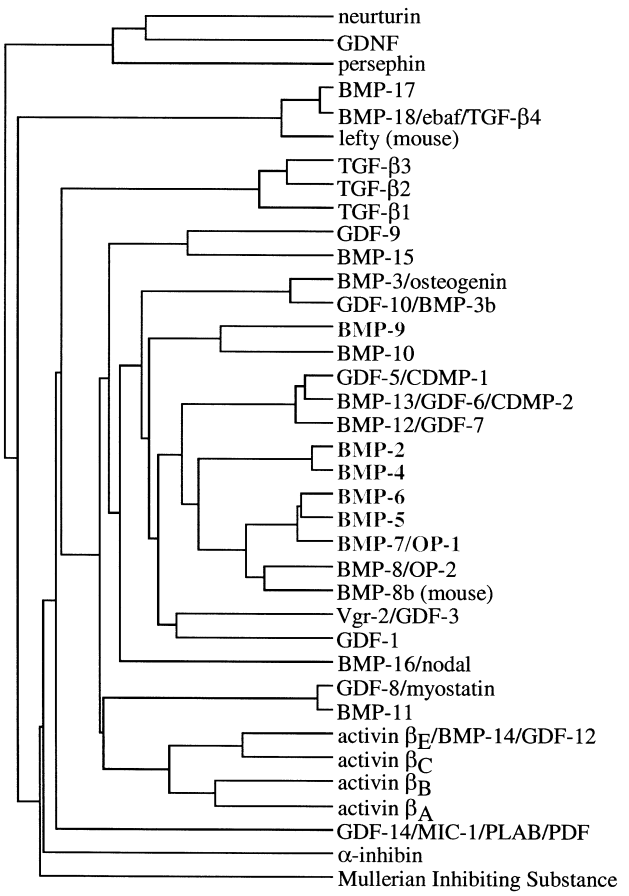

Figure 1 Relationships of BMP/TGF-β gene family members shown as a comparison of their amino acid identities from the first conserved cysteine residue. Osteogenic BMPs are shown in gray. Figure was generated using the GCG program PileUp.

efforts are based on our understanding of the role of BMPs in formation of the embryonic skeleton and maintenance of bone mass in adults.

Therapeutic Applications of Bone Morphogenetic Proteins

Potential therapeutic applications for BMPs are wide ranging and include (1) replacement for bone grafting materials in orthopedic settings, (2) replacing bone grafts for dental applications, and (3) enhancing fixation of prosthetic metal implants. While only small amounts of the osteoinductive BMPs are needed to produce substantial amounts of bone, delivery of these potent molecules must be highly controlled, and BMPs are typically applied with a matrix or carrier. The matrix then aids in defining the shape and volume of the bone being induced and results in the retention of BMP at the site of application long enough for the cellular response to the protein to be initiated. Matrices can be composed of a variety of substances, and the physical form of the material can be optimized for the particular therapeutic application. Materials most commonly studied include collagen (the primary structural protein component of bone and many other tissues), hyaluronic acid, allograft (either mineralized or demineralized), calcium phosphate materials (hydroxyapatite or tricalcium phosphate), and synthetic polymers such as polylactide or poly (lactide-co-glycolide).

Applications of BMPs in Orthopedic Surgery

Large segmental long bone defect models have been extensively utilized to evaluate the osteoinductive potential of BMPs. Advantages of these models include their inability to heal spontaneously (hence, they are "critical-sized"), ease of radiographic evaluation of the bone-induction process, and the ability to unequivocally evaluate the histologic and biomechanic properties of the induced bone. For example, both rhBMP-2 and BMP-7/OP-1 have been shown to heal large defects in species including rats, rabbits, dogs, and monkeys (reviewed in Rosen and Wozney, 2000). These studies have substantiated the potent osteoinductive capacity of these BMPs. In addition, they have demonstrated that the bone induced by BMP intimately associates with the preexisting surrounding bone and remodels consistent with its location (i.e., in response to the biomechanic environment), assuming the biomechanic properties appropriate for the anatomic site. Interestingly, the bone is only formed in the area where the BMP implant was placed, and appears to be self-limiting. This is likely to be due to removal of the inductive substance from the site, as well as upregulation of inhibitory molecules in response to the factor.

Spinal fusion procedures currently utilize large amounts of autogenous bone graft and hence provide an opportunity to evaluate BMPs as a bone graft substitute. RhBMP-2 and BMP-7/OP-1 have been tested successfully in preclinical animal models of both intertransverse process and interbody (in conjunction with interbody fusion cage devices) fusion. For example, rhBMP-2 has been shown to yield successful spinal fusions in rabbit, dog, and monkey models by radiographic, mechanic, and histologic criteria (Boden *et al.,* 1996; David *et al.,* 1999; Sandhu *et al.,* 1995; Sandhu, 1996; Schimandle *et al.,* 1995;). In these studies, BMPs appear to perform equal to, or superior to, autograft. In some cases, fusions form earlier and with higher frequency when BMP is used. Clinical studies utilizing BMPs in spinal fusion applications are ongoing. A small (14 patient) study has been published in which rhBMP-2 was compared to autograft in interbody spinal fusions (Boden *et al.,* 2000). Similar to the preclinical studies, the fusion rate and frequency induced by BMP were at that of autograft. This suggests a significant medical benefit of the use of BMP, as the additional operative procedure of autograft harvest (and associated morbidity) is avoided.

Fracture Repair

Because BMPs are likely physiologic mediators of fracture repair, they are attractive potential therapeutics when insufficient repair process occurs, and perhaps even to

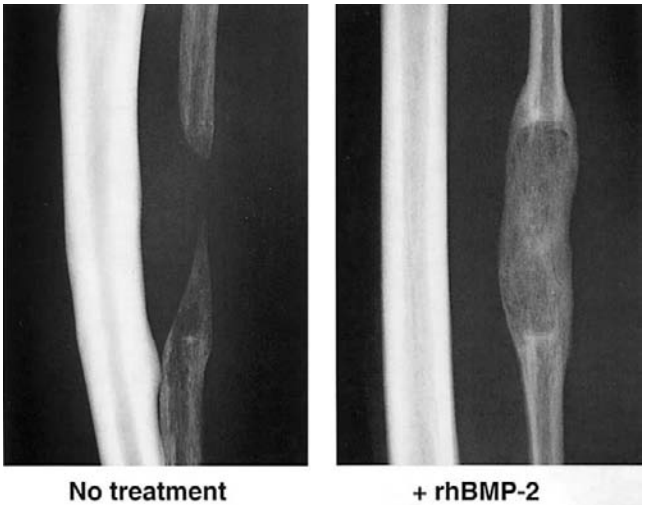

Figure 2 Healing of a critical-sized segmental defect in monkeys by rhBMP-2. Two-centimeter full-thickness defects were created in the fibulae of cynomolgous monkeys. Defects were implanted with rhBMP-2 with a calcium phosphate cement (right) or left untreated (left). Radiographs detail healing after 16 weeks. Note complete regeneration of bone in the rhBMP-2-treated defect compared with minimal bone formation in the untreated control.

accelerate fracture repair. Several animal studies have demonstrated the ability of BMP-2 and BMP-7/OP-1, with or without carriers, to accelerate fracture repair in rabbit, goat, and dog models (Bouxsein *et al.,* 1999; Popich *et al.,* 1997; Turek *et al.,* 1997; Welch *et al.,* 1997). For example, BMP-2 was shown to decrease the time to healing by 35–50% in a rabbit osteotomy model, as assessed by biomechanic criteria. Interestingly, histologic analysis suggested that the addition of exogenous BMP both increased the amount of fracture callus present and accelerated the maturation of the callus, i.e., its conversion from a soft cartilaginous callus to a bridging bony callus (Fig. 2).

Clinical studies have also been performed with BMPs as therapeutics for fracture repair. RhBMP-2/OP-1 combined with a particulate bone matrix carrier has been evaluated in established tibial nonunions. An additional study has evaluated rhBMP-2 with a collagenous sponge carrier in acute tibial fractures. While initial reports on these studies appear promising, full publications on the results of these studies are not available at the time of this writing.

Dental and Craniofacial Applications of BMP Therapeutics

In the dental area, there is a need for bone regeneration to fill tooth extraction sockets and replace bone lost due to periodontal disease. In addition, augmentation of the alveolar bone that has decreased in volume with age is required for the placement of dental implants and subsequent dental restoration. Preclinical studies have documented the ability of BMPs to regenerate large segmental defects in the jaws of rats, dogs, and monkeys, suggesting that BMP has substantial potential for the augmentation of alveolar bone (Boyne *et al.,* 1996; Toruimi *et al.,* 1991). Augmentation of maxillary bone by rhBMP-2 has been shown in goat and monkey models, following implantation in the maxillary sinus (Hanisch *et al.,* 1997a; Margolin *et al.,* 1998; McAllister, 1998; Nevins *et al.,* 1996). In addition, vertical augmentation of mandibular bone by rhBMP-2 has been shown in dog models (Cochran *et al.,* 1999; Hanisch *et al.,* 1997b; Sigurdsson *et al.,* 1997). In both settings, the bone appears to behave as native bone and to support placement and osseointegration of dental implants. These studies have led to a series of clinical studies evaluating rhBMP-2, in combination with a collagen sponge matrix, to augment alveolar bone, including in sinus floor augmentation procedures (Boyne *et al.,* 1997). In these studies, bone, induction was observed by CT scans by 16 weeks after implantation of the BMP. Histologic analysis of core biopsies of the induced bone, taken at the time of implant placement (approximately 6 months postapplication), confirmed the formation of normal bone with extensive vascularization. As predicted from the animal studies, the new bone is capable of supporting placement of dental implants and remodels consistent with the biomechanic forces placed on it. Preliminary studies with OP-1 in sinus floor augmentation procedures have yielded mixed results, with variable amounts of bone induction and some inflammation, perhaps associated with the carrier matrix material (Groeneveld *et al.,* 1999; van den Bergh *et al.,* 2000).

The inflammatory process associated with periodontal disease results in the loss of bone surrounding teeth, as well as the loss of attachment structures. Thus, successful treatment of this condition requires not only regeneration of bone, but also tissues such as cementum and the periodontal ligament. Interestingly, the application of single BMPs, including both BMP-7/OP-1 and BMP-2, has been shown to regenerate periodontal tissues in canine and monkey models (Giannobile *et al.,* 1998; Ishikawa *et al.,* 1994; Ripamonti *et al.,* 1996; Sigurdsson *et al.,* 1995; Wikesjo *et al.,* 1999). Clinical studies with BMPs for periodontal regeneration are also being carried out, although publications on these studies are limited.

BMP Gene Therapy

Gene therapy entails the vectoring of a gene into cells which then synthesize the protein product desired. The gene of interest may be introduced using either just DNA or a vectoring system such as a virus. The gene may be introduced directly into the body, or into cells that are infected *ex vivo,* and then reintroduced into the body. One of the major challenges of this therapy is the continued expression of the transgene; expression typically decreases rapidly with time. Because only transient expression of a BMP is necessary (and desirable) to result in bone induction, it provides a special opportunity for gene therapy. Adenoviral vectors carrying a BMP gene (BMP-2 , BMP-7, or BMP-9) have been used directly in animal models to enhance fracture and

create spinal fusions (Alden *et al.,*1999; Baltzer *et al.,* 2000; Franceschi *et al.,* 2000; Helm *et al.,* 2000; Musgrave *et al.,* 1999).

Bone marrow mesenchymal cells or cell lines have also been infected *ex vivo* with BMP-carrying adenoviral vectors (Lieberman *et al.,* 1998, 1999; Mason *et al.,* 2000; Riew *et al.,* 1998). Upon reintroduction into animals, these cells then have been shown to induce bone and repair both bone and cartilage defects. Finally, direct application of DNA consisting of a BMP gene construct has been shown to enhance long bone repair in a rat model (Fang *et al.,* 1996). Together, these studies suggest the potential for use of BMP-based gene therapies. The technical difficulties and safety considerations with current gene therapy modalities need to be carefully considered relative to the traditional application of the BMP proteins.

BMP Biology

Progress in the therapeutic utility of BMP has been paralleled by increases in our understanding of the roles of individual BMPs during formation of the embryonic skeleton and in the role endogenous BMPs play in skeletal homeostasis in adults.

Role of BMPs on Formation of the Embryonic Skeleton

Localization of BMPs to developing skeletal structures provides primary evidence for the roles of these proteins in skeletal patterning and skeletal cell differentiation. Data obtained from *in situ* hybridization studies confirm that transcripts for BMP-2 through BMP-7, and GDF5–7 are all present in the developing embryo at sites and times consistent with their involvement in mesenchymal condensation and cartilage differentiation (reviewed in Gamer and Rosen, 2000).

While many of the upstream signals that control the expression of individual BMPs at specific sites still need to be defined, data from many laboratories suggest that BMPs, fibroblast growth factors (FGFs), and sonic hedgehog (Shh) interact in a hierarchial way to pattern limb skeletal elements (Bitgood and McMahon, 1995; Laufer *et al.,* 1994; Niswander and Martin, 1993). BMP-2 and -4 transcripts are found in the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA), two important signaling centers (Lyons *et al.,* 1990) that define limb patterning. At these locations, BMP-2 and -4 are thought to act downstream of FGF and Shh signals. Unfortunately, mice carrying null mutations for BMP-2 or BMP-4 die early in embryogenesis before limb patterning occurs (Winnier *et al.,* 1995; Zhang and Bradley, 1996), providing us with little information on specific roles for BMP-2 and -4 in the early limb.

BMP-7 (OP-1) is expressed in the early limb bud in a manner consistent with involvement in early limb formation. Mice lacking BMP-7 survive until birth and display only mild limb skeleton abnormalities (Dudley *et al.,* 1995; Luo *et al.,* 1995). The absence of a strong skeletal phenotype in BMP-7 knockouts may be due to the ability of BMPs to compensate for each other, and BMP-2 and -4 are still present in the limbs of BMP-7 null mice (Dudley and Robertson, 1997). Data from mice carrying null mutations for both BMP-7 and BMPRIB support the idea that BMPs have overlapping functions in skeletal development (Yi *et al.,* 2000).

A clear example of BMPs directing the differentiation of specific skeletal structures is in the GDF5 null mouse, which exhibits a brachypodism phenotype. bp/bp mice display shortening of the long bones of the appendicular skeleton, reduction in the number of digits in the paws, and misshapen bones in both the front and hind feet (Gruneberg and Lee, 1973; Storm *et al.,* 1994). In these mice, expression of GDF5 transcripts normally occurs at all sites where skeletal malformations take place. The lack of GDF5 signals results in failure of joint morphogenesis between individual bones and in alterations in the size and shape of the mesenchymal condensations themselves. These changes are paralleled in humans where mutations in the GDF5 gene result in joint dysmorphogenesis at sites where GDF5 is normally expressed, and in this instance the skeletal anomalies can be directly related to the expression pattern of GDF5 (Polinkovsky *et al.,* 1997; Thomas *et al.,* 1997; Weinstein *et al.,* 2000).

BMP-6 expression appears to mark chondrocytes actively undergoing hypertrophy, and misexpression experiments using the chick limb have established a temporal relationship between the expression of BMP-6 and Indian hedgehog (Ihh) in chondrocyte hypertrophy and replacement by bone (Vortkamp *et al.,* 1996). It is surprising then that BMP-6 null mice appear to have normal skeletons at birth, suggesting that BMP-6 is not required for endochondral ossification to occur (Solloway *et al.,* 1998).

In the axial skeleton, BMP-5 appears to play a central role in formation of the cartilaginous structures of the outer ear, the sternum, and the ribs. Mice lacking a functional BMP-5 gene display the short ear (se) phenotype, with characteristic defects in each of these structures (King *et al.,* 1994). These defects are evident early in skeletal development, as condensations that give rise to affected structures are altered in size and shape. BMP-5 transcripts can also be found in other skeletal elements where no effect of the loss of BMP-5 is observed (Kingsley *et al.,* 1992). Here, the presence of other osteogenic BMPs may compensate for the absence of BMP-5.

The ability to remove specific BMPs and observe skeletal development in mice has clarified the role of BMP-3 in the skeleton. Much historical information on BMP-3 exists; it is the most abundant BMP in bone matrix, and highly purified osteogenic fractions from bone, named osteogenin by Reddi, display bone inductive activity (Luyten *et al.,* 1992; Sampath *et al.,* 1987). However, efforts to show that recombinant BMP-3 induces bone formation have been unsuccessful (Takao *et al.,* 1996). Most surprisingly, examination of the phenotype of BMP-3 null mice revealed that

the loss of BMP-3 during embryogenesis does not affect skeletal patterning, as the size, shape, and location of all skeletal elements appear normal (Daluski *et al.,* 2001). Adult BMP-3 null mice display increased bone density, with a trabecular volume twice that of wild-type littermates at 5–6 weeks of age. While the cellular basis for this phenotype is currently under investigation, preliminary data suggest that BMP-3 can antagonize BMP-2 activity by acting through the activin receptor pathway to modulate BMP-2-mediated transcriptional events. Careful evaluation of the role of BMP-3 in adult bone should help determine the usefulness of modulating endogenous levels of BMP-3 for regulation of bone mass.

GDF11, a recently described BMP family member, is expressed during mouse embryogenesis in a pattern consistent with its involvement in skeletal patterning and differentiation (Gamer *et al.,* 1999; Nakashima *et al.,* 1999). While GDF11 protein shows no osteoinductive activity in the rat ectopic assay (K. Cox, personal communication), mice lacking functional GDF11 exhibit extensive axial skeletal patterning defects, as well as palate abnormalities. This mutant skeletal phenotype is thought to be due to broad homeotic transformation of vertebrae to more anterior developmental fates, resulting in additional lumbar and thoracic vertebrae and the complete absence of a tail (McPherron *et al.,* 1999). Detailed analysis of GDF11 transcripts in the chick limb show that it is highly expressed in limb bud mesenchyme, but is excluded from the central core of prechondrogenic condensations. Ectopic addition of GDF11 to developing limbs results in foreshortening of the limbs through inhibition of both chondrogenesis and myogenesis (Gamer *et al.,* 2000). These observations suggest additional roles for BMP-like molecules as negative regulators of skeletal patterning.

Tooth formation also appears to be BMP mediated, and specific BMPs have been localized in early dental epithelium, in dental mesenchyme, and finally during terminal differentiation of both odontoblasts and ameloblasts (Thesleff *et al.,* 1995; Thesleff and Nieminen, 1996; Vanio *et al.,* 1993).

In addition to the examination of BMP expression, localization of BMP receptors has provided us with clues to BMP responsive cell types during skeletal development. In the chick, BMPRIB is expressed in precartilaginous condensations, whereas BMPRIA is expressed at low levels throughout the limb mesenchyme, with higher levels seen in regions of cell fate specification in the posterior distal aspect of the limb (Kawakami *et al.,* 1996; Zou *et al.,* 1997). At later stages, BMPRIA is highly expressed in the prehypertrophic chondrocytes (Zou *et al.,* 1997). The BMP type II receptor is also found in precartilaginous condensations, as well as in the AER and interdigitial mesenchyme (Kawakami *et al.,* 1996). Studies with dominant-negative and constitutively active BMP receptors in chick have revealed critical roles of BMP signaling in chondrogenesis and skeletal patterning (Enomoto-Iwamoto *et al.,* 1998; Zhou and Niswander, 1996). In addition, data from studies in which BMPRIB has been inactivated in mice show that significant receptor function overlap exists and that loss of BMPRIB results in small alterations in skeletal structures, suggesting that BMPRIB has broadly overlapping functions with other BMP receptors during skeletal development (Baur *et al.,* 2000; Yi *et al.,* 2000).

Developmental analysis of the function of BMP antagonists suggests that they guard against BMP activity during formation of the skeleton. Inactivation of the noggin gene in mice results in hyperplasia of cartilage condensations and failure of joint formation (Brunet *et al.,* 1998). This phenotype is reminiscent of that seen in humans with missense mutations in noggin. Both proximal symphalangism and multiple synostosis syndrome, autosomal-dominant disorders, are characterized by joint fusion caused by the union of two individual bones (Gong *et al.,* 1999). Gain-of-function studies in the chick limb have also shown that too much noggin blocks BMP activity and inhibits normal skeletal chondrogenesis (Capdevila *et al.,* 1998; Pathis *et al.,* 1999). *In vitro* studies using primary fetal osteoblasts have shown that BMP-2 induces noggin expression in target cells, creating a negative feedback loop, which tightly controls BMP activity (Gazzerro *et al.,* 2000).

The physiological role of other BMP antagonists such as chordin, follistatin, and members of the Dan family in skeletal patterning and skeletal cell differentiation has also suggested that these proteins act to dampen BMP activity at sites where it is expressed (Gamer and Rosen, 2000).

Osteogenesis in the Adult Skeleton

While determination of the size, shape, and location of skeletal elements is an embryonic event, the adult skeleton undergoes continuous turnover, a process known as bone remodeling. Removal of existing bone and its replacement by new bone is carried out in a highly controlled manner and requires the differentiation of both osteoblasts and osteoclasts from precursors located in the bone marrow environment (Manolagas and Jilka, 1995). The exact nature of the signals that control remodeling remains to be established, but it is likely that both osteoblast and osteoclast precursors are affected by systemic and local signals and by mechanical stimulation (Rodan, 1998).

BMPs have been proposed to be the local signals that induce commitment of mesenchymal stem cells resident in bone marrow into osteoprogenitors and osteoblasts (Abe *et al.,* 2000; Manolagas and Weinstein, 1999). Several lines of evidence make this hypothesis an attractive one. Much data exist to show that BMPs are present in bone matrix in a form that allows for their release or presentation to marrow stromal cells, cells that can differentiate into osteoblasts in response to BMPs (Rosen *et al.,* 1996). Osteoblasts have also been shown to synthesize and secrete BMPs both *in vitro* and *in vivo,* suggesting that once BMPs initiate MSC differentiation, a positive feedback loop is created, allowing for the production of additional BMP signals (Suzawa *et al.,* 1999). The BMP antagonists noggin and gremlin are

present in bone and are made by osteoblasts (Gazzarro *et al.,* 1998; Pereira *et al.,* 2000), suggesting that local activation of MSC may be highly controlled. It has also been reported that BMP-1, the procollagen C propeptide, is able to release BMPs from collagenous matrix, providing another way that endogenous BMPs may be made available in a site-specific manner during the remodeling process (Wardle *et al.,* 1999). Finally, bone formation and bone resorption during remodeling may be linked through BMPs, as BMPs are thought to regulate the transcription of several osteoblast-specific transcription factors, which in turn may regulate transcription of RANK ligand, a signal important for the differentiation of hematopoietic progenitors into osteoclasts (Manolagas and Weinstein, 1999). If this is the case, BMPs may be involved in every step of the remodeling process.

These tantalizing links between BMPs and bone remodeling have become the focus of much research and may lead to new therapeutic approaches to osteopenia. Of fundamental importance in designing treatments that utilize site-specific activation of endogenous BMPs is the identification of which BMPs are available to affect bone remodeling and which cell types at each step of the remodeling process are the targets of these signals. Once the BMPs necessary for remodeling are identified, it will be necessary to determine whether factors that are known to change the balance in remodeling toward formation or resorption do so through effects on these BMPs. *In vitro* studies have shown that estrogens and glucocorticoids, two agents known to affect remodeling, increase BMP-6 synthesis by osteoblasts (Boden *et al.,* 1997; Ricard *et al.,* 1998). However, mice in which the BMP-6 gene has been removed through homologous recombination in ES cells have no apparent skeletal defects and do not develop osteopenia, suggesting that BMP regulation of remodeling is likely to be complex (Solloway *et al.,* 1998).

It will also be of great interest to determine whether the decrease in MSC present in bone marrow that occurs with aging is related to the changes in levels of specific BMPs found in bone matrix in older animals (D'Ippolito *et al.,* 1999). If BMPs are required for MSC survival, as well as MSC differentiation, changing the BMP content of bone matrix and bone marrow may have important clinical benefits. A hint that this may be the case has been provided by the production of transgenic mice in which noggin, a potent BMP antagonist, is overexpressed using the bone-specific osteocalcin promoter. These mice have osteopenia and are prone to fracture, suggesting that a reduction in osteogenic BMPs available to the postnatal skeleton reduces overall bone formation and bone healing (Devlin *et al.,* 2000).

New bone formation also occurs in the adult skeleton during the process of fracture repair, when osteoblast precursors resident in marrow and periosteum differentiate into osteoblasts in a highly regulated manner (Rosen and Thies, 1992). BMPs have been shown to be present at fracture repair sites, and several lines of evidence support the role of BMPs in this process. First, all of the cell types that synthesize new bone during fracture healing have been shown to be targets for BMPs *in vitro* and to possess BMP receptors *in vivo* (Bostrom *et al.,* 1995). In addition, immunohistochemical identification of BMP-2 and -4 at the fracture site in rats suggests that BMPs may be released from bone matrix at the time of fracture and are then able to act on target cells in the area or recruit cells to the fracture site, where they differentiate into osteoblasts (Nakase *et al,* 1994). Alternatively, BMPs may be synthesized by osteoblasts resident in bone and activated by other cytokines released during the fracture healing process. While we still do not know which BMPs are absolutely required for fracture repair, data from the *short ear mouse,* a naturally occurring null for BMP-5, show that mice lacking BMP-5 have a reduced capacity to repair rib fractures as adults (Green, 1958). The availability of animal models in which specific BMPs, BMP receptors, and BMP antagonists have been removed should allow us to address this issue and to begin to understand what regulates endogenous BMP production during fracture healing.

Another circumstance in which the adult skeleton produces new bone is during distraction osteogenesis, a method of bone lengthening that takes advantage of the inherent capacity of bone to repair after breaking (Paley, 1988). In a clinically controlled setting, the bone to be lengthened is broken and the two opposing ends are increasingly separated from each other over the course of days or weeks. The end result of this engineered trauma is bone regeneration tailored to the distance of bone separation (Yasui *et al.,* 1997). We are just beginning to understand the molecular and cellular events that form the basis of distraction osteogenesis, and from these initial studies, it seems likely that BMPs are important mediators of bone formation at the distraction site. Data demonstrate temporal and spatial localization of mRNAs for BMPs in a rat model of distraction osteogenesis and link the mechanical stress/tension needed for successful distraction osteogenesis with BMP gene expression (Sato *et al.,* 1999). It is likely that mechanical forces influence BMP gene expression at sites of distraction osteogenesis through a yet to be understood process and that BMPs are components of the complex signals required during several stages of the repair. The precise link between mechanical stress and BMP gene expression remains to be discovered, as do the other signaling pathways that must interact with BMPs to produce the cascade of events that results in temporally delayed bone formation. Understanding what regulates the temporal differences in bone formation seen between distraction osteogenesis and normal fracture healing, that are thought to be BMP-mediated events, should provide insight into how BMPs affect the rate of repair.

Data from Mundy *et al.* (1999) identified statins as a class of previously unknown bone anabolic agents. Preliminary studies suggest that statins promote bone formation *in vivo* by specifically activating the BMP-2 promoter in osteoblasts and osteoprogenitor cells. These findings provide the rationale for the development of systemic agents

that would promote bone formation via activation of BMP-2 transcription at skeletal sites, creating a new therapeutic modality for osteoporosis.

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CHAPTER 51

Bone Morphogenetic Protein Receptors and Actions

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Introduction: BMPs

Bone morphogenetic proteins (BMPs) were originally identified as proteins that induce the formation of bone and cartilage tissues when implanted at ectopic sites in rats (Wozney *et al.,* 1988; Reddi, 1998). *In vitro,* BMPs have potent effects on the regulation of growth and differentiation of chondroblast and osteoblast lineage cells. Moreover, BMPs have been shown to be multifunctional cytokines with a wide range of biological activities in various cell types, including epithelial cells, mesenchymal cells, monocytes, and neuronal cells (see Chapter 50).

BMPs belong to a larger superfamily, known as the transforming growth factor-β (TGF-β) superfamily, which includes TGF-βs, activins and inhibins, and Müllerian inhibiting substance (MIS). More than a dozen molecules have been identified in the BMP subfamily; these can be classified into several subgroups based on similarities in structure and function. BMP-2 and BMP-4 are highly similar and form one subgroup (BMP-2/4 group). BMP-5, BMP-6, BMP-7 (also termed osteogenic protein-1; OP-1), and BMP-8 (OP-2) form another subgroup (OP-1 group). Growth/differentiation factor-5 (GDF-5, also termed cartilage-derived morphogenetic protein-1, CDMP-1), GDF-6 (CDMP-2, also termed BMP-13), and GDF-7 (BMP-12) belong to a third subgroup (GDF-5 group) (Fig. 1) (Kawabata and Miyazono, 2000). Most of the members of the BMP-2/4 and OP-1 groups have been shown to induce formation of bone and cartilage tissues *in vivo,* whereas those of the GDF-5 group induce cartilage and tendon-like tissues. In contrast, certain other BMPs appear not to induce formation of bone and cartilage tissues *in vivo.* Myostatin (also termed GDF-8) plays a central role in muscle formation, but probably not in bone formation.

In this chapter, mechanisms for the signal transduction by BMPs, mainly those in the BMP-2/4, OP-1, and GDF-5 subgroups, are described and compared to the TGF-β signaling system. Although the signaling mechanisms of serine/threonine kinase receptors and Smads have been intensively studied in invertebrates, i.e., in *Drosophila* and *C. elegans* (Zimmerman and Padgett, 2000), BMP signaling pathways in mammals will be the principal focus of this chapter.

BMP Receptors

Serine/Threonine Kinase Receptors

Members of the TGF-β superfamily bind to two distinct types of serine/threonine kinase receptors, termed type I and type II receptors (Heldin *et al.,* 1997). Both type II and type I receptors are essential for signal transduction. TGF-β and activin ligands bind to type II receptors independently of type I receptors, whereas type I receptors can bind these ligands only in the presence of type II receptors. In contrast, BMPs weakly bind to type II receptors independently of type I receptors, but their binding affinity is dramatically increased when both types of receptors are present (see below) (Liu *et al.,* 1995; Rosenzweig *et al.,* 1995). The type II receptor transphosphorylates the type I receptor, which transmits intracellular signals.

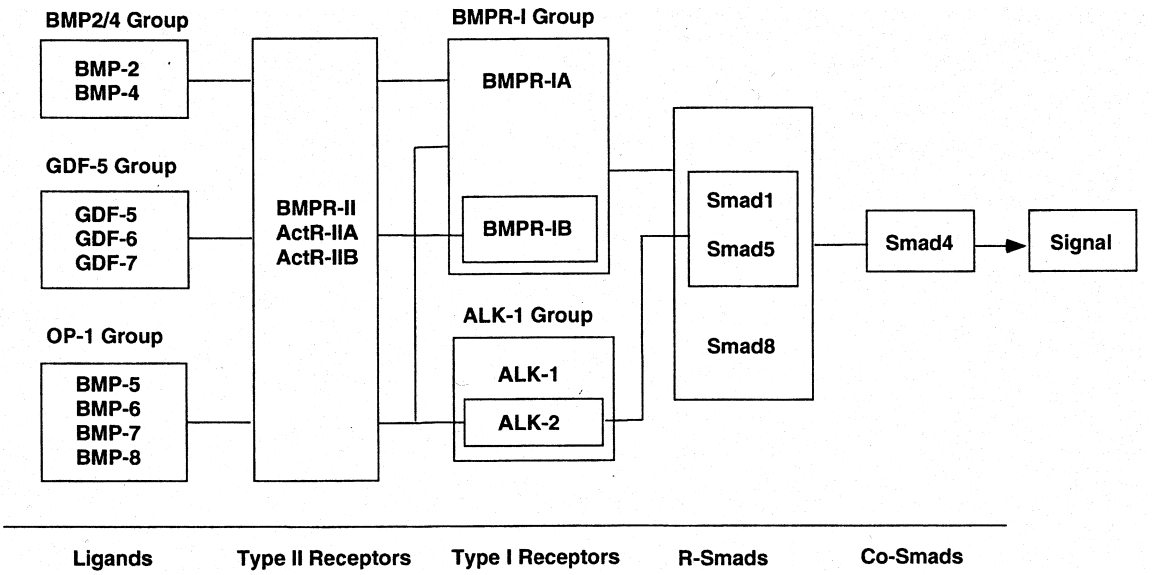

Figure 1 Relationships between BMPs, type II and type I receptors, and Smad proteins in signal transduction. Among the receptors of the ALK-1 group, only ALK-2 serves as a receptor for the BMPs in the OP-1 group. Similarly, BMPR-IB, but not BMPR-IA, preferentially functions as a type I receptor for GDF-5. Of the BMP-R-Smads, Smad1 and Smad5 are activated by ALK-2, whereas all three BMP-R-Smads are activated by BMPR-IA and BMPR-IB.

The structures of the type I and type II receptors are schematically shown in Fig. 2A. They have a relatively short extracellular domain, a single membrane-spanning domain, and an intracellular domain containing a serine-/threonine kinase domain. The extracellular domains have several conserved cysteines, which are important for the formation of characteristic three-dimensional structures (Greenwald *et al.,* 1999; Kirsch *et al.,* 2000a). In addition to the serine/threonine kinase domain, the intracellular domains of type I receptors, but not those of type II receptors, have a characteristic GS domain (glycine and serine-rich domain) located N-terminal to the serine/threonine kinase domains. In the kinase domain, type I receptors have a characteristic structure, denoted L45 loop, between kinase subdomains IV and V. The C-terminal tails of type I receptors are very short (less than 9 amino acids), whereas those of type II receptors are more than 24 amino acids long. The C-terminal tail of BMPR-II is much longer (530 amino acids) than other type II receptors. Although the functional importance of the C-terminal tail of BMPR-II is unknown, mutations in this domain have been found in some patients with familial primary pulmonary hypertension (PPH) (see below) (IPPH Consortium, 2000; Deng *et al.,* 2000).

BMP Type I Receptors

Seven type I receptors, termed activin receptor-like kinases (ALKs) 1 through 7, have been identified in mammals. ALKs can be classified into three subgroups, i.e., the BMPR-I group, the ALK-1 group (Fig. 1), and the TβR-I group (Kawabata and Miyazono, 2000). ALK-3 and ALK-6 are very similar to each other (BMPR-I group), and are also denoted BMP type IA and type IB receptors (BMPR-IA and BMPR-IB), respectively. The kinase domains of BMPR-IA and BMPR-IB have 85% amino acid sequence identity. Functional differences between the intracellular domains of BMPR-IA and BMPR-IB have not been determined. ALK-5, ALK-4, and ALK-7 are structurally similar to each other (TβR-I group), and ALK-5 and ALK-4 serve as TGF-β and activin type I receptors (TβR-I and ActR-IB), respectively. Ligands for ALK-7 have not yet been identified. ALK-1 and ALK-2 are structurally similar to each other (ALK-1 group), and they are distantly related to the other type I receptors. ALK-1 is highly expressed in endothelial cells and binds TGF-β in these cells (Oh *et al.,* 2000). ALK-2 is a type I receptor for certain BMPs, including BMP-6 and BMP-7 (ten Dijke *et al.,* 1994; Macías-Silva *et al.,* 1998; Ebisawa *et al.,* 1999). Despite the low degree of similarity between the receptors of the ALK-1 group and those of the BMPR-I group, they transduce similar intracellular signals.

BMPR-IA is expressed in various types of cells, including human foreskin fibroblasts, MC3T3-E1 osteoblasts, C2C12 myoblasts, and ROB-C26 osteoprogenitor cells (ten Dijke *et al.,* 1994; Nishitoh *et al.,* 1996; Ebisawa *et al.,* 1999). ALK-2 is also widely expressed in various cells, including human foreskin fibroblasts, MC3T3-E1 cells, and C2C12 cells. In contrast, expression of BMPR-IB is restricted to certain cell types, including ROB-C26 cells and glioblastoma cell lines.

Members of the BMP family bind with different affinities to BMPR-IA, BMPR-IB, and ALK-2, overexpressed in mammalian cells. Under physiological conditions, BMP-2 and BMP-4 bind to BMPR-IA and BMPR-IB (ten Dijke *et al.,* 1994). In contrast, BMP-6 and BMP-7 bind strongly

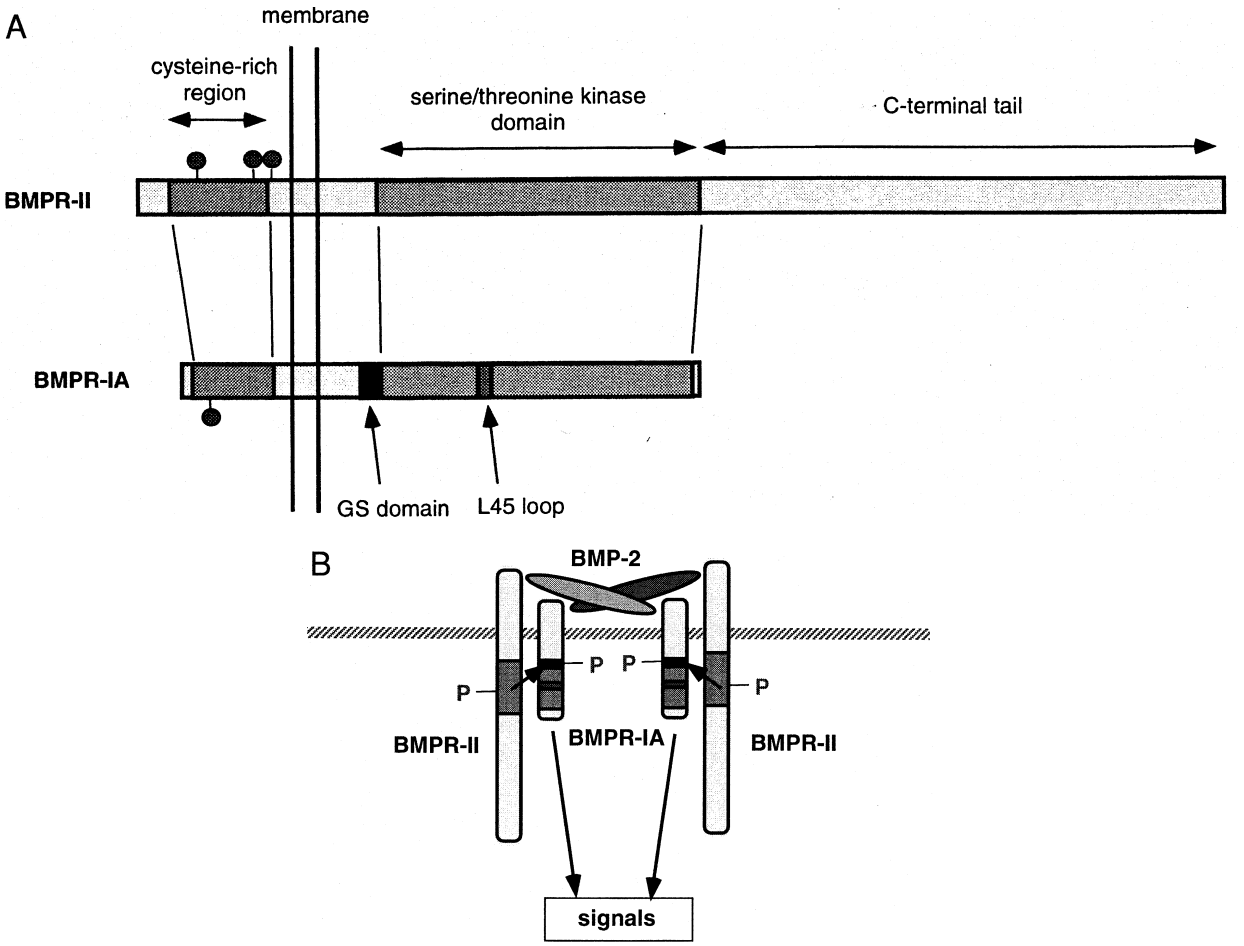

Figure 2 BMP type II and type I receptors. (A) Structure of BMPR-II and BMPR-IA. ActR-IIA and ActR-IIB have similar structures, but shorter C-terminal tails than BMPR-II. BMPR-IB and ALK-2 have overall structures similar to that of BMPR-IA. Closed circles indicate N-glycosylation sites. (B) BMP-2 has an elongated structure, and binds to BMPR-IA and BMPR-II through wrist and knuckle epitopes, respectively. BMPR-II transphosphorylates the GS domain of BMPR-IA, and BMPR-IA kinase transduces intracellular signals.

to ALK-2, and weakly to BMPR-IB (Ebisawa *et al.,* 1999). GDF-5 preferentially binds to BMPR-IB, but not to other receptors (Nishitoh *et al.,* 1996). In addition to members of the BMP family, MIS binds to BMPR-IB together with its specific type II receptor, MISR-II (Gouédard *et al.,* 2000). Thus, BMP type I receptors are shared by certain other members of the TGF-β superfamily. Although ALK-2 was originally identified as a type I receptor for activin (ActR-I), under physiological conditions it functions as a receptor for BMPs but not for activins.

Truncated forms of BMPR-IA and BMPR-IB, lacking the intracellular domains, or kinase inactive forms with mutations in the ATP-binding sites, have been widely used to study the functions of BMPs. BMPs induce differentiation of C2C12 cells into osteoblast-like cells, but in the presence of the truncated form of BMPR-IA, cells fail to become osteoblasts, differentiating instead into mature myocytes (Namiki *et al.,* 1997). In chick limbs, BMPs induce apoptosis in interdigital tissues, since truncated forms of BMPR-IA and BMPR-IB induce web formation in these regions (Zou and Niswander, 1996; Yokouchi *et al.,* 1996).

The two unique structures in the intracellular domain, i.e., GS domain and L45 loop, are observed only in type I receptors, but not in type II receptors. Mutations in the GS domains (see below) lead to constitutive activation of type I receptors. The constitutively active BMP type I receptors, including those of the BMPR-I group and the ALK-2 group, stimulate osteoblast differentiation *in vitro* (Fujii *et al.,* 1999). The L45 loop located between kinase subdomains IV and V of type I receptors is most important for the determination of intracellular signaling.

BMP Type II Receptors

Three receptors, i.e., BMPR-II, ActR-IIA, and ActR-IIB, serve as type II receptors for BMPs. BMPR-II is specific for BMPs, whereas ActR-IIA and ActR-IIB are shared by activins and BMPs. In contrast to type I receptors, these type II receptors appear to bind most BMPs.

Two alternatively spliced variants of BMPR-II have been identified (Rosenzweig *et al.,* 1995; Liu *et al.,* 1995). The long form of BMPR-II is composed of 1038 amino

acids, and includes the long C-terminal tail rich in serines and threonines. The short form lacks the long C-terminal tail, but its expression is rare in most tissues. The long and short versions have been reported to be functionally indistinguishable in *Xenopus* assays (Ishikawa *et al.,* 1995); however, nonsense mutations in the C-terminal tail have been found in some patients with familial PPH, suggesting that this region plays an important role in signaling activity (IPPH Consortium, 2000; Deng *et al.,* 2000).

BMPR-II binds ligands only weakly by itself, but its ligand-binding affinity is increased in the presence of type I receptors. ActR-IIA and ActR-IIB bind activins and BMPs, but the binding affinities of ActR-IIA/IIB to BMPs are lower than those to activins (Yamashita *et al.,* 1995). ActR-IIA/IIB binds activins with high affinity in the absence of type I receptors. Similar to BMPR-II, however, ActR-IIA/IIB binds BMPs with high affinity only in the presence of type I receptors (Rosenzweig *et al.,* 1995; Liu *et al.,* 1995).

BMPR-II and ActR-IIA/IIB are widely expressed in various tissues. BMPR-II is expressed in skeletal muscle, heart, brain, and lung, and plays an important role in mesoderm formation during early embryogenesis (Beppu *et al.,* 2000).

Molecular Mechanism of BMP Receptor Activation

BMP Binding to Receptor Extracellular Domains

The extracellular domains of BMP type I receptors interact with ligands even in the absence of type II receptors. Since a soluble form of BMPR-IA lacking the transmembrane and intracellular domains binds BMPs in the absence of type II receptors, it may be useful as an antagonist of BMPs (Natsume *et al.,* 1997). The binding affinity of BMP-2 for soluble BMPR-IA is about 1 nM, and that of BMPR-IB is about 11 nM (Kirsch *et al.,* 2000b). In contrast, binding affinities for the extracellular domains of BMPR-II and ActR-IIA are only 100 and 50 nM, respectively.

Two receptor-binding motifs have been identified in BMP-2 (Kirsch *et al.,* 2000b) (Fig. 2B). A large epitope 1 (termed "wrist epitope") is a high-affinity binding site for BMPR-IA, and a smaller epitope 2 (termed "knuckle epitope") is a low-affinity binding site for BMPR-II. The wrist epitopes from both monomers contribute to binding to BMPR-IA, whereas the knuckle epitope from only one monomer is involved in the binding to BMPR-II. The wrist and knuckle epitopes are very closely located to each other, possibly facilitating interaction between BMPR-IA and BMPR-II in activation of the receptor kinases. Interestingly, certain BMP-2 mutants in the knuckle epitope (Ala-34 and Leu-90), but not in the wrist epitope, function as antagonists of BMP-2, since these mutants bind to BMPR-IA with the wrist epitope, but fail to interact with BMPR-II through the mutated knuckle epitope.

The extracellular domain of ActR-IIA has a structure termed three-finger toxin fold, composed of three pairs of β-strands projecting from the palm domain with a conserved scaffold of disulfide bridges (Greenwald *et al.,* 1999). The extracellular domains of ActR-IIA and BMPR-IA have very similar three-dimensional configurations but with some differences (Kirsch *et al.,* 2000a). The BMPR-IA extracellular domain consists of two β-sheets and one α-helix (helix α1); the latter is missing in ActR-IIA, and may serve as a key element in type I receptors for specific ligand-binding.

Activation of Serine/Threonine Kinases

Small fractions of type II and type I receptors are present as preexisting homodimers as well as heterodimers on the cell surface in the absence of ligand stimulation (Gilboa *et al.,* 2000). All BMP receptors form oligomers, including type II/type I heteromers (BMPR-II/BMPR-IA and BMPR-II/BMPR-IA) and type II/type II and type I/type I oligomers (BMPR-II/BMPR-II, BMPR-IA/BMPR-IA, BMPR-IB/BMPR-IB, and BMPR-IA/BMPR-IB). Ligand binding increases significantly receptor hetero- and homooligomerization, except for BMPR-II homodimers. Receptor oligomerization may also induce conformational alterations of these receptor molecules.

The type II receptor kinase is constitutively active in the absence of ligand. Upon ligand binding, the type II receptor kinase transphosphorylates the GS domain of type I receptor, resulting in the activation of type I receptor kinases (Fig. 2B). The intracellular substrates are then activated by the type I kinase (Wrana *et al.,* 1994).

Phosphorylation of the GS domain is a critical event in signal transduction by the serine/threonine kinase receptors. Crystallographic analysis of the intracellular domain of TβR-I revealed that the inactive conformation of the TβR-I kinase is maintained by physical interaction between the GS domain, the N-terminal lobe, and the activation loop of the kinase (Huse *et al.,* 1999). Upon phosphorylation of the GS domain by TβR-II, the receptor is converted to an active conformation. Mutations of Thr-204 in TβR-I and the corresponding Gln in BMP type I receptors to acidic amino acids (Asp or Glu) lead to constitutive activation of the type I receptors (Wieser *et al.,* 1995). The constitutively active type I receptors transmit intracellular signals in the absence of ligands or type II receptors, indicating that type I receptors are a downstream component of the type II receptor signaling pathways and that they determine the specificity of the intracellular signals. However, truncation of the C-terminal tail of BMPR-II was found in some familial PPH patients, suggesting that yet unidentified signals may be transduced through BMPR-II (IPPH Consortium, 2000; Deng *et al.,* 2000).

FKBP12 is a binding protein for immunosuppressants such as FK506 and rapamycin. It binds to the Leu–Pro sequence in the GS domain of type I receptors, and negatively regulates the activity of type I receptors (Wang *et al.,*

1996; Chen *et al.,* 1997). FKBP12 thus prevents spontaneous activation of type I receptors by type II receptors.

The L45 loop of the N lobe in the type I receptor protrudes from the kinase domain (Huse *et al.,* 1999) and specifically interacts with intracellular substrates, e.g., Smads (Feng and Derynck, 1997). Amino acid sequences of the L45 loop are conserved in each type I receptor subgroup, but diverge between different subgroups. The L45 loop of the BMPR-I group is more similar to that of the TβR-I group than that of the ALK-1 group; however, receptors of the BMPR-I and ALK-1 groups activate a similar set of Smads, while those of the TβR-I group activate a distinct set of Smads.

PSEUDORECEPTOR: BAMBI

Xenopus BAMBI is a pseudoreceptor for serine/threonine kinase receptors with a high degree of sequence similarity to the human *nma* gene product (Onichtchouk *et al.,* 1999). BAMBI/Nma is structurally related to type I serine/threonine kinase receptors, but the former does not have the intracellular domain. BAMBI stably interacts with various type I and type II receptors and inhibits signaling by these receptors. Since the expression of BAMBI and *nma* is induced by BMP and TGF-β (Onichtchouk *et al.,* 1999; Akiyoshi *et al.,* 2001), BAMBI/Nma may regulate BMP/TGF-β signaling via a negative feedback loop.

Mutations of BMP Receptors *in Vivo*

During embryogenesis, BMPR-IA is ubiquitously expressed, while expression of BMPR-IB is more limited. In adult tissues BMPR-IB is observed only in brain. BMPR-IB and BMPR-IA are expressed in glioblastoma (Yamada *et al.,* 1996), and their expression correlates very well with the malignancy grade. Since BMPs induce differentiation of neuroepithelial cells into astrocytes, BMPs may play an important role in preventing the progression of this tumor.

BMPs play essential roles during embryogenesis, and mice lacking BMP-2 or BMP-4 exhibit defects in mesoderm formation during early embryonic stages. Similarly, gene targeting of *BMPR-IA* or *BMPR-II* leads to defects in mesoderm formation. Homozygous *BMPR-IA* mutant mice die by E9.5 due to defects in mesoderm formation during gastrulation (Mishina *et al.,* 1995). *BMPR-II* null mice also die by E9.5, and their phenotype is very similar to that of *BMPR-IA*$^{-/-}$ mice (Beppu *et al.,* 2000).

BMPR-IB-deficient mice are viable, but exhibit short limbs and abnormal digit cartilage, similar to *GDF-5* null mice (Baur *et al.,* 2000; Yi *et al.,* 2000). Analysis of *GDF-5*; *BMPR-IB* double mutant mice revealed that GDF-5 is a physiological ligand for BMPR-IB *in vivo,* although other receptors may also be involved in the GDF-5 signaling pathway. In addition, analysis of *BMP-7*; *BMPR-IB* double knockout mice revealed that BMP-7 and BMPR-IB may act in part in overlapping pathways.

Some, but not all, *ActR-IIA*-deficient mice exhibit hypoplasia of the mandible and other skeletal abnormalities (Matzuk *et al.,* 1995). Defects in the reproductive system and decrease in the secretion of follicle stimulating hormone (FSH) are also observed in these mice. *ActR-IIB* null mice exhibit complicated cardiac defects, and abnormalities in lateral asymmetry and vertebral patterning (Oh and Li, 1997). ActR-IIA and ActR-IIB also play important roles in the formation of the endocrine pancreas (S. K. Kim *et al.,* 2000).

Primary pulmonary hypertension is characterized by stenosis of precapillary pulmonary arteries. Proliferation of endothelial cells and smooth muscle cells is observed in the pulmonary arteries of PPH and leads to high pulmonary artery pressure. Familial PPH occurs as an autosomal dominant disorder with reduced penetrance. Mutations in the *BMPR-II* gene are found in some familial PPH patients (IPPH Consortium, 2000; Deng *et al.,* 2000). Mutations can be observed in various regions of this gene, including the extracellular domain, transmembrane domain, intracellular domain, and even the C-terminal tail. BMP signaling may thus play an important role in the maintenance of vascular integrity in the pulmonary arteries.

Intracellular Signaling

Signaling by Smad proteins

STRUCTURE OF SMADS

Three Classes of Smads Smads are the major signal transducers for the serine/threonine kinase receptors (Heldin *et al.,* 1997; Miyazono *et al.,* 2000). Activated type I receptor kinases phosphorylate receptor-regulated Smads (R-Smads). R-Smads then form a complex with common-partner Smads (Co-Smads) and translocate to the nucleus. The oligomeric Smad complexes regulate the transcription of target genes through interaction with various transcription factors and transcriptional coactivators or corepressors. Inhibitory Smads (I-Smads) negatively regulate the action of R-Smads and/or Co-Smads.

Eight different Smads have been identified in mammals. They can be classified into three subclasses as described above, i.e., R-Smad, Co-Smad, and I-Smad. R-Smads are subdivided into those activated in BMP signaling pathways (BMP-R-Smads) and those in TGF-β/activin signaling pathways (TGF-β-R-Smads). BMP-R-Smads include Smad1, Smad5, and Smad8, while Smad2 and Smad3 are TGF-β-R-Smads. Smad4 is the only Co-Smad in mammals, shared by both the BMP and the TGF-β/activin signaling pathways. Smad6 and Smad7 are I-Smads. Smad1 and Smad5 have been shown to induce the differentiation of C2C12 myoblasts into osteoblasts, a process facilitated by the addition of Smad4 (Fujii *et al.,* 1999). Moreover, osteoblast differentiation by Smad1/5 is facilitated upon nuclear translocation of R-Smads induced by ligand or receptor activation.

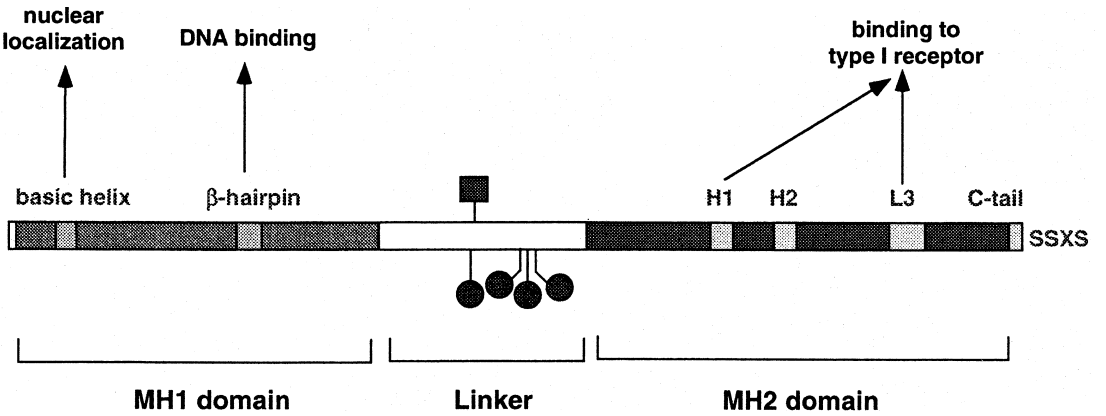

Figure 3 Structure of R-Smads. Helix 2 (H2) and the C-terminal region are important for specific interaction with DNA-binding protein FAST1, but other portions of R-Smads may also be able to interact with various DNA-binding proteins. Circles in the linker region indicate the PXS/TP (or S/TP) motif, and the square indicates the PY motif.

Smads have highly conserved N- and C-terminal regions termed Mad homology (MH) 1 and MH2 domains, respectively, which are bridged by a linker region of variable length and amino acid sequence (Fig. 3). MH2 domains are found in all three classes of Smads, whereas MH1 domains are conserved only in R-Smads and Co-Smads. The N-terminal regions of I-Smads highly diverge from those of the other Smads. R-Smads also have a characteristic Ser-Ser-Val/Met-Ser sequence (SSXS motif) at the C-terminal ends. The SSXS motif is phosphorylated by the type I receptors, resulting in the activation of R-Smads for signaling.

Smad1, Smad5, and Smad8 are structurally highly similar to each other, and the functional differences between them are unknown. However, Smad1 and Smad5, but not Smad8, are activated by BMP-6 and BMP-7 (Ebisawa *et al.,* 1999), whereas all three BMP-R-Smads are activated by BMP-2 (Aoki *et al.,* 2001). It has been reported that Smad1 and Smad5 are activated by TGF-β in certain cell types (X. Liu *et al.,* 1998); however, it is currently unknown whether activation of Smad1/5 occurs directly by TβR-I or through other receptors (e.g., ALK-1).

MH2 Domain The MH2 domain, composed of about 200 amino acids, have various important functions. It is responsible for (1) interaction with receptors, (2) binding to SARA (Smad anchor for receptor activation), (3) oligomer formation with other Smads, (4) interaction with various DNA-binding proteins, and (5) transcriptional activation. In the absence of receptor activation, the MH2 and MH1 domains physically interact with each other, and repress each other's function. Upon phosphorylation of the SSXS motif by receptors, this interaction is eliminated, and R-Smads become activated.

A region composed of 17 amino acids, termed the "L3 loop," protrudes from the surface of the molecule and interacts with the L45 loop of type I receptors (Chen *et al.,* 1998; Shi *et al.,* 1997; Wu *et al.,* 2000). The amino acid sequences of the L3 loop are conserved in BMP-R-Smads and in TGF-β-R-Smads, but diverge between these two groups. In addition to the L3 loop, the α-helix H1 in the MH2 domain, composed of eight amino acids, is also required for the interaction of BMP-R-Smads with type I receptors of the ALK-1 group (Chen and Massagué, 1999). The α-helix H2 and the C-terminal tail of the MH2 domain of R-Smads may interact with specific DNA-binding proteins and determine the specificity of the downstream signaling pathways (Fig. 3).

MH1 Domain The MH1 domains, composed of approximately 130 amino acids, are responsible for (1) binding to specific DNA sequences, (2) interaction with certain DNA-binding proteins, (3) nuclear translocation, and (4) repression of the function of MH2 domains. The direct-DNA binding of Smads occurs through an 11-amino-acid "β-hairpin loop," which protrudes from the surface of the molecule (Shi *et al.,* 1998). Although the structure of the β-hairpin loop is conserved in R-Smads and Co-Smad in mammals, only Smad4 and Smad3 bind to the characteristic Smad-binding element (AGAC or GTCT sequence) (Zawel *et al.,* 1998). In contrast, BMP-R-Smads bind to the AGAC/GTCT sequence only weakly and bind instead to the GCCGnCGC sequence with relatively high affinity (Kim *et al.,* 1997; Kusanagi *et al.,* 2000). Smad1 was shown to bind to the GCAT motif in the *Xenopus Xvent.2B* promoter (Henningfeld *et al.,* 2000).

The C-terminal half of α-helix 2 in the MH1 domain, located N-terminal to the β-hairpin loop, is rich in basic residues (Lys-40-Lys-Leu-Lys-Lys; termed "basic helix") (Shi *et al.,* 1998). The basic helix of R-Smads, but not that of Co-Smad, serves as a nuclear localization signal through interacting with importin β (Xiao *et al.,* 2000a,b).

Linker Region The linker regions of R-Smads contain four copies of PXS/TP (or S/TP) motifs which are phosphorylated by MAP kinases (Kretzschmar *et al.,* 1997). When these motifs are phosphorylated, R-Smads become unable to translocate into the nucleus.

The PPXY sequence (known as the PY motif) interacts with proteins containing WW domains. A PY motif is found

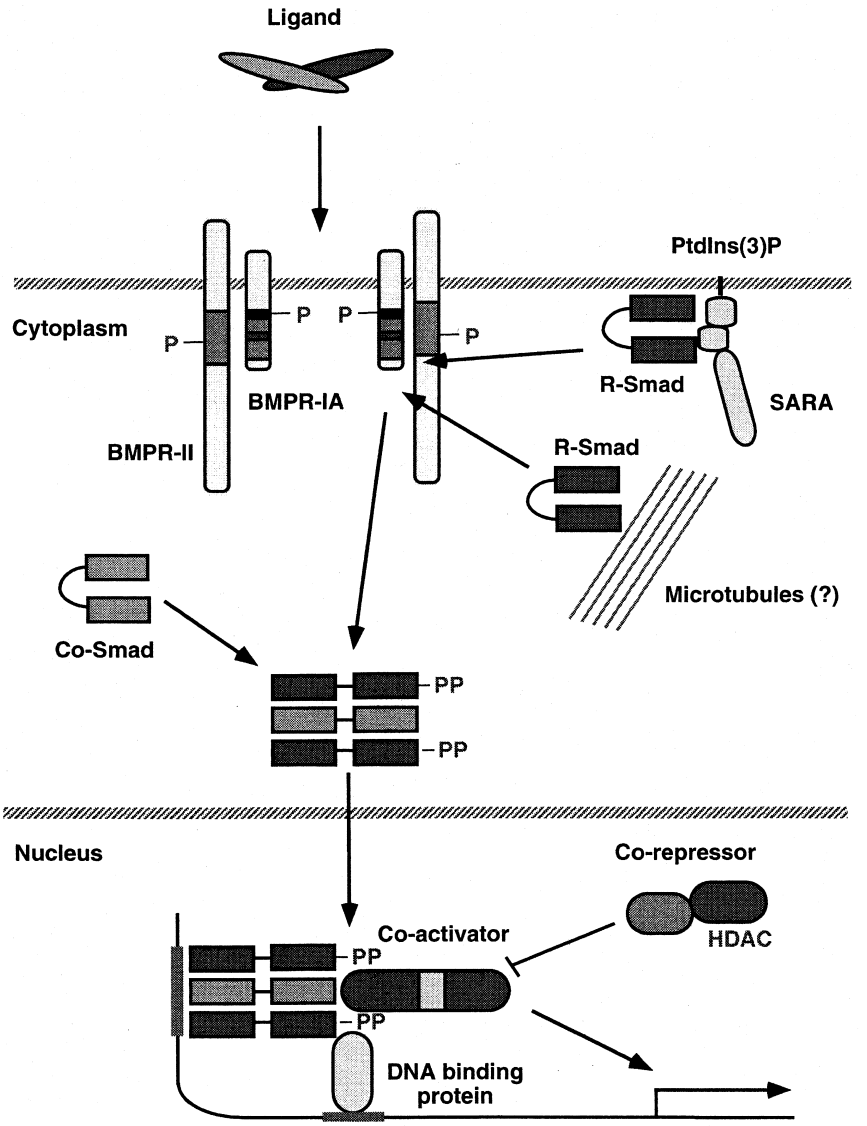

Figure 4 Mechanism of activation of Smad signaling pathway. R-Smads exist in the cytoplasm through interaction with membrane anchoring protein SARA. TGF-β-R-Smads have been shown to interact with microtubules, but it is not known whether BMP-R-Smads also interact with the microtubular network. Transcriptional corepressors inhibit the transcription induced by transcriptional coactivators, but transcriptional corepressors that specifically interact with BMP-R-Smads have not been identified.

in all Smads except Smad4 and Smad8. Smurf1, a member of the Hect family of E3 ubiquitin ligases, interacts with Smad1 and Smad5 through the PY motif, and degrades them in a ligand-independent manner (Zhu *et al.,* 1999).

An alternatively spliced variant of Smad8, lacking 37 amino acids in the linker region, has been observed (Watanabe *et al.,* 1997). Whether there is a functional difference between the long and short versions of Smad8 remains to be determined.

SMAD SIGNALING IN THE CYTOPLASM

R-Smads exist in the cytoplasm bound to membrane-associated proteins or to microtubules (Fig. 4). Although a Smad2/3-interacting protein SARA (Smad anchor for receptor activation) has been identified (Tsukazaki *et al.,* 1998), SARA-like molecule(s) that anchor BMP-R-Smads to the cell surface or intracellular membranes have not been identified. β-Tubulin interacts with Smads 2, 3, and 4 (Dong *et al.,* 2000), but interaction of BMP-R-Smads to the microtubular network has not been demonstrated.

SARA contains a phospholipid interaction domain, termed the FYVE domain, which targets SARA to the plasma membrane. SARA presents R-Smads to type I receptors, and facilitates their activation (Tsukazaki *et al.,* 1998). R-Smads transiently interact with and become phosphorylated by activated type I receptors. The interaction between type I receptors and R-Smads is determined by the L45 loop of type I receptors and L3 loop and α-helix H1

of the MH2 domains of R-Smads (Fig. 3) (Wu *et al.,* 2000). Phosphorylation occurs at the last two serine residues in the SSXS motif of the R-Smads. R-Smads then form heteromeric complexes with Co-Smads, possibly composed of two molecules of R-Smads and one molecule of Co-Smad (Kawabata *et al.,* 1998).

Nuclear translocation of R-Smads occurs independently of Co-Smads, whereas Co-Smads require R-Smads for nuclear translocation (Watanabe *et al.,* 2000). The basic helix in the MH1 domains of R-Smads is masked in unstimulated cells; after activation by the type I receptors, the basic helix is exposed, and interacts with importin β, leading to nuclear translocation of R-Smads (Xiao *et al.,* 2000a,b). Isolated MH2 domains are also actively transported into the nucleus, although the mechanisms by which this occurs remain to be determined (L. Xu *et al.,* 2000).

SMAD SIGNALING IN THE NUCLEUS

In the nucleus, Smads regulate transcription of target genes through (1) direct binding to specific DNA sequences, (2) interaction with other DNA-binding proteins, and (3) recruitment of transcriptional coactivators and/or corepressors (Fig. 4).

Instead of the characteristic GTCT/AGAC sequence specific for Smad3 and Smad4, BMP-R-Smads bind to a GC-rich sequence (GCCGnCGC motif) found in the promoter region of *Smad6* (Isihida *et al.,* 2000). Although direct DNA-binding of Smads is important for transcriptional activation of certain genes, it is not required for other genes.

Smads interact with various DNA-binding proteins, and this interaction may be a critical event in the exhibition of specific effects of TGF-β and BMPs in different types of cells. Although many DNA-binding proteins, including FAST1, AP-1 complex, TFE3, Sp1, and vitamin D receptor, interact with TGF-β-R-Smads, only several DNA-binding proteins have been reported to interact with BMP-R-Smads. Runx is a family of transcription factors that regulate various biological events, including hematopoiesis and osteogenesis. Three mammalian isoforms of Runx, Runx1 through -3, have been identified. Mice lacking the *Runx2* gene (also termed Cbfa1 or polyomavirus enhancer binding protein-αA; PEBP2αA) exhibit complete loss of bone formation. BMP-R-Smads physically interact with Runx2 upon activation by BMP receptors (Hanai *et al.,* 1999; Zhang *et al.,* 2000). Runx2 and BMP-R-Smads cooperatively activate the transcription of target genes, and facilitate the osteoblast differentiation of osteoprogenitor cells. Runx thus functions as a nuclear target of Smads in specific tissues.

OAZ is a DNA-binding protein containing 30 zinc-finger domains. OAZ interacts with BMP-R-Smads in response to BMP-2 stimulation, and activates the transcription of *Xvent.2* through direct DNA-binding (Hata *et al.,* 2000). OAZ has also been shown to be a transcriptional partner of Olf-1/EBF in olfactory epithelium and pre-B lymphocytes. OAZ is thus a transcription factor with dual functions, utilizing distinct clusters of zinc-fingers for the BMP-Smad pathway and Olf signaling pathway.

Zinc-finger transcription factors of the Gli family play an important role in hedgehog signaling. A C-terminally truncated version of Gli3, but not the full-length Gli protein, interacts with Smads 1 through 4 (F. Liu *et al.,* 1998). This interaction occurs in the absence of ligands, and dissociation occurs upon ligand stimulation. Since the C-terminally truncated Gli3 is observed in Pallister–Hall syndrome and polydactyly type A, the biological significance of the Smad–Gli3 complex is an important question to be answered.

In addition to the transcription factors that positively regulate the transcription of target genes, certain DNA-binding proteins act as transcriptional repressors in the absence of BMP/TGF-β stimulation, and this repression may be relieved after interaction with Smads upon ligand stimulation (Miyazono *et al.,* 2000). A homeodomain protein Hoxc-8 binds to the promoter region of the *osteopontin* gene and represses its transcription. Upon BMP stimulation, Smad1 interacts with Hoxc-8, which then dissociates from the DNA-binding element (Shi *et al.,* 1999). Another homeodomain protein, SIP1, a member of the δEF1/Zfh family (Verschueren *et al.,* 1999), has been suggested to repress the expression of the *brachyury* gene in a fashion similar to Hoxc-8. SIP1 interacts with all R-Smads; whether SIP1 acts as a target for BMP-R-Smads under physiological conditions remains to be determined.

p300 and CBP (CREB binding protein) have histone acetyl transferase (HAT) domains, which upregulate gene transcription by loosening nucleosomal structure and by increasing the accessibility to the general transcription machinery. p300 and CBP interact with various transcription factors; they interact with Smad1, -2, and -3 upon ligand stimulation and enhance Smad-dependent transcription of target genes (Miyazono *et al.,* 2000; Massagué and Wotton, 2000). SNIP1, a nuclear protein with forkhead-associated (FHA) domain, interacts with Smads 1, -2, and -4 (R. H. Kim *et al.,* 2000). SNIP1 inhibits complex formation between Smad4 and p300, and suppresses the transcriptional activation induced by TGF-β.

Transcriptional co-repressors, including TGIF, c-Ski, and SnoN, recruit histone deacetylases (HDACs) to Smad complexes (Miyazono, 2000). In contrast to p300/CBP, they induce nucleosomal condensation, and consequently repress the transcription of target genes. TGIF, c-Ski, and SnoN preferentially interact with TGF-β-R-Smads. However, c-Ski represses BMP signaling *in vivo* (Wang *et al.,* 2000).

TARGET GENES FOR BMPS

In contrast to the TGF-β/activin signaling system, target genes regulated by BMP-R-Smads have not been fully determined. *Tlx-2* is a target gene for BMPs. *Tlx-2* is a homeobox gene related to human *Hox11* and is expressed in the primitive streak of mouse embryos (Tang *et al.,* 1998). *Xvent.2* is another BMP-target gene, which is also a homeobox gene and mediates the effects of BMP-4 in *Xenopus* embryos (Candia *et al.,* 1997). Smad6 and Smad7 in mammals and Daughters against Dpp (Dad) in *Drosophila* are induced by BMP signaling. BMP-R-Smads directly activate

transcription from the *Smad6* promoter (Ishida *et al.,* 2000) and from the *Xenopus Xvent.2* promoter (Henningfeld *et al.,* 2000). *Id* gene products are negative regulators of basic helix–loop–helix transcription factors, including MyoD. *Id1* through *-3* are immediate–early response targets of BMPs in embryonic stem cells (Hollnagel *et al.,* 1999). Transcription of the *osteopontin* gene is regulated by BMPs through direct binding of Hoxc-8 to a specific DNA-binding element (Shi *et al.,* 1999).

REGULATION OF SMAD SIGNALING

I-Smads I-Smads, i.e., Smad6 and Smad7, function as antagonists of R-Smad/Co-Smad signaling (Fig. 5). I-Smads interact with type I receptors activated by type II receptors through their MH2 domains. Unlike R-Smads, however, they do not dissociate from type I receptors, and thus prevent the activation of R-Smads (reviewed in Miyazono *et al.,* 2000). Smad6 has also been reported to form a complex with Smad1 and compete with Smad4 for oligomer formation. As a third mechanism, Smad6 has been reported to interact with a transcription factor Hoxc-8, and thereby inhibit the interaction between Smad1 and Hoxc-8 and repress the transcription of the *osteopontin* gene induced by Smad1 (Bai *et al.,* 2000). Smad7 inhibits both TGF-β and BMP signaling, whereas Smad6 preferentially represses BMP, but not TGF-β or activin, signaling.

In mammalian cells, Smad7 is located in the nucleus, and exported to the cytoplasm upon TGF-β stimulation (Itoh *et al.,* 1998). The mechanism and functional importance of the nuclear export of Smad7 are unknown. Smad6 is diffusely expressed in both the cytoplasm and the nucleus. Whether Smad6 is also exported to the cytoplasm remains to be determined.

Expression of I-Smads is regulated by various stimuli, including growth factors, mechanical stress, interferon-γ, and NF-κB signaling (reviewed in Miyazono, 2000). Most

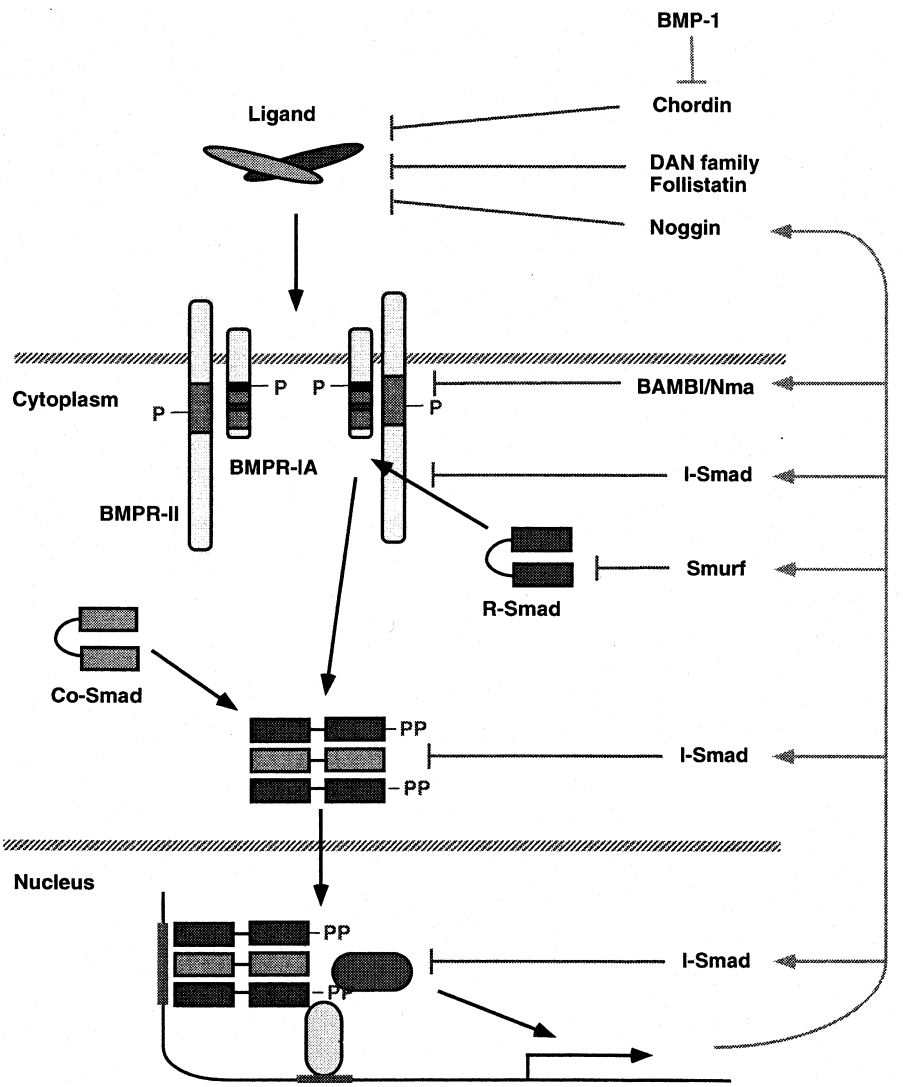

Figure 5 Regulation of BMP signaling pathways. BMP signaling is negatively regulated by extracellular antagonists, pseudoreceptor BAMBI/Nma, E3 ubiquitin ligase Smurf, and I-Smads. Expression of these regulatory molecules is induced by BMP or TGF-β signaling.

importantly, expression of I-Smads is strongly induced by TGF-β/activins and BMPs; transcription of Smad7 and Smad6 has been shown to be induced by direct effects of TGF-β-R-Smads and BMP-R-Smads, respectively (Nagarajan *et al.,* 1999; Ishida *et al.,* 2000). R-Smad/Co-Smad and I-Smad thus form a negative feedback loop for regulation of TGF-β superfamily signaling.

Proteasome-Dependent Degradation of Smads R-Smads are degraded by the ubiquitin–proteasome pathway in ligand-independent and ligand-dependent fashions (Miyazono, 2000). Protein ubiquitination is triggered by E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin-ligases. Of these, E3 ligases are most important for specific recognition and degradation of target proteins. Smurf1 is a Hect family E3 ligase that interacts with BMP-R-Smads. This interaction occurs between two copies of the WW motif in Smurf1 and the PY motif in the linker region of BMP-R-Smads (Zhu *et al.,* 1999). Smurf1 degrades BMP-R-Smads in a ligand-independent fashion. Since Smurf1 may regulate the cellular pool of BMP-R-Smads, cells become more sensitive to TGF-β/activins than to BMPs in the presence of large amounts of Smurf1.

After nuclear translocation and transcriptional activation, R-Smads may be degraded by the ubiquitin-proteasome pathway (Lo and Massagué, 1999; Fukuchi *et al.,* 2001). This ligand-dependent degradation of R-Smads may be important for irreversible termination of TGF-β/BMP signaling.

Cross-Talk with Other Signaling Pathways BMP signaling is regulated negatively and positively by various signaling pathways, including MAP kinase and STAT pathways. The Erk MAP kinase pathway is activated by peptide growth factors. Erk phosphorylates the serines and threonines in the PXT/SP (or T/SP) motif, four copies of which are present in the linker regions of R-Smads. R-Smads phosphorylated by Erk are unable to translocate into the nucleus, and thus signaling by BMPs and TGF-β is inhibited by peptide growth factors through the action of Erk MAP kinase (Kretzschmar *et al.,* 1997).

Leukemia inhibitory factor (LIF) and BMP-2 synergistically induce astrocytic differentiation of neuroepithelial cells (Nakashima *et al.,* 1999). LIF activates STAT3 through gp130 and JAKs, and BMP-2 activates BMP-R-Smads. Although direct interaction between STAT3 and BMP-R-Smads is not observed, they are indirectly associated through binding to p300, which results in cooperative transcriptional activation of target genes.

IN VIVO ABNORMALITIES ASSOCIATED WITH *SMAD* GENES

Smad4 null mice die before E7.5 with gastrulation defects and an abnormal visceral mesoderm, which are similar to the phenotypes of *BMP-4*- and *BMPR-IA*-deficient mice (Sirard *et al.,* 1998). Mutations of the human *Smad4/DPC4* gene are frequently found in pancreatic cancer, biliary tract cancer, metastatic colon cancer, and juvenile polyposis (reviewed in Miyazono *et al.,* 2000). Homozygotic loss of the *Smad4* gene together with *Apc* mutations results in progression of intestinal tumors (Takaku *et al.,* 1998). *Smad4* heterozygous mice develop gastric polyposis 6–12 months after birth, which eventually progresses to gastric cancer as a result of loss of heterozygosity (Takaku *et al.,* 1999; X. Xu *et al.,* 2000).

Smad5-deficient mice exhibit abnormalities in angiogenesis with dilated vessels and decreased number of smooth muscle cells surrounding vessels (Yang *et al.,* 1999; Chang *et al.,* 1999). The phenotype of *Smad5*-null mice is similar to that of the *ALK-1* null mice as well as those of *TGF-β-*, *TβR-II-*, or *endoglin*-deficient mice. Since ALK-1 binds TGF-β in endothelial cells and activates BMP-R-Smads (Oh *et al.,* 2000), Smad5 might be a major signal transducer for TGF-β in endothelium. Smad5 is also required for development of left–right asymmetry (Chang *et al.,* 2000).

Smad6-null mice exhibit cardiac defects with abnormal valve formation and outflow tract septation (Galvin *et al.,* 2000). Some *Smad6*-null mice survive through adulthood, but exhibit aortic ossification and elevated blood pressure, indicating that Smad6 is required for development and homeostasis of the cardiovascular system.

Other Signaling Pathways

In addition to the Smad pathways, BMP-2 and GDF-5 have been shown to activate the p38 MAP kinase pathway (Iwasaki *et al.,* 1999; Nakamura *et al.,* 1999; Kimura *et al.,* 2000). The p38 MAP kinase activated by BMPs induces neurite outgrowth from PC12 pheochromocytoma cells, chondrocyte differentiation of ATDC5 mouse teratocarcinoma cells, and apoptosis in mouse hybridoma MH60 cells. Erk MAP kinases have also been shown to be activated by BMP-2 in certain cells. These non-Smad pathways activated by BMPs may play important roles in modulation of effects of Smads on cellular proliferation and differentiation.

TAK1 has been reported to act as a downstream component of BMP and TGF-β and activate the SAPK/JNK and p38 MAP kinase pathways. BMPR-IA directly interacts with XIAP, which activates TAB1-TAK1. However, activation of TAK1 by TGF-β or BMPs may occur only under certain conditions; under physiological conditions, TAK1 may serve as a downstream target of interleukin-1 (Ninomiya-Tsuji *et al.,* 1999).

Extracellular Regulators of BMPs

BMP Antagonists

The biological activities of BMPs are tightly regulated by extracellular antagonists (Fig. 5). A wide variety of extracellular antagonists of the TGF-β superfamily exist, and are divided into two different types: those that directly bind ligands (ligand-binding antagonists) and others that compete with ligands for binding to specific receptors (pseudoligand-type antagonists) (Miyazono, 2000). Lefty-1, Lefty-2, and

zebrafish Antivin are typical pseudoligand-type antagonists, which interfere with binding of Nodal and activin-like factors to activin type II receptors; however, it is unknown whether Lefty-1/Lefty-2/Antivin block the activity of BMPs *in vivo.*

Most of the antagonists of BMPs are ligand-binding type antagonists, including Noggin, Chordin, Cerberus and its related proteins, and Follistatin (Miyazono, 2000; Massagué and Chen, 2000). Cerberus and its related proteins, including Gremlin, Caronte, and DAN, contain a conserved cystine-knot motif, and are collectively referred to as the DAN family. BMP antagonists other than those of the DAN family do not exhibit structural similarity to each other.

These BMP antagonists have distinct expression profiles and different affinities with various BMP isoforms. Noggin antagonizes BMP-2, BMP-4, and GDF-6 efficiently, but BMP-7 less efficiently. Caronte functions as an antagonist of BMP-4, BMP-7, and Nodal. Noggin competes with Cerberus, Gremlin, and DAN for binding to BMP-2, indicating that the binding sites in BMPs are shared by these antagonists (Hsu *et al.,* 1998). Cerberus binds to several cytokines, including BMPs, nodal-like factors, and Wnt, through independent binding sites in its molecule (Piccolo *et al.,* 1999). Follistatin is a potent antagonist of activins, but also antagonizes certain BMPs both *in vitro* and *in vivo.*

Limb development is regulated by various BMP antagonists, including Noggin, Chordin, Gremlin, and Follistatin (McMahon *et al.,* 1998; Merino *et al.,* 1999). Noggin is also involved in hair-follicle induction. In *Xenopus* embryos, Noggin and Chordin are expressed in Spemann's organizer and induce neural tissues and dorsalize ventral mesoderm. These BMP antagonists may thus play a central role in the formation of morphogen gradients during early embryogenesis.

Certain BMP antagonists may exert their effects through a negative feedback loop. Expression of Noggin is induced by BMPs in osteoprogenitor cells (Gazzero *et al.,* 1998). Other cytokines, including TGF-β, also induce expression of Noggin, indicating that expression of BMP antagonists is regulated by BMP signaling itself, as well as through crosstalk of signals.

BMP-1 Is a BMP Activator

BMP-1 does not belong to the TGF-β superfamily, but is a procollagen C-proteinase that cleaves procollagens and induces accumulation of extracellular matrix (Kessler *et al.,* 1996). BMP-1 homologs are present in *Drosophila* (*Tolloid*) and in *Xenopus* (*Xolloid*); studies of Tolloid and Xolloid suggest that BMP-1 may be an activator of BMPs, since it releases active BMPs from inactive complexes by cleavage of Chordin (Piccolo *et al.,* 1996; Marques *et al.,* 1997).

Conclusion

BMPs play multiple roles in various tissues including bone, cartilage, neurons, heart, kidney, and lung. Signaling by BMPs is transduced by serine/threonine kinase receptors and Smad proteins. BMP signaling is tightly regulated by various mechanisms including extracellular antagonists, pseudoreceptors, and I-Smads. BMPs thus exhibit a diverse array of biological activities in various tissues. Further understanding of the BMP signaling pathways can aid the pharmacological regulation of biological activities of BMPs in various tissues.

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CHAPTER 52

Colony-Stimulating Factors

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Introduction

Between bone and bone marrow exists a close anatomical relationship, and several lines of evidence suggest a reciprocal interaction between the two tissues. On the one hand, bone marrow contributes the precursors of the cells that provide the cellular basis of bone remodeling. The bone resorbing cell, the osteoclast, stems from a hemopoietic progenitor, the colony-forming unit granulocyte/macrophage (CFU-GM) (Hagenaars *et al.,* 1991; Hattersley *et al.,* 1991a; Kurihara *et al.,* 1990). The bone-forming cell, the osteoblast, and the osteocyte are derived from mesenchymal progenitors of the marrow stromal fibroblasts, the colony-forming unit fibroblasts (CFU-F) (Owen, 1985; Owen and Friedenstein, 1988).

On the other hand, bone seems to exert a positive influence on hemopoiesis. In developing bone, osteoclasts resorb and invade the calcified cartilage rudiment. As a result, the primitive marrow cavity is formed and hemopoiesis is initiated. Cells of the osteoblastic lineage control osteoclast recruitment and activity through synthesis of factors acting directly on progenitors and mature osteoclasts (Chambers, 1992; Suda *et al.,* 1995; Yasuda *et al.,* 1998). This way osteogenic cells are essential for the process of invasion of the bone rudiment by osteoclasts and endothelia and the consequent establishment of hemopoiesis.

Bone cells may support hemopoiesis by providing factors essential for hemopoiesis. In marrow, multipotential hemopoietic stem cells proliferate and differentiate into lineage-restricted hemopoietic progenitors that eventually give rise to the terminally differentiated cells of the myeloid series. This progression occurs under the influence of macromolecules called colony-stimulating factors (CSFs) (Metcalf, 1984). The CSFs are glycoproteins initially characterized by their ability to stimulate *in vitro* the clonal proliferation of hemopoietic multipotential stem cells and/or mono- or bipotential progenitors in semisolid (agar or methylcellulose) medium. In some cases, however, CSFs are also able to commit hemopoietic cells to a single myeloid lineage. In addition, they may also induce the functional activity of mature, specialized myeloid cells and maintain their survival. This group of CSFs includes interleukin-3 (IL-3, also named multi-CSF), granulocyte/macrophage-CSF (GM-CSF), granulocyte-CSF (G-CSF), and CSF-1 (also named macrophage-CSF, M-CSF).

CSFs are ideal candidate factors for being synthesized by osteogenic cells to modulate locally, as described above, both osteoclast recruitment and marrow hemopoiesis. In this chapter, we shall exclusively describe the role of CSFs in modulating osteoclast development and activity. Other cytokines and interleukins have been shown to have intrinsic and/or synergistic effects on general hemopoiesis and/or osteoclast development. However, these factors were not identified originally based on their capacity to induce clonal proliferation of hemopoietic cells and for this reason they are not considered as CSFs. Their role in the local control of bone cell physiology is extensively described in other chapters of this book.

Colony-Stimulating Factor-1

Colony-stimulating factor-1 (CSF-1), also called macrophage colony-stimulating factor, was originally described as the growth factor for cells of the mononuclear phagocyte system (MNPS) and for osteoclasts. The cytokine binds to a single class of high-affinity cell-surface receptors that are encoded by the proto-oncogene c-*fms* and belong to the

receptor tyrosine kinase family. Expression of c-*fms* is considered a marker for the cells of the MNPS (Felix *et al.,* 1994; Stanley, 1994). Within this chapter, the effects exerted by the cytokine on the cells of this lineage as well as the mechanisms involved will be discussed.

Molecular Biology and Biochemistry of CSF-1

CSF-1 is the product of a single-copy gene, localized in humans on chromosome 1 and in mice on chromosome 3. Size and structure of the gene, spanning ≈120 kb and containing 10 exons, 8 of which encode translated sequences, are well conserved among the species. Several transcripts, derived from a common RNA precursor by alternative splicing, encode either a secreted or a membrane-bound form of the cytokine.

Transcripts encoding the secreted or the membrane-bound form of CSF-1 differ in alternative usage of exon 6 sequences encoding amino acid sequences responsible for posttranslational processing of the protein. Besides a transmembrane domain, present in all transcripts, exon 6 encodes a stretch of amino acids containing a proteolytic cleavage site, which is hydrolyzed to release the N-terminal peptide from the transmembrane domain. This process occurs within the secretory vesicles of the cells, and the cleavage product is rapidly secreted by exocytosis. In the absence of the proteolytic cleavage site, the protein remains anchored in the membrane and, after fusion of the secretory vesicles with the cell membrane, becomes exposed on the cell surface, from where it is slowly released (for review, see (Sherr and Stanley, 1990)).

All translation products contain an amino-terminal signal peptide of 32 amino acids. Furthermore, a hydrophobic 23-amino-acid region is followed by a charged "stop transfer" sequence, characteristic for many membrane-spanning proteins. The CSF-1 peptide is cotranslationally glycosylated and remains integrated in the membrane of the endoplasmic reticulum, the amino-terminal end facing the lumen. In the Golgi, the protein is O-glycosylated and the N-linked carbohydrate molecules are further processed. The secreted forms of the cytokine can be further modified by the attachment of a glycosaminoglycan side chain.

The CSF-1 precursor protein for the secreted cytokine is synthesized as a 554-amino-acid precursor, which is processed to yield a disulfide-linked homodimer of 223 amino acids per chain. The membrane-bound form of CSF-1 is synthesized as a 256-amino-acid precursor protein, which, in turn, is processed to a homodimer of 150 amino acids per chain.

Expression and Regulation of CSF-1

Several groups have demonstrated the essential role of CSF-1 in the formation of osteoclasts (Felix *et al.,* 1990a; Kodama *et al.,* 1991; Wiktor-Jedrzejczak *et al.,* 1990; see Chapter 7). The involvement of the cytokine in the regulation of acute bone resorption, however, is much less defined. The main question is whether CSF-1 constitutes an essential component of the hemopoietic microenvironment, essential but not exerting regulatory functions, or whether the cytokine is involved in the acute regulation of bone resorption and is modulated by osteotropic hormones and cytokines. The regulation of the biological activity of CSF-1 is further complicated by the fact that the cytokine is synthesized either as a secreted or as a membrane-bound molecule (Stanley, 1994). Whether the two molecular forms of the growth factor exert different biological actions or whether the difference is merely based on spatial distribution is yet an open question. The membrane-bound form of CSF-1 is more efficient *in vitro* than soluble CSF-1 in promoting osteoclast formation (Takahashi *et al.,* 1991a). This may be attributed to the proximal relationship of other membrane-associated factors that influence osteoclastogenesis, namely receptor activator of NF-κB ligand (RANKL; see Chapter 7).

CSF-1 has been shown to be produced constitutively by mesenchymal cells, among them osteoblasts, and by activated macrophages *in vitro* (Stanley, 1994). The secretion is stimulated by parathyroid hormone (PTH), IL-1, and tumor necrosis factor (TNF)-α (Felix *et al.,* 1989; Sato *et al.,* 1986; Shiina-Ishimi *et al.,* 1986; Weir *et al.,* 1993). In contrast, expression of the cytokine is tightly controlled *in vivo.* Thus, the expression of CSF-1 is dramatically increased in mice during early pregnancy (Bartocci *et al.,* 1986), where CSF-1 is induced by 17β-estradiol and progesterone in uterine glandular epithelial cells (Daiter *et al.,* 1992; Pollard *et al.,* 1987). In bone, expression of CSF-1 is correlated temporally and spatially with osteoclast formation and bone resorption (Hofstetter *et al.,* 1995). Together, these observations suggest that the expression of CSF-1 is tightly controlled under physiological conditions. Despite these observations, relatively little is known concerning the regulation of the expression of CSF-1 by hormones and cytokines *in vivo.* As mentioned above, estrogen deficiency, through the induction of the synthesis of IL-1 and TNF-α by marrow cells may induce the expansion of a subpopulation of stromal cells high in CSF-1 production (Kimble *et al.,* 1996). These increased levels of CSF-1 support the formation of osteoclasts, causing the osteopenia. The levels of CSF-1 were also reported to be elevated after injections of lipopolysaccharide (LPS) into mice (Roth *et al.,* 1997), and by this mechanism, the mobilization and activation of mononuclear phagocytes in the inflammatory response is facilitated.

Further evidence for the regulation of the expression of CSF-1 was provided by *in vitro* studies on organ or cell cultures. In organ cultures of fetal rat long bones, PTH and PTH-related peptide (PTHrP) were shown to induce an increase in bone resorption (Weir *et al.,* 1996). By using models of bone resorption that are dependent on the formation of new (fetal metacarpals) or on the activation of existing osteoclasts (fetal radii), it was possible to distinguish the effect of CSF-1 on recruitment (proliferation, differentiation) and on osteoclast activity.

Since CSF-1 is synthesized as a membrane bound and as a secreted molecule, it has been speculated that these molecular forms may exert different biological activities or may be of different efficiencies in their biological actions (Takahashi *et al.,* 1991a). Subsequently it was shown that the cell surface form of CSF-1 is sufficient to induce the formation of osteoclasts from bone marrow cells (Yao *et al.,* 1998). This implies the possibility that the two molecular forms are differentially regulated. Indeed, various groups described hormones, e.g., PTH, 1,25$(OH)_2$ vitamin D_3, and dexamethasone, cytokines such as TNF-α, or mechanical stimuli such as pressure to stimulate expression of the membrane-bound form of CSF-1 while the synthesis of the secreted molecule is not affected (Rubin *et al.,* 1997, 1998, 1996; Yao *et al.,* 1998). Since the different forms of CSF-1 are encoded by different mRNAs that are derived from a common hnRNA precursor by differential splicing, the regulation of the expression of membrane-bound and secreted CSF-1 occurs at the posttranscriptional level. The mechanisms that allow for this regulation, however, are as yet not known.

A considerable effort was devoted to elucidate the transcriptional control of CSF-1 expression. When the partial promoter of the murine CSF-1 gene was cloned, it was found to contain consensus sequences for a number of different transcription factors (Harrington *et al.,* 1991). From cell culture studies it has become evident that TNF-α is most effective in regulating CSF-1 expression in cells of the osteoblast lineage. An important signal transduction pathway induced by TNF-α involves the members of the NF-κB family of transcription factors. The importance of NF-κB family members in bone metabolism was evident when double knockout mice, deficient in the NF-κBs p50 and p52, expressed an osteopetrotic phenotype, caused by an absence of osteoclasts (Iotsova *et al.,* 1997). The requirement for NF-κB in CSF-1 expression was demonstrated using osteoblastic cells derived from p50 knockout mice. These cells failed to react to treatment with TNF-α by increasing the synthesis of CSF-1, as is the case in osteoblasts from wild-type controls (Yao *et al.,* 2000). Somewhat contradictory, however, was the finding that the NF-κB consensus sequence of the CSF-1 promoter was not critically involved in the stimulation of CSF-1 expression by TNF-α (Isaacs *et al.,* 1999; Rubin *et al.,* 2000). In stromal ST-2 cells, inactivation of NF-κB still allowed for TNF-α-induced expression of the cytokine. Removal of the NF-κB consensus sequence increased basal expression of CSF-1, but still allowed further stimulation by TNF-α. Thus it is clear that NF-κB transcription factors are critically involved in osteoclastogenesis and also that exposure to TNF-α induces the synthesis of CSF-1, but the interrelationship of the two is not yet understood. Another transcription factor that was shown to be involved in the transcription of the CSF-1 gene was AP-1 (Konicek *et al.,* 1998). Most important was the finding that the expression of the cytokine in different cell types is governed by common and by cell-type-specific *trans*-acting factors, allowing for a tight temporal and spatial regulation. As mentioned above, however, presently it is still not clear whether CSF-1 is involved in the acute regulation of bone resorption or whether the growth factor acts as a constitutive component of the hemopoietic microenvironment. Support for the latter results from recent studies in which IL-1, IL-3, IL-6, IL-7, IL-10, GM-CSF, G-CSF, bFGF, and TGF-β did not effect CSF-1 expression by cultured human bone marrow stromal cells (Besse *et al.,* 2000).

CSF-1 in Postmenopausal Osteoporosis

CSF-1, together with RANKL (also known as osteoclast differentiation factor [ODF], osteoprotegerin ligand [OPGL], stromal osteoclast forming activity [SOFA], TNF superfamily 11 [TNFSF-11], and TNF-related activation-induced cytokine [TRANCE]) (Anon, 2000), is one of the two essential growth factor for osteoclasts (Quinn *et al.,* 1998; see Chapter 7). It was not clear, however, whether CSF-1 would contribute in a critical way to the observed bone loss under conditions of estrogen deficiency, and many studies focused on its role in the bone loss induced by estrogen deficiency. Estrogen has been shown in one culture system of osteoblast-like cells to stimulate the synthesis of osteoprotegerin (OPG), a decoy receptor for RANKL (Hofbauer *et al.,* 1999, 2000), and to suppress signal transduction through RANK (receptor activator of NF-κB) (Shevde *et al.,* 2000). It was more difficult to elucidate the regulation of CSF-1 expression by estrogen.

Several years ago it was reported that peripheral blood monocytes from osteoporotic women (Pacifici *et al.,* 1987) or from women after oophorectomy (Pacifici *et al.,* 1991) secrete elevated levels of IL-1 and TNF-α. An increase in the production of these cytokines, and their critical role in increased bone loss, was confirmed in various animal studies (Kimble *et al.,* 1997; Kitazawa *et al.,* 1994; Pacifici *et al.,* 1987; Srivastava *et al.,* 1995). CSF-1 was first implicated in bone loss after estrogen deficiency, when it was found that after ovariectomy, a subpopulation of stromal cells developed that synthesized increased levels of CSF-1 (Kimble *et al.,* 1996). This stimulation was brought about by the action of the monocyte products IL-1 and TNF-α on stromal cells. Further studies on the mechanism of action of IL-1 and TNF-α on the expression of CSF-1 demonstrated a role for the early response gene *egr-1*. Under estrogen deficiency, *Egr-1* becomes phosphorylated. In its phosphorylated state, *Egr-1* cannot interact with the transcription factor SP-1, causing an excess of free SP-1 to stimulate CSF-1 production via the SP-1 site in the CSF-1 promoter (Srivastava *et al.,* 1998). In mice deficient in *Egr-1,* bone turnover is high, but estrogen deficiency fails to stimulate bone resorption further (Cenci *et al.,* 2000a), again demonstrating the role of the transcription factor in the upregulation of CSF-1 expression.

The above-described studies on the effect of estrogen deficiency on CSF-1 expression do not take into account the possibility that the expression of the two main molecular forms, membrane bound versus secreted, may be affected as

well. Indeed it was shown in cultures of human bone marrow cells (Sarma *et al.,* 1998) and in ovariectomized rats (Lea *et al.,* 1999) that the expression of the membrane-bound form of CSF-1 was increased under conditions of estrogen deficiency, an effect that was reversed by treatment with estrogen.

In the recent past, the cross-talk between cells of the immune defense and bone has been shown to be very important, in particular in a disease like rheumatoid arthritis. Thus, activated T cells were shown to secrete RANKL *in vitro* and to support osteoclast formation (Horwood *et al.,* 1999) and *in vivo* (Kong *et al.,* 1999). Most interesting, osteoclastogenesis induced by T cells is counteracted by interferon-γ, another T-cell product (Takayanagi *et al.,* 2000). This T-cell–bone connection, however, is not only important in inflammatory and immunologic situations, but has been shown to be relevant in estrogen deficiency as well. Thus, not only cells of the monocyte/macrophage lineage may mediate the effect of estrogen deficiency on bone by the production of TNF-α and IL-1, but T lymphocytes contribute to bone loss through the synthesis of TNF-α as well (Cenci *et al.,* 2000b). The critical role of T cells in this process was further underlined by the fact that bone wasting is not observed in athymic *nu/nu* mice (Cenci *et al.,* 2000b) and in Rag1 mice that are deficient in both T and B lymphocytes (Spanopoulou *et al.,* 1994).

The Role of CSF-1 in the Formation of Osteoclasts

Animals defective in the production of growth factors represent ideal models for investigating the effects of these proteins during development and function of organs and cells. One such model is the murine osteopetrotic mutant strain *op,* which is devoid of biologically active CSF-1 (Felix *et al.,* 1990b; Wiktor-Jedrzejczak *et al.,* 1990), the defect being caused by a point mutation within the coding region of the CSF-1 gene (Yoshida *et al.,* 1990). The phenotype of *op/op* mice is characterized by a virtual absence of osteoclasts (Marks and Lane, 1976). Treatment of these animals with daily injections of recombinant human CSF-1 induced osteoclast formation and bone resorption, proving that the deficiency in CSF-1 is indeed the cause of the osteopetrosis in *op* mice (Felix *et al.,* 1990a; Kodama *et al.,* 1991).

The dependence of osteoclast formation on CSF-1 was further corroborated in organ and in cell culture systems. In cultures of ^{45}Ca-prelabeled fetal metatarsals, CSF-1 stimulated osteoclastogenesis and release of ^{45}Ca. This effect was blocked by irradiation of the bone rudiments, indicating an action of CSF-1 on proliferating osteoclast precursors. No effect on bone resorption by the cytokine was observed in cultured fetal radii, in which bone resorption depends on the activation of mature osteoclasts (Corboz *et al.,* 1992). This data suggests an effect of CSF-1 on recruitment of osteoclasts rather than on the activation of mature cells. In cultures of ^{45}Ca-prelabeled fetal metatarsals from *op/op* mice, CSF-1 alone had a slight effect only on the release of ^{45}Ca. Bone resorption was stimulated, however, when the rudiments were cultured in the presence of CSF-1 plus PTH and $1,25(OH)_2D_3$ (Morohashi *et al.,* 1994). Thus, in this system, CSF-1 was required but not sufficient for the formation of osteoclasts. The process of osteoclast formation may be initiated by CSF-1 inducing the proliferation of osteoclast precursors. PTH and $1,25(OH)_2D_3$ presumably acted on osteoblast/stromal cells to induce RANKL expression.

In vitro, osteoclasts are formed in cocultures of osteoblasts or stromal cells and hemopoietic precursor cells in the presence of $1,25(OH)_2D_3$ (Takahashi *et al.,* 1988). Cocultures of hemopoietic precursors and osteoblastic cells from *op/op* or +/? littermates gave rise to osteoclasts, when the mesenchymal cells as the source for CSF-1 were derived from phenotypically normal +/? animals. When the osteoblasts were derived from *op/op* animals, osteoclast formation was observed upon addition of high concentrations (up to 100 ng/ml) of exogenous CSF-1 only. In these experiments, it was irrelevant whether the hemopoietic precursor cells were derived from *op/op* or +/? animals, respectively (Takahashi *et al.,* 1991a).

The coculture system can be divided into an early phase in which proliferation of osteoclast precursors takes place and a late phase of differentiation and fusion. While $1,25(OH)_2D_3$ is essential only during the second phase of the culture, CSF-1 was found to be required during the proliferation and differentiation/fusion phases (Tanaka *et al.,* 1993). Neutralizing antibodies against GM-CSF did not affect osteoclast formation. Precursor cells grown in the presence of GM-CSF or interleukin-3, however, are capable of forming osteoclasts in coculture with osteoblasts (Takahashi *et al.,* 1991b). These cytokines may therefore support proliferation of early precursors, but are not able to replace CSF-1 at later stages of development. Somewhat contradictory was the finding that fusion of osteoclast precursors in long-term bone marrow cultures is not blocked by anti-CSF-1 antibodies, demonstrating the independence of this step in osteoclast formation from the cytokine (Biskobing *et al.,* 1995).

The notion that the late stages of osteoclast formation and of bone resorption are independent of the presence of CSF-1 was supported by some *in vivo* data. To induce osteoclast formation in osteopetrotic *op/op* mice, a single injection of the growth factor proved to be sufficient (Kodama *et al.,* 1993). This observation allows the conclusion that CSF-1 is essential to initiate proliferation of early osteoclast precursors, while the later steps of differentiation and fusion may be governed by other factors (see Chapter 7).

Data similar to those obtained with the *op/op* mouse were obtained with the osteopetrotic rat *tl* mutant. Phenotypically, *tl* rat and *op/op* mice are similar, in both cases osteopetrosis being caused by the virtual absence of osteoclasts. Furthermore, in both mutants, a reduction of femoral, peritoneal, and pleural macrophages was observed. Since the *tl* rat was not cured by marrow transplantation, and since the increase in CSF-1 activity after treatment with endotoxin was substantially smaller in sera from *tl/tl* rats than in sera from

phenotypically normal littermates, it was proposed that the osteopetrosis in *tl/tl* rats is also due to CSF-1 deficiency. Indeed, upon injection of CSF-1 into *tl/tl* rats, osteoclastogenesis was induced and bone resorption was restored (Marks *et al.,* 1992). Furthermore, osteoclast formation was induced in neonatal metatarsals from *tl/tl* rats when cultured in the presence of CSF-1, PTH, and $1,25(OH)_2D_3$ (Peura and Marks, 1995), as was described previously for the murine *op/op* mutant (Morohashi *et al.,* 1994).

Expression of the CSF-1 Receptor in Bone

Osteoclast precursors and mature osteoclasts are among the target cells for CSF-1, since these cells were shown to contain transcripts encoding the receptor (Hofstetter *et al.,* 1992; Weir *et al.,* 1993), as well as to bind the cytokine (Hofstetter *et al.,* 1995).

Expression of c-*fms* is reliant on the transcription factor PU.1 (DeKoter *et al.,* 1998). PU.1 controls the development of granulocytes, macrophages, and B and T lymphocytes. Granulocytic precursors deficient in PU.1 cannot differentiate with CSF-1 due to the lack of c-*fms* expression, and hence the PU.1 null mice do not have osteoclasts and are osteopetrotic (Tondravi *et al.,* 1997).

The identification of RANKL will further aid in the identification of signaling pathways involved in osteoclast formation and the potential for cross-talk between c-*Fms* and RANK signaling. RANKL and CSF-1 alone are sufficient to differentiate hemopoietic cells to osteoclasts and many studies have now been performed in stromal cell-free systems. However, the search for a cell line with the potential to differentiate to a *bona fide* osteoclast is still paramount to address many fundamental questions for osteoclast formation, differentiation and activation. The RAW264.7 cells have been recognized as a potential cell line that fulfils many of the functions of the osteoclast following differentiation in response to RANKL (Burgess *et al.,* 1999). However, a degree of caution must be exercised with this cell line. This cell line is CSF-1 independent and it is not known whether the independence is provided by a mutated c-*fms* allowing constitutively active receptor signaling or is a consequence of dysregulation of other control mechanisms. Due to the CSF-1-independent nature of this cell line, effects of agents determined for this cell line should be confirmed in other hemopoietic cell lines, such as C7 (Miyamoto *et al.,* 1998) or in populations of hemopoietic cell populations.

While CSF-1 action on osteoclast precursors is clearly established, the effect on mature osteoclasts is less clear. The resorptive activity of disaggregated osteoclasts on bone slices was inhibited by exogenously added CSF-1 (Hattersley *et al.,* 1988). Further analysis revealed that, besides supporting survival of mature osteoclasts, CSF-1 acts as a chemotactic agent and induces spreading and migration in these cells (Fuller *et al.,* 1993; see Chapter 7). As a consequence, reducing the proportion of resorbing cells inhibited total resorptive activity. By this mechanism, CSF-1 would regulate both the quantity and the spatial pattern of resorption. Strengthening this hypothesis is the observation that in fetal rat long bones in culture, addition of antibodies against CSF-1 led to an increase of PTH-stimulated bone resorption (Weir *et al.,* 1996).

Biological Effects of the Molecular Forms of CSF-1

As has been described above, osteoblasts and hypertrophic chondrocytes express CSF-1 *in vivo.* These cells are in close proximity to osteoclast precursors and mature osteoclasts, which express binding sites for the cytokine. These findings suggest that locally produced and acting forms of the growth factor might modulate bone resorption by regulating osteoclastic development and activity.

CSF-1 is synthesized either as a rapidly secreted molecule, a portion of which is posttranslationally modified to a proteoglycan (Price *et al.,* 1992; Suzu *et al.,* 1992), or as a membrane-bound protein. The proteoglycan form, shown to bind to collagen type V, could be integrated in the extracellular matrix and has been shown to be present in bone (Ohtsuki *et al.,* 1993, 1995). This proteoglycan, together with the membrane-bound CSF-1, can confer a locally restricted biological action. *In vitro,* osteoblasts have been shown to synthesize each of these various molecular forms of the growth factor (Felix *et al.,* 1996). However, little is known yet about the biological activities of the different forms of the cytokines as well as the regulation of their expression.

Recent studies concentrated on the regulation of the various molecular forms of CSF-1 by bone active hormones and cytokines. In primary murine osteoblasts, $1,25(OH)_2D_3$ increased the levels of transcripts encoding the membrane-bound and the secreted forms of the cytokine, respectively (Rubin *et al.,* 1996). Also PTH and TNF-α were found in human osteoblast-like cells to increase all CSF-1 transcripts (Yao *et al.,* 1998). Thus, no specific regulation of either the membrane-bound or the secreted molecular forms of the cytokine has yet been demonstrated.

Accumulating evidence suggests that the molecular forms of CSF-1 exert distinct biological effects on their respective target cells. Injections of the cytokine into *op/op* animals did not completely reverse the osteopetrotic phenotype; a residual subepiphyseal osteosclerosis persisted (Sundquist *et al.,* 1995). Furthermore, CSF-1 injections into *op/op* animals restore some populations of tissue macrophages, while others were only partially or not at all responsive (Cecchini *et al.,* 1994). These results indicate that circulating CSF-1 cannot fully replace endogenous cytokine production. This may be due to limited access to the sites where CSF-1 is required or to the possibility that production and efficiency of membrane- and matrix-bound and secreted CSF-1 may differ at various sites. Furthermore, new roles for CSF-1 were indicated when the membrane-bound molecule was found to not only act as a growth factor, but to mediate intracellular signal transduction (Zheng *et al.,* 2000).

The data obtained using the coculture of osteoblasts and spleen cells point in the same direction. Only high

concentrations of CSF-1 were able to support osteoclast formation when the osteoblasts were derived from *op/op* animals, suggesting that soluble CSF-1 may not be the most efficient molecular form of the cytokine to support this process (Takahashi *et al.,* 1991a).

Resolution of the Osteopetrotic Phenotype in *op/op* Mice

Osteopetrotic *op/op* mice undergo an age-dependent hemopoietic recovery (Begg *et al.,* 1993; Marks and Lane, 1976; Wink *et al.,* 1991). After 6 weeks of age, an increase in the marrow space available for hemopoiesis is observed, and at 22 weeks of age, marrow cavity and cellularity were comparable with normal. Since the gene encoding CSF-1 is mutated in the *op/op* strain, the recovery cannot be due to an age-dependent expression of this factor, suggesting osteoclast formation via a CSF-1 independent regulatory pathway. However, based on the complex posttranscriptional processing of CSF-1 transcripts, an incompletely penetrant phenotype, due to a leaky mutation causing a delay of normal development, cannot be excluded (Hume and Favot, 1995). If, on the other hand, CSF-1 would be partially redundant, a candidate growth factor to replace CSF-1 may be GM-CSF, although cytokine production, besides CSF-1, is not impaired in *op/op* mice (Felix *et al.,* 1995; Wiktor-Jedrzejczak *et al.,* 1990). Injections of GM-CSF alone did not reverse the osteopetrotic phenotype in *op/op* animals (Wiktor-Jedrzejczak *et al.,* 1994), but when GM-CSF was injected together with IL-3, the osteopetrosis was corrected (Myint *et al.,* 1999). Furthermore, bone resorption and hemopoietic recovery proceeded with the same kinetics in *op/op* mice and in GM-CSF/CSF-1 double knockout animals (Nilsson *et al.,* 1995), negating an essential role of GM-CSF in the recovery process. Thus, the formation of osteoclasts and the reconstitution of bone resorption in growing *op/op* animals require proliferation, differentiation, and activation of macrophages and osteoclasts by alternative pathways, independent of CSF-1 and GM-CSF. Although CSF-1 and RANKL both are essential growth factors for cells of the osteoclast lineage (Quinn *et al.,* 1998), in *op/op* mice, the formation of osteoclasts can proceed without CSF-1, albeit with lower efficiency.

VEGF and TGF-β in the Formation of Osteoclasts

A step toward the elucidation of the mechanisms leading to the resolution of the osteopetrotic phenotype in *op/op* mice was recognized when a single injection of vascular endothelial growth factor (VEGF) was found to resolve the osteopetrotic phenotype in *op/op* mice (Niida *et al.,* 1999). The authors suggest that VEGF is produced in *op/op* mice at levels sufficient for the survival and functioning of mature osteoclasts, but not for the recruitment of these cells at maximal levels, resulting in an osteopetrotic phenotype that is resolved with time. The hypothesis for a potential role of VEGF in the regulation of bone resorption was further strengthened by the finding that the factor acts chemotactically on osteoclasts and their precursors (Engsig *et al.,* 2000). Since VEGF is synthesized by hypertrophic chondrocytes (Gerber *et al.,* 1999), is integrated in the extracellular matrix of bone and cartilage, and can be released by metalloproteinases (Bergers *et al.,* 2000), VEGF seems to be suited to act as a growth factor for the cells of the osteoclastic lineage.

Another factor gaining interest as a growth factor for osteoclasts is transforming growth factor-β (TGF-β). The effect of TGF-β on the formation of osteoclasts and on bone resorption has been controversial, with stimulatory and inhibitory effects being described. An essential role of TGF-β in the inhibition of bone resorption by estrogen was suggested when it was found that inhibition of bone resorption by estrogen *in vivo* and *in vitro* was due to the induction of apoptosis in osteoclasts by the factor (Hughes *et al.,* 1996). Recent data, however, demonstrated the ability of TGF-β to enhance the formation of osteoclasts. The factor prevents osteoclast precursors to become irreversibly committed to the macrophage lineage in the presence of CSF-1. In the presence of TGF-β, the cells retain their potential to differentiate into osteoclasts in response to RANKL (Fuller *et al.,* 2000; Sells Galvin *et al.,* 1999). The three factors CSF-1, TGF-β, and RANKL may therefore fulfil specific roles in the formation of osteoclasts, CSF-1 supporting proliferation, differentiation, and survival of hemopoietic precursors, TGF-β blocking the precursors developing into macrophages, and RANKL inducing the cells to develop into mature osteoclasts. On the mature cells, the actions of the three cytokines vary—TGF-β causing apoptosis, CSF-1 supporting survival and migration and RANKL being required for survival and activation (Jimi *et al.,* 1999).

The differential actions of TGF-β to be inhibitory in cocultures, yet stimulatory in RANKL- and CSF-1 treated hemopoietic cultures indicated that the inhibitory actions of TGF-β might result from its actions on the osteoblast. Indeed this appears to be so. TGF-β acts to increase osteoblast OPG production while decreasing RANKL production (Quinn *et al.,* 2001; Takai *et al.,* 1998). The independence of OPG involvement to inhibit osteoclast formation was demonstrated using cocultures established from osteoblasts and hemopoietic cells from OPG null mice. In such cultures, TGF-β was still inhibitory and recombinant RANKL could rescue osteoclast formation, although osteoclast formation was not equivalent to cultures that had not been treated with TGF-β.

Although GM-CSF alone does not reverse the osteopetrotic phenotype in *op/op* mice, the cytokine nevertheless plays an essential role in the development of parts of the mononuclear phagocytic system. Some populations of macrophages are normal at birth and during life in *op/op* mice and are not affected by injections of CSF-1 (Cecchini *et al.,* 1994). These monocytes, which are involved in inflammatory and immune responses, appear to depend on GM-CSF rather than CSF-1 (Naito *et al.,* 1991;

Wiktor-Jedrzejczak *et al.,* 1992). Macrophages involved in organogenesis and tissue turnover (osteoclasts might be included in this group) require the presence of CSF-1 for their development and survival. Absence of CSF-1 blocks development of precursor cell populations at an early stage of differentiation.

The fact that deficiency in CSF-1 leads to a lack of both macrophages and osteoclasts suggest the presence of a common precursor requiring CSF-1 for proliferation and/or differentiation. Only later do these cells reach a branching point after which they are committed to differentiate into macrophages or osteoclasts. Mice deficient in c-*Fos,* a component of the dimeric transcription factor activator protein AP-1, express an osteopetrotic phenotype caused by the absence of osteoclasts, as is the case for *op* mice (Wang *et al.,* 1992). In these animals, however, macrophages are normal or even more numerous than normal. The c-*Fos* protein subsequently was shown to be an essential regulator of osteoclast-lineage determination (Grigoriadis *et al.,* 1994). Since only osteoclasts are affected by the lack of c-*Fos,* the precursors are already committed to develop into osteoclasts at the time they require the transcription factor. Thus, they are beyond the branching point separating the differentiation pathways of the macrophage and osteoclast lineages. Subsequently, it was shown that RANKL induces the expression of *Fra-1* in a c-*Fos*-dependent manner, thereby establishing a link between RANK signaling and the defect in c-*fos* knockout mice. Further transfection of *Fra-1,* unlike c-*fos,* into osteoclast precursor cells increased osteoclast formation (Owens *et al.,* 1999). This suggested that *Fra-1* may be limiting in osteoclast formation or that it fulfills roles distinct from those of c-*Fos.*

Effects of CSF-1 on Mature Osteoclasts and Signal Transduction

The effects exerted by CSF-1 on macrophages are extensively studied, as are the mechanisms involved in signal transduction in these cells. Ligand activation of the receptor kinase serves as the proximal signal that eventually elicits the mitogenic response. CSF-1 is required during the G1 phase of the cell cycle for mononuclear cells to enter S phase, but the growth factor is not necessary during S, G2, and M phases. Withdrawal of the growth factor induces the cells to enter a quiescent state and leads to cell death. In osteoclasts CSF-1 induces, as in macrophages, cell spreading. The cytokine increases migration of osteoclasts, this way decreasing the number of resorbing cells and as a consequence decreasing total resorptive activity (Fuller *et al.,* 1993). In addition to inducing migration of osteoclasts, CSF-1 induces the fusion of mature cells as well (Amano *et al.,* 1998). In summary, CSF-1 supports proliferation, differentiation, fusion, and survival of progenitors and mature osteoclasts. The cytokine, however, does not activate the bone resorptive activity of the mature cells (Jimi *et al.,* 1999).

Upon binding of CSF-1 to c-*Fms,* the receptor dimerizes noncovalently and autophosphorylates by activation of its intrinsic tyrosine kinase activity (Hamilton, 1997). After phosphorylation, a number of intracellular proteins can bind to the receptor, propagating specific signal cascades and cellular responses. Subsequently, the receptor becomes internalized, dephosphorylated, multiubiquinylated, and degraded (Lee *et al.,* 1999; Wang *et al.,* 1999).

As mentioned above, binding of CSF-1 induces cell spreading both in macrophages and in osteoclasts. This effect is brought about by cytoskeletal rearrangements, involving c-*Src*-dependent tyrosine phosphorylation (Insogna *et al.,* 1997) and translocation of phosphatidylinositol-3-kinase (PI-3K) from the cytoplasm to the cell membrane (Grey *et al.,* 2000; Palacio and Felix, 2001). Although the spreading can be blocked by the specific PI-3K inhibitor wortmannin (Amano *et al.,* 1998), deficiency in the monocyte specific PI-3K isoform PI-3Kγ does not affect the bone phenotype (Hirsch *et al.,* 2000). In c-*src*-deficient mice, which are characterized by an osteopetrotic phenotype due to an osteoclastic failure to form a ruffled border (Boyce *et al.,* 1992), the autophosphorylation of c-*Fms* is not affected, while the phosphorylation of an as yet unidentified protein of 85–90 kDa is significantly reduced, suggesting an abnormal phosphorylation of down-stream targets (Insogna *et al.,* 1997).

Other signal transduction pathways induced by CSF-1 involve *Cbl* (Lee *et al.,* 1999; Ota *et al.,* 2000), which is important for degradation of c-*Fms.* Furthermore, mitogen-activated protein kinases and phosphatases (Lee and States, 2000; Valledor *et al.,* 1999) are activated, finally leading to regulation of cell cycle progression by cyclins (Dey *et al.,* 2000). This short summary of signal transduction pathways induced by CSF-1 upon binding to c-*Fms* indicates the complexity of signaling that is required for the fulfilment of the multiple roles the growth factor exerts on the cells of the osteoclast lineage.

Interleukin-3 (IL-3: Multicolony-Stimulating Factor)

IL-3, which is also called multicolony-stimulating factor, has the broadest target specificity for any of the known cytokines. IL-3 can stimulate proliferation and differentiation of basophils, erythroid cells, macrophages, mast cells, megakaryocytes and neutrophils. It induces primitive pluripotential stem cells to proliferate and differentiate to erythroid and myeloid cells.

IL-3 is a glycoprotein and is produced in high amounts by activated T lymphocytes, raising the possibility of its involvement in rheumatoid arthritis or other autoimmune diseases affecting bone.

The receptor for IL-3 is a member of the hemopoietin receptor superfamily. The receptors for IL-3, IL-5, and GM-CSF are heterodimeric and each uses a common β chain. The α chains of these receptors confer specificity for their cognate ligand, although the affinities for the ligand–α chain interaction are low. The common β chain for

these receptors has no affinity for the ligands and is responsible for signal transduction following ligand binding to the α chain and recruitment of the β chain. In humans only one common β chain has been described, while in the mouse the β chain has been duplicated such that it carries two copies that are over 95% identical. The murine β chains appear to be redundant and can compensate for each other if mutated (Nishinakamura *et al.,* 1995). Although the receptor activation results in signal transduction and JAK/STAT activation (Bagley *et al.,* 1997; Woodcock *et al.,* 1997), the ability of IL-5 to internalize along with its receptor (Jans *et al.,* 1997) raises the potential that other members of this family (IL-3 or GM-CSF) might undergo a similar process.

The expression of IL-3 by osteoblasts is well documented (Birch *et al.,* 1993; Bost *et al.,* 2000; Walsh *et al.,* 2000); however, little is known of the action of IL-3 on osteoblasts. IL-3 has been demonstrated to have no effect of osteoblast proliferation or in the production of NO synthase (Riancho *et al.,* 1995; Stock *et al.,* 1998).

Because of the ability of IL-3 to stimulate the proliferation and differentiation of hemopoietic stem cells, it would be expected that IL-3 would affect osteoclast formation. Any action of IL-3 on hemopoietic stem cells would cause the proliferation of basophils, erythroid cells, macrophages, mast cells, megakaryocytes, neutrophils, and osteoclasts. IL-3 has been reported to induce murine bone marrow cells to differentiate into osteoclasts (Barton and Mayer, 1989; Hattersley and Chambers, 1990), and a neutralizing antibody to IL-3 inhibited this differentiation (Barton and Mayer, 1989). In contrast to these findings, IL-3 also can inhibit this process (Shinar *et al.,* 1990) and has no direct effects on mature osteoclasts to regulate resorptive activity (Barton and Mayer, 1990).

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

Biochemistry of GM-CSF

The principal role of GM-CSF consists in the stimulation of the growth of granulocytes and macrophages, although the cytokine may modulate osteoclastogenesis as well (see below). GM-CSF is synthesized by a number of different cell types, including endothelial cells, fibroblasts, macrophages, osteoblasts, stromal cells, and T lymphocytes. Cross species GM-CSF is not well conserved, with human and murine sharing only 56% amino acid identity and each is unable to substitute for the other to confer biological activity. GM-CSF contains two potential N-linked glycosylation sites, and both murine and human GM-CSF is glycosylated, although glycosylation is not required for activity.

The GM-CSF receptor, like that of IL-3, is a heterodimer composed of an α and a β chain; the β chain is common for both IL-3 and GM-CSF. GM-CSF can bind to the α chain with low affinity; however, signal transduction is conferred by the β chain. Upon binding the α chain, the β chain is recruited and the relatively low-affinity GM-CSF–α chain interaction is converted to one of a high-affinity state, permitting signal transduction. Several cellular targets of GM-CSF-induced tyrosine phosphorylation have been identified, including the β chain, JAK2, MAP kinase, raf, shc, and STAT5A, and STAT5B (Mui *et al.,* 1995a,b).

Much information has been gained on the action of GM-CSF since the production of GM-CSF-deficient and GM-CSF receptor β chain-deficient mice. Further, resolution of the interacting amino acids of GM-CSF with a neutralizing antibody has shed light on the topology of the receptor binding site of the β chain of the GM-CSF receptor (Rossjohn *et al.,* 2000). It is anticipated that the structure of the ligand–receptor complex would be solved in the immediate future.

GM-CSF Actions in Bone

In response to lipopolysaccharide, PTH, or TNF-α, GM-CSF production by osteoblasts is enhanced (Felix *et al.,* 1991). T lymphocytes on exposure to IL-18, a cytokine whose expression is elevated in mature osteoblasts, demonstrate enhanced GM-CSF production (Udagawa *et al.,* 1997). The contribution of locally produced GM-CSF to bone remodeling and turnover is controversial (see below), and the activity of GM-CSF on osteoblasts is unknown.

The effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) on osteoclast formation vary considerably according to the culture system employed. The addition of GM-CSF to murine cocultures, where the hematopoietic cells were pretreated with GM-CSF before being cultured with normal osteoblastic cells, stimulates osteoclast formation (Takahashi *et al.,* 1991b). Furthermore, in cells derived from human subjects, a combination of GM-CSF, IL-1, and IL-3 has been reported to stimulate the formation of multinucleated cells from $CD34^+$, stromal cell antigen negative ($Stro\text{-}1^-$) cells (Matayoshi *et al.,* 1996). Annexin II, a calcium-dependent phospholipid binding protein, induces osteoclast formation and bone resorption (Takahashi *et al.,* 1994). The mechanism by which Annexin II increases osteoclast formation involves the production of GM-CSF by T cells in response to Annexin II, followed by the expansion of the CFU-GM stem cells (Menaa *et al.,* 1999). Although these data suggest that GM-CSF may well enhance osteoclast formation, its actions are not independent of CSF-1 and GM-CSF can not substitute for CSF-1 to facilitate osteoclast formation. In cocultures of spleen cells from wild-type mice with primary osteoblast derived from *op/op* mice, neither GM-CSF nor IL-3 supported osteoclast formation (Hattersley *et al.,* 1991b). While injection of GM-CSF in *op/op* mice was able to compensate in part for the absence of CSF-1 in the development of macrophages, it was unable to play a similar role in osteoclast differentiation (Wiktor-Jedrzejczak *et al.,* 1994). The effects of GM-CSF in *op/op* mice was restricted to stimulating macrophages participating in immune defense,

but was without action on resident tissue macrophages, which are dependent on CSF-1 (Wiktor-Jedrzejczak *et al.,* 1994). These differential effects suggest the existence of at least two populations of macrophages, one dependent on GM-CSF and the other on CSF-1 (Cecchini *et al.,* 1994; Wiktor-Jedrzejczak *et al.,* 1992).

GM-CSF has also been reported to inhibit osteoclast formation *in vitro* using a variety of assay systems. GM-CSF was found to dose-dependently inhibit osteoclast formation in murine coculture assays of primary osteoblasts with spleen cells (Gillespie and Horwood, 1998; Horwood *et al.,* 1998; Udagawa *et al.,* 1997), in murine spleen cultures stimulated with RANKL and CSF-1 (as described in Chapter 7; Quinn *et al.,* 1998)), and in human peripheral blood monocyte cultures treated with RANKL and CSF-1 (Quinn *et al.,* 1998). The effects of GM-CSF action were limited to the first 3 days of coculture of primary osteoblasts with spleen cells, indicative of GM-CSF action during the proliferative phase of osteoclast formation.

When GM-CSF was exogenously added to murine bone marrow culture, it suppressed osteoclast-like cell formation induced by $1,25(OH)_2D_3$. These effects were abrogated by the addition of neutralizing anti-GM-CSF antibodies (Hattersley and Chambers, 1990). Both mouse and human osteoclast formation, supported by SaOS-4/3 cells that express the PTH/PTHrP receptor, were inhibited by either adding an antibody against M-CSF or by adding GM-CSF (Matsuzaki *et al.,* 1999). Dexamethasone, a glucocorticoid implicated in osteoclast formation, inhibits endogenous production of GM-CSF in murine bone marrow culture, consistent with GM-CSF acting as a negative regulator of osteoclast formation (Shuto *et al.,* 1994). Human osteosarcoma cell lines, MG-63 and SaOS-2, both produce G-CSF and GM-CSF and osteoblasts were suggested to play a central role in the hemopoietic microenvironment as basal producers of these cytokines (Taichman and Emerson, 1996).

The differentiation of human blood monocytes/macrophages in response to CSF-1 or GM-CSF is starting to be addressed at the molecular level using serial analysis of gene expression (Hashimoto *et al.,* 1999). Of note, monocyte-derived chemokine and prostaglandin synthases were induced in response to CSF-1. Quite surprisingly, CSF-1 and GM-CSF induced the expression of legumain, an asparaginyl endopeptidase, that has been shown to inhibit osteoclast formation (Choi *et al.,* 1999). The ability of CSF-1 to promote osteoclast formation, as well as potentially inhibit this process by the induction of an inhibitor, provides a further mechanism for exquisite control for this process.

Although GM-CSF does not permit the formation of osteoclasts, it is probably not a true inhibitor of the process but is involved in macrophage lineage commitment more than permitting osteoclast differentiation.

Consistent with a role for local GM-CSF production, interleukin-18 (IL-18) was found to be a potent inhibitor of osteoclast formation *in vitro.* IL-18 enhances the production of both IFN-γ and GM-CSF, both of which are potent inhibitors of osteoclast formation (Gillespie and Horwood, 1998; Udagawa *et al.,* 1997). IL-18 was shown to be expressed by mature osteoblastic cells and to enhance the production of GM-CSF by T cells (Horwood *et al.,* 1998; Udagawa *et al.,* 1997). In turn, the T cell-derived GM-CSF was sufficient to inhibit osteoclast formation, as was demonstrated using cultures using cells from GM-CSF null or GM-CSF receptor null mice. Thus, GM-CSF production by osteoblast/stromal cells, T cells, or fibroblastic cells may influence osteoclast formation.

Although the *in vitro* data demonstrating that GM-CSF can alter osteoclast formation is quite compelling, there is little *in vivo* data indicating a central role for GM-CSF to either enhance or inhibit osteoclast formation during normal bone turnover. Null mice for either GM-CSF, GM-CSF receptor, IL-18, or interleukin-1 β converting enzyme, which is responsible for processing IL-1 and IL-18, display normal bone architecture (Kuida *et al.,* 1995; Nishinakamura *et al.,* 1995; Robb *et al.,* 1995; Stanley *et al.,* 1994; Takeda *et al.,* 1998) and display no evidence of osteoporosis or osteopetrosis. This differs markedly from what would be predicted from the *in vitro* data. However, in pathological conditions such as arthritis the IL-18/GM-CSF pathway may have a profound effect in the establishment of arthritis and ultimately the bone destruction associated with this condition. The involvement of GM-CSF or IL-18 in collagen-induced arthritis (CIA) has been demonstrated in GM-CSF-deficient (Campbell *et al.,* 1998) or IL-18-deficient (Wei *et al.,* 2001) mice, respectively. GM-CSF or IL-18 null mice develop markedly reduced incidence or severity (hyperplasia, infiltration and bone erosion) of arthritis compared with their respective heterozygous or wild-type mice. While administration of GM-CSF or CSF-1 exacerbates synovial hyperplasia, joint inflammation, and bone erosion (Bischof *et al.,* 2000). Such findings argue that the proinflammatory roles of IL-18 or GM-CSF are responsible for the enhanced severity of arthritis observed in these CIA models. Since IL-18 can induce the expression of both GM-CSF and IFN-γ, the contribution of each of these to the onset and development of arthritis is not known. Recent data, however, suggests that IFN-γ production in arthritis may protect the bone by counteracting the excess synthesis of RANKL by activated T cells (Takayanagi *et al.,* 2000). Further, evidence is accumulating for GM-CSF- or IFN-γ-independent actions of IL-18 that are particularly evident in the presence of IL-12. IL-18 with IL-12 act in synergy to inhibit osteoclast formation *in vitro,* and this occurs independently of GM-CSF or IFN-γ action (Horwood *et al.,* 2001). As with the conflicting observations derived from *in vitro* studies with GM-CSF and IL-18 relative to the severity of arthritis seen in CIA in GM-CSF or IL-18 deficient mice, IL-12 and IL-18 coadministration promotes arthritis in mice with established CIA (Gracie *et al.,* 1999; Leung *et al.,* 2000). Additionally, anti-IL-12 therapy alone, or in combination with anti-TNF-α, suppresses murine collagen-induced arthritis (Butler *et al.,* 1999). This probably results from the proinflammatory roles of IL-12 and IL-18, both of

which have been reported to enhance synovial hyperplasia and increase cellular infiltration when administered in the murine CIA model (Leung *et al.,* 2000).

The only animal model in which IL-18 has been shown to have a protective role to prevent bone loss is a mouse model of breast cancer metastasis to bone. IL-18 administration reduced osteolysis and hypercalcemic effects of MDA-MB-231 cells following intracardiac delivery and subsequent metastasis in bone (Nakata *et al.,* 1999). Given that IL-18 acts on T lymphocytes to promote IFN-γ and GM-CSF production and that GM-CSF can be a potent inhibitor, it is tempting to speculate that IL-18-induced GM-CSF inhibits osteoclast formation, resulting in reduced osteolysis and hypercalcemia, in a manner akin to the *in vitro* situation. It has been established that breast cancer cell cytokines such as PTHrP facilitate osteoclast formation by enhancing and inhibiting osteoblastic RANKL and OPG production, respectively (Thomas *et al.,* 1999; see Chapter 61). Thus the activation and inhibition of osteoclast formation/activity is a complex one in pathological conditions where unique interrelationships among hemopoietic cells, osteoblasts, breast cancer cells, stromal cells and cells of the immune system become apparent. The relative contribution of CSFs in the maintenance, proliferation, differentiation, and cellular responses of each of these cell types needs to be considered to determine their ultimate role in modifying the skeleton.

Granulocyte Colony-Stimulating Factor (G-CSF)

Biochemistry of G-CSF

Granulocyte colony-stimulating factor (G-CSF) is principally involved in the regulation of neutrophil function and their maturation. G-CSF is a glycoprotein of ~25 kDa, and the human and murine forms are 73% identical in their primary amino acid sequence and cross-react biologically. Activated macrophages, endothelial cells, fibroblasts, and osteoblasts produce G-CSF.

The G-CSF receptor is a member of the hemopoietic growth growth-factor receptor family. Its structure resembles that of the leukemia inhibitor factor (LIF), IL-6, and oncostatin, M receptors and gp 130. Unlike these receptors which function as heterodimers or heterotrimers, the active form of the G-CSF receptor is as a homodimer.

Effects of G-CSF on the Functions of Osteoblasts and Osteoclasts

Several reports document effects of G-CSF to enhance osteoclast formation *in vivo;* however, there is little indication that G-CSF has a direct effect on osteoclastogenesis *in vitro.* Hemopoietic cells treated with G-CSF *in vitro* differentiate along the granulocyte lineage, and do not commit to the osteoclast lineage. However, when osteoclast progenitors established under myeloid and lymphoid conditions were treated with G-CSF, expansion of osteoclast colony-forming units was noted (Lee *et al.,* 1997). Support for such a role for G-CSF *in vivo* results from administration of G-CSF to mice, and transgenic mice overexpressing G-CSF (Takahashi *et al.,* 1996; Takamatsu *et al.,* 1998). G-CSF administration in animal models (mouse and rat) resulted in osteoporosis, as a consequence of two mechanisms — dramatically increased osteoclast numbers and reduced bone formation (Soshi *et al.,* 1996; Takamatsu *et al.,* 1998). Similarly, osteoporosis/osteopenia is frequently described in patients with congenital neutropenia treated with G-CSF (Bishop *et al.,* 1995; Yakisan *et al.,* 1997). It is unknown whether the osteoporosis/osteopenia in these patients directly results from G-CSF therapy or is a consequence of the neutropenia. Anabolic steroid and bisphosphonate therapy in one such patient resulted in increased bone mass (Bishop *et al.,* 1995).

Although osteoblasts synthesize G-CSF, the action of G-CSF on osteoblast function is not extensively documented. Rats administered with G-CSF resulted in inhibition of endosteal bone formation, while periosteal formation was unaffected (Soshi *et al.,* 1996). This may be attributed to the excessive number of granulocytes being produced in the endosteum, or differential actions of G-CSF on these osteoblastic populations.

Conclusions

In the recent past, our understanding of the recruitment and the activation of osteoclasts have vastly improved. Animal and cell culture models helped to identify critical steps in the regulation of the differentiation pathway from the hemopoietic precursor cell to the mature osteoclast and to understand the interplay of factors collaborating in the regulation of bone resorption. In this chapter, the contribution of CSFs to the formation and activation of osteoclasts has been reviewed.

Hemopoietic cells are the principal targets of CSFs, and as such the principal bone cell type affected by CSF action is the osteoclast. The exquisite modulation of osteoclast formation, differentiation, and activation is fundamental for the process of bone resorption. Each of the CSFs, i.e., CSF-1, GM-CSF, IL-3, and G-CSF, is expressed by osteoblasts and may impinge upon osteoclast differentiation. Each CSF exerts its unique effects on the proliferation, differentiation, activation, and survival of hemopoietic cells. The interplay between these factors, along with other cytokines and growth factors, will ultimately dictate proliferation, differentiation, lineage commitment, and apoptosis of the target cells.

The osteoblast is often considered the "CSF factory of bone" due to its ability to produce CSF-1, a critical factor to the proliferation and survival of the osteoclast. The fundamental role of CSF-1 in osteoclast formation was recognized as a consequence of the *op* mouse, which is devoid of functional CSF-1. In these mice, osteoclast formation does not

occur and as a consequence the mice are osteopetrotic. The osteopetrotic phenotype was almost completely reversed by CSF-1 administration, inducing the formation of osteoclasts and restoration of bone resorption. However, a subepiphyseal osteosclerosis persisted, suggesting that the soluble form of CSF-1 does not account for all the biological effects of the cytokine. Several growth factors have been described to coexist as secreted and membrane-bound molecules (stem cell factor, RANKL, TNF-α), and it is probable that these molecular forms fulfil specific biological roles.

Treatment of osteopetrotic op mice with a combination of GM-CSF and IL-3 or with VEGF could restore the bone defect associated with CSF-1 deficiency. Thus it has become evident that the lack of CSF-1 can be compensated for by other factors. It is not clear, however, whether the combination of GM-CSF and VEGF administration to *op* mice could fully restore the CSF-1 functions. It is unlikely that these agents when combined would fully rescue the *op*/*op* phenotype; however, combined they should rectify peripheral macrophage development and enhance osteoclast formation, although hitherto unrecognized phenotypes may develop.

The demonstration that osteoclast formation and survival is dependent upon the action of CSF-1, and that osteoclasts express *c-fms,* provides substantial evidence that osteoclasts and macrophages are derived from common precursors. Consistent with this, osteoclasts can be derived from macrophage-rich populations or macrophage-like cell lines such as RAW264.7 or C7. Such a common progenitor (CFU-GM) would differentiate in response to G-CSF or CSF-1 to a CFU-G or CFU-M progenitor, respectively (Fig. 1, see also color plate). The CFU-G progenitor is considered to be the precursor for myeloblasts and neutrophils, while monocytes, macrophages and osteoclasts would be derived from the CFU-M progenitor. However, we need to be mindful of the potential for plasticity between these different pathways.

Although CSF-1 and RANKL are crucial for osteoclast development, their action cannot be considered in isolation. Both CSF-1 and RANKL are widely distributed (Kartsogiannis *et al.,* 1999; Stanley, 1994) and this should facilitate osteoclast formation in a nonrestrictive manner. Indeed fibroblasts from a variety of organs have the capacity to support osteoclast formation and in a number of pathologies multinucleate TRAP expressing cells have been reported at sites distal to bone (Quinn *et al.,* 2000). Thus, appropriately primed hemopoietic cells juxtaposed to RANKL- and CSF-1-expressing cells or exposed to membrane shed forms may have the potential to differentiate along the osteoclast linage. Such an assumption requires that inhibitors of osteoclast formation (see Chapter 7) are abundant at these sites. Thus crucial to the development of osteoclasts at extraskeletal sites, is the presence of active CSF-1 and RANKL. Excess OPG, the decoy receptor for RANKL, at such sites would retain cells to differentiate along the macrophage pathway. Conversely, appropriate combinations of CSFs may permit cells to be maintained as a stem cell population to differentiate along other lineages such as granulocytes or macrophages. A striking example for

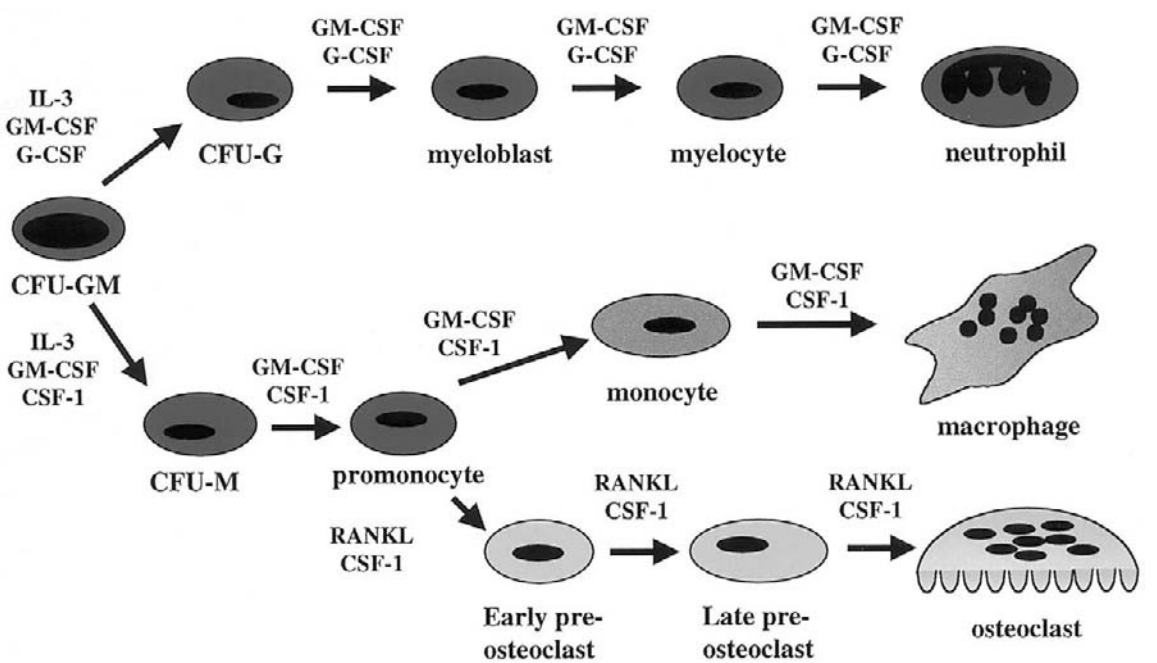

Figure 1 The lineage of osteoclasts. Osteoclasts, macrophages, and neutrophils potentially arise from a common stem cell known as the granulocyte-macrophage colony forming unit (CFU-GM). Differentiation toward a myelocyte and neutophil is dependent upon the action of G-CSF and GM-CSF. The initial pathway of osteoclast differentiation from the macrophage colony-forming unit (CFU-M) is identical to that of the monocyte/macrophages, but the final pathway is different and diverges from that of the monocytes. Both macrophage and osteoclast differentiation pathways are dependent upon the action of CSF-1. (See also color plate.)

this hypothesis is the possible role of TGF-β in the formation of osteoclasts. Marrow cells, in the presence of CSF-1 and TGF-β are maintained in their capacity to develop into osteoclasts upon exposure to RANKL, while in the absence of TGF-β, the cells loose their responsiveness to RANKL and become committed to the macrophage lineage.

Similar control mechanisms also may well exist in bone to facilitate coupled bone formation and bone resorption. Cytokines and growth factors are produced by cells in bone and may act as the soluble form via the extracellular fluid, but they may also be integrated into the extracellular matrix and act as "homing" molecules or regain their biological activity after release. In this context, some cytokines may contribute constitutively to the microenvironment suitable for cell differentiation and/or survival, while others are acutely regulated and direct cell development along specific pathways.

The activities of the CSFs in the bone microenvironment, in concert with other factors, would thus permit maintenance of appropriate cell numbers for each hemopoietic lineage.

The previously hypothesized but undocumented role of T- and B-lymphocytes and fibroblastic cells in normal bone remodeling in estrogen deplete and replete states, and in pathological bone remodeling are being characterized. The contribution of CSFs expressed by these cells, as well as other factors that may modulate osteoclast formation or activity needs to be considered. This is particularly true for pathologies such as prostate and breast cancer metastases to bone and rheumatoid arthritis. Since prostate and breast cancers produce CSF-1, both would be predicted to provide an environment for osteolysis. Prostate cancers and some breast cancers have the propensity to develop osteoblastic/osteosclerotic metastasis; however, osteolysis is a prerequisite for the subsequent growth of the metastases.

In rheumatoid arthritis, the T lymphocyte has a pivotal role in the establishment and onset of arthritis. Coupled with the production of RANKL by activated T cells, is a concomitant production of a number of CSFs that may provide a conducive environment for osteoclast formation. Simultaneously with the production of osteoclastogenic factors and cytokines, T cells have been shown to secrete factors inhibiting the formation of osteoclasts as well. The cytokine and/or CSF production by T lymphocytes is most certainly augmented by further production of cytokines and growth factors from synovial fibroblasts that become hyperplastic.

The identification of RANKL/OPG/RANK pathway to control osteoclast differentiation has permitted the expansion of experimental cell systems used to address osteoclast formation and facilitated a means to identify new control mechanisms. However, appropriately primed hemopoietic cells could predispose and commit osteoclasts to the action of RANKL or TNF-α. The interdependence of TNF-α with RANKL for osteoclast formation have recently been highlighted (Lam *et al.,* 2000). The requirement of TNF-α priming of hemopoietic cells prior to RANKL exposure shows parallels with the actions of TGF-β.

Future research will undoubtedly focus on the process of osteoclast formation and the cross-talk between the osteoclast-inductive pathways in response to RANKL and CSF-1 signaling. How do inhibitory agents impinge upon CSF-1 signaling? What is the relative contribution of CSFs from cellular sources during cancer or immuno-induced osteolysis? Is CSF-1 production modulated prior to or during osteoporosis? However the most important issue to address in this context is the molecular basis for the interrelationship and interdependence of the various cells in the bone microenvironment in their ability to influence bone remodeling.

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CHAPTER 53

Local Regulators of Bone

IL-1, TNF, Lymphotoxin, Interferon-γ, IL-8, IL-10, IL-4, the LIF/IL-6 Family, and Additional Cytokines

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The bone remodeling cycle is highly regulated by a variety of factors (i.e., hormones, cytokines, growth factors, and physical force). These are produced both locally and systemically and act in concert to direct local rates of bone turnover. Recent studies of the mechanisms regulating bone remodeling have concentrated on the role of cytokines since these factors appear to have crucial roles in both normal and pathologic bone cell function. A variety of cytokines that were originally identified by their ability to regulate immune and hematopoietic cells are believed to be produced in the bone microenvironment either spontaneously or in response to specific stimuli. It has become apparent that during both health and disease, the production of cytokines by cells in the bone microenvironment and the responses of bone cells to these cytokines are regulated in a highly ordered manner. It is hypothesized that the spectrum of cytokines that are produced in bone defines the responses of bone cells to a particular state and predicts the subsequent development of normal or pathologic bone remodeling. Diseases of bone where cytokines are believed to play an important role include osteoporosis, Paget's disease, and the effects of malignancy on bone. Studies of the production of cytokines in bone and the responses of bone cells to these cytokines may provide insights into the mechanisms that regulate the development of these diseases and could lead to new therapies for these conditions. The following is a broad overview of the actions of a number of cytokines on bone and the mechanisms by which bone cells respond to these cytokines.

Effects of Interleukin-1 on Bone

Interleukin-1 (IL-1) is a multifunctional cytokine with a wide variety of activities. It is a family of two active peptides (IL-1α and IL-1β) that are encoded by two separate gene products. Both forms of IL-1 have identical activities and potencies (Dinarello, 1991). IL-1 is the first polypeptide mediator of immune cell function that was shown to regulate bone resorption (Gowen *et al.,* 1983; Lorenzo *et al.,* 1987) and formation (Canalis, 1986), and it is the major activity that had previously been identified as osteoclast activating factor (OAF) (Dewhirst *et al.,* 1985). IL-1 is the most potent stimulator of bone resorption yet identified (Lorenzo *et al.,* 1987). It also increases prostaglandin synthesis in bone (Lorenzo *et al.,* 1987), an effect that may account for some of its resorptive activity since prostaglandins themselves are potent resorption stimuli (Klein and Raisz, 1970). The ability of IL-1 to stimulate bone resorption *in vivo* and *in vitro* requires that inducible nitric oxide synthase be present (Van't Hof *et al.,* 2000). Like a variety of other resorption stimuli, IL-1 increases receptor activator of nuclear factor κB ligand (RANKL) production in stromal/osteoblastic cells (Hofbauer *et al.,* 2001) by a mechanism that may require STAT3 activation (O'Brien *et al.* 1999). It also paradoxically stimulates OPG production in a human osteosarcoma cell line (Vidal *et al.,* 1998) and it directly enhances the resorptive activity of mature osteoclasts (Jimi *et al.,* 1999) by a mechanism that may involve activation of nuclear factor κB (NF-κB) (Miyazaki *et al.,* 2000).

Osteoclast-like multinucleated giant cell formation in cultured bone marrow is stimulated by IL-1 through a prostaglandin-dependent mechanism (Akatsu *et al.,* 1991). Hence, IL-1 may be involved in the differentiation of osteoclasts from hematopoietic progenitor cells, and this appears to be an additional mechanism by which it regulates bone resorption. IL-1 is a potent bone resorption stimulus *in vivo* (Sabatini *et al.,* 1988). Its effects on bone formation appear to be mainly inhibitory (Canalis, 1986), although it does stimulate DNA synthesis in both bone organ cultures and primary cultures of human bone cells (Canalis, 1986; Gowen *et al.,* 1985).

IL-1 is produced by bone organ cultures. However, the cells responsible for this production are not clearly identified (Lorenzo *et al.,* 1990b). It appears that both hematopoietic and mesenchymal/osteoblastic cells can produce IL-1 and its production is enhanced when both cell types are cocultured together (Haynes *et al.,* 1999). Macrophages are a likely source of IL-1 in bone marrow (Horowitz *et al.,* 1989), and osteoblast-like cells from human adult bone can also produce IL-1β *in vitro* (Keeting *et al.,* 1991).

A natural inhibitor to IL-1 has been identified (Arend *et al.,* 1990). This peptide, IL-1 receptor antagonist (IL-1ra), is an analog of IL-1 that binds but does not activate IL-1 receptors. IL-1ra blocks the ability of IL-1 to stimulate resorption and PGE_2 production in bone organ cultures (Seckinger *et al.,* 1990). Increased release of IL-1ra has recently been shown in the conditioned medium of cultured peripheral blood monocytes (PBM) from either normal or osteoporotic postmenopausal women when levels are compared with cultures of PBM from premenopausal women or postmenopausal women who are treated with estrogen (Pacifici *et al.,* 1993).

There are two known receptors for IL-1, type I and type II (Dinarello, 1993a,b). All known biologic responses to IL-1 appear to be mediated exclusively through the type I receptor (Sims *et al.,* 1993). Postreceptor signaling through the type I receptor involves sphingomyelin breakdown and production of ceramide (Kolesnick and Golde, 1994) in addition to activation of NF-κB (Jimi *et al.,* 1996). The type II IL-1 receptor appears to have little or no agonist activity rather it functions as a decoy receptor that prevents the activation of IL-1 type I receptor (Colotta *et al.,* 1993). In addition, type II IL-1 receptor can be released and circulate in serum as a soluble binding protein that inhibits IL-1 interactions with the type I receptor (Dinarello, 1993a,b). Type II IL-1 receptor may also synergize with IL-1ra to inhibit activation of the type I IL-1 receptor by IL-1 (Burger *et al.,* 1995).

Production of IL-1 appears to be involved in the development of osteoporosis. Increased IL-1 bioactivity has been found in the conditioned medium of PBM from some patients with a high-turnover form of this disease (Pacifici *et al.,* 1987). One group has found that *in vivo* estrogen treatment reduced the amount of IL-1 that was released from cultured PBM (Pacifici *et al.,* 1989). However, not all studies have confirmed this finding (Hustmeyer *et al.,* 1993; Stock *et al.,* 1989). In a related study, greater bone resorbing activity was found in lipopolysaccharide (LPS)-stimulated, PBM-conditioned medium from ovariectomized women than in PBM-conditioned medium from premenopausal or estrogen treated postmenopausal women (Cohen-Solal *et al.,* 1993). Neutralization studies showed this activity to result from both IL-1 and tumor necrosis factor (TNF)-α production *in vitro.* Measurements of IL-1 in the serum of pre- and postmenopausal women have produced conflicting results. One study showed that IL-1 levels were increased 30 days after ovariectomy (Fiore *et al.,* 1994), but others failed to find a correlation between serum IL-1α, IL-1β, or IL-1ra levels and indices of bone turnover in either pre- or postmenopausal women (McKane *et al.,* 1994) or between osteoporotic women and normals (Khosla *et al.,* 1994). Because modulation of cytokine production and/or responses in the bone marrow microenvironment may be the key mechanism by which estrogens modulate bone cell function, recent studies have focused on the ability of estrogen withdrawal to regulate IL-1 production in bone marrow. Increased production of a number of cytokines including IL-1α has also been identified in the conditioned medium of bone marrow cultures from postmenopausal women who had discontinued estrogen replacement within 1 month compared with similar studies of premenopausal controls (Bismar *et al.,* 1995). Two groups have demonstrated in mice that IL-1α biologic activity in bone marrow serum increases after ovariectomy but levels of IL-1α protein do not (Miyaura *et al.,* 1995; Kawaguchi *et al.,* 1995).

In vivo administration of IL-1ra inhibited the bone loss that occurred in ovariectomized rats (Kimble *et al.,* 1994). This effect was most pronounced after 4 weeks and was much less at earlier times. In addition, treatment of mice for 2 weeks after ovariectomy with IL-1ra decreased the ability of marrow cell cultures to form osteoclasts *in vitro* and inhibited the excretion of pyridinoline cross-links (a marker of *in vivo* bone resorption) (Kitazawa *et al.,* 1994).

IL-1 has also been implicated as one mediator of the hypercalcemia that accompanies some forms of cancer. Production of IL-1 occurs in myeloma cells that are cultured *in vitro* (Carter *et al.,* 1990) and correlates with their ability to stimulate bone resorption and hypercalcemia *in vivo.* In addition, the uncoupling of bone resorption and bone formation, which is characteristic of the lytic bone lesions in myeloma, is reproduced by local *in vivo* infusions of IL-1 (Boyce *et al.,* 1989).

Effects of Tumor Necrosis Factor on Bone

Like IL-1, TNF is a family of two related polypeptides (α and β) that are products of separate genes (Beutler and Cerami, 1986; Paul and Ruddle, 1988). TNF-α and -β have similar biologic activities and are both potent stimulators of bone resorption (Bertolini *et al.,* 1986; Lorenzo *et al.,* 1987) and inhibitors of bone collagen synthesis (Bertolini *et al.,* 1986; Canalis, 1987).

In vivo, TNF-α injections increased the serum calcium of mice (Tashjian *et al.,* 1987), and similar effects were seen with TNF-β (Garrett *et al.,* 1987). In a more detailed study, Chinese hamster ovary (CHO) cells that were genetically engineered to release large amounts of active TNF-α peptide were injected into nude mice (Johnson *et al.,* 1989). These animals became hypercalcemic within 2 weeks. Bone histomorphometry demonstrated a 10-fold increase in the number of osteoclasts in their bones compared with controls (animals injected with CHO cells that contained an empty vector). In addition, the percentage of the bone surface undergoing active resorption was similarly increased in animals receiving the TNF-producing cells.

The effects of TNF on resorption appear to be mediated by its effects on osteoclasts since osteoclast number increased after TNF treatment of bones (Johnson *et al.,* 1989) and because resorption stimulated by TNF was inhibited by calcitonin (Stashenko *et al.,* 1987). Like IL-1, TNF-stimulated induction of osteoclast-like cells formation in bone marrow culture (Pfeilschifter *et al.,* 1989) is mediated by increases in RANKL expression (Hofbauer *et al.,* 1999). However, in addition to increasing RANKL expression TNF also stimulates osteoprotegerin (OPG) in osteoblastic cell models (Hofbauer *et al.,* 1998). There is currently a controversy as to whether TNF can stimulate osteoclast formation from precursor cells in the absence of RANKL. Some authors have demonstrated this activity (Kobayashi *et al.,* 2000; Azuma *et al.,* 2000). However, others have found TNF to directly stimulate the differentiation of osteoclast precursor cells into osteoclasts only in the presence of RANKL (Lam *et al.,* 2000). The synergistic effects of TNF on RANKL-mediated osteoclastogenesis are mediated by activation of the TNF-receptor 1 (p55) (Zhang *et al.,* 2000)

In bone organ cultures, TNF stimulates DNA synthesis (Canalis, 1987). However, in an osteoblast-like osteosarcoma cell line, ROS 17/2.8, TNF did not stimulate DNA synthesis but did inhibit collagen synthesis (Nanes *et al.,* 1989). Furthermore, addition of hydroxyurea, an inhibitor of DNA synthesis, to primary rat osteoblast-enriched cultures did not alter the inhibitory effects of TNF on collagen synthesis (Centrella *et al.,* 1988). Hence, the effects that TNF has on cell replication do not appear linked to its effects on collagen synthesis. *In vitro,* TNF directly inhibits the differentiation of osteoblast precursor cells into mature osteoblasts (Gilbert *et al.,* 2000). This effect appears to involve production of nitric oxide and peroxynitrite (Hikiji *et al.,* 1997, 2000). Osteoblast apoptosis (programmed cell death) can also be stimulated by TNF (Jilka *et al.,* 1998) and this response also may be mediated by nitric oxide production (Damoulis and Hauschka, 1997).

In contrast to the inhibitory effects that continuous treatment with TNF has on collagen synthesis in bone, transient treatment with TNF for 24 hr causes a rebound increase in collagen synthesis in primary cultures of osteoblast-like cells from rat calvaria (Centrella *et al.,* 1988). Posttranscriptional regulation of message translation may also be involved in the effects that TNF has on collagen synthesis (Centrella *et al.,* 1988). TNF-α is produced by human osteoblast-like cell cultures (Gowen *et al.,* 1990) and its production is stimulated by IL-1, GM-CSF, and lipopolysaccharide but not by PTH, $1,25(OH)_2$ vitamin D_3, or calcitonin. Like RANKL, effects of TNF on osteoblastic cells are mediated by stimulation of NF-κB activity (Ali *et al.,* 1999; Yao *et al.,* 2000).

As with IL-1, TNF binds to two cell surface receptors, the TNF receptor 1 or p55, and the TNF receptor 2 or p75 (Fiers, 1993). In contrast to IL-1, both receptors transmit biologic responses. There appear to be interactions between the TNF receptor 1 and TNF receptor 2 (Tartaglia *et al.,* 1993), and for many responses, activation of both receptors is necessary to produce a full biologic effect (Vandenabeele *et al.,* 1995). However, some effects can be induced by selective activation of either receptor (Sheehan *et al.,* 1995). Mice deficient in the TNF receptor 1 and TNF receptor 2 have been made by ES cell knockout techniques (Rothe *et al.,* 1993; Erickson *et al.,* 1994). These animals appear healthy and breed normally, but lack normal immune responses and apoptotic mechanisms.

Estrogens are reported to modulate TNF production *in vitro* in human osteoblast cultures by one group (Rickard *et al.,* 1992). However, another group failed to find an effect of estrogen treatment on TNF protein production in human osteoblast-like cell cultures (Chaudhary *et al.,* 1992). Spontaneous production of TNF in cultured peripheral monocytes from women who had recently undergone ovariectomy was increased compared with levels from cells that were assayed preovariectomy (Pacifici *et al.,* 1991). As mentioned above, bone-resorbing activity in LPS-stimulated peripheral blood monocyte-conditioned medium (CM) is increased in cultures from postmenopausal women when compared with CM from premenopausal or postmenopausal women that were treated with estrogen and this effect relies on TNF in the CM to some degree (Cohen-Solal *et al.,* 1993). Kimble *et al.* (1994) found that like the effect of *in vivo* administration of IL-1ra, treatment of mice with soluble TNF receptor, an inhibitor of TNF action, reduced the ability of ovariectomy to decrease bone mass (Kimble *et al.,* 1995a). Interestingly, the most potent inhibition of the effects of estrogen withdrawal on bone mass was seen in rats that were treated with both IL-1ra and soluble TNF receptor. In a related study, it was demonstrated that mice overexpressing soluble TNF receptor 1, which binds TNF and inhibits its action, did not have increased bone loss after ovariectomy (Ammann *et al.,* 1995). Recently, mice lacking mature T-lymphocytes were found to not lose bone mass after ovariectomy and this effect appeared to be related to T-lymphocyte-mediated production of TNF (Cenci *et al.,* 2000).

TNF-β has been implicated as one cause of the effects that hematological malignancies have on bone (Garrett *et al.,* 1987). A tumor cell line was established from a patient with multiple myeloma who had developed hypercalcemia and osteolytic bone lesions. This cell line was found to contain detectable steady-state levels of TNF-β mRNA and to produce TNF-β *in vitro.* Furthermore, an antibody to TNF-β blocked some but not all of the *in vitro* bone resorbing activity that was present in the conditioned

medium from these cells and from other established myeloma cell lines. Like IL-1, TNF stimulates bone resorption while inhibiting bone formation and mimics the *in vivo* responses of bone to hematological malignancy (Bertolini *et al.,* 1986).

TNF has also been linked to the resorptive effects that nonhematologic malignancies have on bone. In one report, a squamous carcinoma cell line was shown to release a factor that stimulated monocyte cultures to produce TNF-α. This tumor did not itself produce TNF. However, implantation of the tumor into nude mice stimulated bone resorption, hypercalcemia, and leukocytosis that were reversed by a specific anti-TNF-α antibody (Yoneda *et al.,* 1991).

Effects of Interferon-γ on Bone

Interferon (IFN)-γ is another regulatory cytokine with a wide variety of biologic activities. *In vitro* IFN-γ was first shown to inhibit resorption (Gowen and Mundy, 1986; Peterlik *et al.,* 1985). This effect appears to be more specific for the response to IL-1 and TNF since lower concentrations of IFN-γ inhibit the maximum activity of these factors compared with resorption stimulated by PTH or $1,25(OH)_2$vitamin D_3 (Gowen *et al.,* 1986). The actions of IFN-γ on *in vitro* resorption appear to be mediated by its effects on osteoclast progenitor cells. IFN-γ inhibits the ability of $1,25(OH)_2$ vitamin D_3, parathyroid hormone (PTH), IL-1, and RANKL to stimulate the formation of osteoclast-like cells in cultures of human bone marrow (Takahashi *et al.,* 1986; Fox and Chambers, 2000). This effect is mediated at least in part by the ability of IFN to stimulate degradation of TRAF-6 (Takayanagi *et al.,* 2000), an intermediate in RANK signaling (Wong *et al.,* 1999).

IFN-γ is also known to inhibit other hematopoietic functions including erythropoiesis (Mamus *et al.,* 1985). However, it does not affect the resorptive activity of mature osteoclasts (Hattersley *et al.,* 1988). IFN-γ can synergistically augment IL-1 or TNF stimulated nitric oxide (NO) production by cultured osteoblasts (Ralston *et al.,* 1995). NO appears to be a biphasic regulator of osteoclast-mediated bone resorption that stimulates at low concentrations and inhibits at high concentrations (Ralston *et al.,* 1995). It is possible that part of the inhibitory effects of IFN-γ on IL-1- and TNF-mediated resorption results from effects on NO synthesis. IFN-γ together with IL-1 or TNF synergistically stimulates NO production to high levels in bone, which inhibits osteoclast-mediated resorption by inducing apoptosis of osteoclast progenitors and inhibiting osteoclast activity (Van't Hof and Ralston, 1995).

The *in vitro* effects of IFN-γ on collagen synthesis are also inhibitory. In bone organ culture, IFN-γ decreases both collagen and noncollagen protein synthesis (Smith *et al.,* 1987). This effect was not dependent on prostaglandin synthesis (Smith *et al.,* 1987). The effects of IFN-γ on collagen synthesis in the osteoblast-like ROS 17/2.8 cell line are similar to those seen in organ cultures (Nanes *et al.,* 1989).

IFN-γ also inhibits DNA synthesis in bone organ cultures (Smith *et al.,* 1987), in human cultured bone cells with osteoblast characteristics, and in the ROS 17/2.8 cells (Nanes *et al.,* 1989). It also inhibits the stimulatory effects that TNF and IL-1 have on cell replication in bone cultures (Smith *et al.,* 1987) and that TNF has in human osteoblast-like cells (Gowen *et al.,* 1988) and the ROS 17/2.8 osteosarcoma cell line (Nanes *et al.,* 1989). Effects of IFN-γ on osteoblast function appear to involve nitric oxide production (MacPherson *et al.,* 1999).

The effects of IFN-γ on bone *in vivo* are markedly different from its actions *in vitro.* In rats, ip injection of IFN-γ for 8 days induced osteopenia (Mann *et al.,* 1994). In patients who have osteopetrosis and produce defective osteoclasts, administration of IFN-γ stimulates bone resorption and appears to partially reverse the pathology of the disease. These effects are possibly due to the ability of IFN-γ to stimulate osteoclast superoxide synthesis (Key *et al.,* 1992, 1995) or osteoclast formation *in vivo* (Vignery *et al.,* 1990).

Additional Cytokines That Are Produced in Bone or Have Effects on Bone Cell Function

A variety of additional cytokines have been shown to either be produced by bone cells or to have effects on bone cell function. Interleukin-8 (IL-8) and monocyte chemotactic peptide-1 (MCP-1) are members of the chemokine family of cytokines, which is named for the ability of its members to direct the migration of cells to sites of inflammation. Both are produced in bone cells in a regulated manner (Chaudhary and Avioli, 1995; Takeshita *et al.,* 1993; Zhu *et al.,* 1994). More than 20 different chemokines have been described. In addition to IL-8 and MCP-1, these include macrophage inflammatory proteins 1α, 1β, and 2 (MIP-1α, MIP-1β, and MIP-2) and RANTES (Regulated on Activation Normal T-cell Expressed and Secreted) (Horuk, 1994). All contain four conserved cysteine residues and are classified into two categories depending on whether the first two conserved cysteine residues are separated by an intervening amino acid (CXC) or not (CC). IL-8 is a member of the CXC chemokine group while MCP-1 belongs to the CC group. IL-8 has specific neutrophil chemotactic activity. It is produced by human osteoblast-like cells, the human osteosarcoma cell line MG-63, human bone marrow stromal cells and human osteoclasts (Chaudary and Avioli, 1995; Chaudary *et al.,* 1992; Rothe *et al.,* 1998). Production of IL-8 in these cells was augmented by treatment with either IL-1 or TNF and was synergistically increased by their combination. Dexamethasone decreased the production of IL-8 in the cells in response to treatment with IL-1 or TNF. However, *in vitro* estrogen treatment had no effect.

MCP-1, which was originally called JE in mice, is a potent monocyte chemoattractant that is produced by stimulated osteoblasts (Zhu *et al.,* 1994). Like IL-8, IL-1 and TNF strongly stimulated its production in human and murine

osteoblastic cells (Takeshita *et al.,* 1993; Zhu *et al.,* 1994; Graves *et al.,* 1999).

The function of IL-8 and MCP-1 in bone remains to be defined. However, MCP-1 does not appear to be involved in regulating the development or activity of osteoclasts since it did not stimulate the maturation of osteoclast-like cells in bone marrow cultures (Zhu *et al.,* 1994) nor did it affect bone resorption rates in organ cultures (Williams *et al.,* 1992) or in isolated osteoclasts that were cultured on bone slices (Fuller *et al.,* 1995). However, it is possible that it regulates the migration of a common monocyte-osteoclast progenitor cell from the blood or bone marrow to sites of osteoclast development at the bone surface.

Of the other members of the chemokine family that have been examined for their effects on bone cells, MCP-1α and IL-8 were found to stimulate the motility but suppress the resorptive rate of isolated osteoclasts while MIP-1β, MIP-2, and RANTES were without effect (Fuller *et al.,* 1995). However MIP-1α is a potent stimulator of bone resorption that may be involved in the effects of multiple myeloma on bone (Choi *et al.,* 2000).

Interleukin 4, 10, and 13 are members of a group of locally acting factors that have been termed inhibitory cytokines (Burger and Dayer, 1995). These proteins modulate the biosynthesis of proinflammatory cytokines and regulate the production of proinflammatory cytokine antagonists that either bind but do not activate cytokine receptors (IL-1ra) or prevent binding of active cytokines to receptors by forming complexes in solution (soluble type 2 IL-1 receptor or soluble TNF receptors). IL-10 is produced by TH2 cells as well as other cell types including stimulated monocytes. In bone marrow cell cultures, which express a variety of proteins characteristic of osteoblasts including alkaline phosphatase, type I collagen, and osteocalcin, treatment with IL-10 suppressed the production of osteoblastic proteins and prevented the onset of mineralization (Van Vlasselaer *et al.,* 1993). IL-10 also inhibits the formation of osteoclast-like cells in bone marrow cultures without affecting macrophage formation or the resorptive activity of mature osteoclasts (Owens *et al.,* 1996). The inhibitory effects of IL-4 on osteoclastogenesis appears to involve tyrosine phosphorylation of specific intracellular proteins (Hong *et al.,* 2000). *In vitro* both IL-4 and IL-13 inhibit bone resorption by suppressing prostaglandin production in osteoblasts (Onoe *et al.,* 1996). In addition, they have been identified as chemoattractants for osteoblasts *in vitro* (Lind *et al.,* 1995).

Effects of IL-4 on Bone

IL-4 is a 19-kDa pleotropic cytokine secreted by activated TH2 cells and mast cells (Howard *et al.,* 1982). Its major functions include the growth and survival of T helper cells, the activation and growth of B cells, increased expression of class II MHC molecules, and the inhibition of macrophage function (Hart *et al.,* 1989; Stuart *et al.,* 1988). Although IL-4 is not a member of the LIF/IL-6 subfamily of cytokines, it uses a two-chain plasma membrane-expressed receptor similar in many ways to the LIF and IL-11 receptors. Ligand binds to the 140-kDa IL-4 receptor, which is a single polypeptide chain of 800 amino acids that confers both specificity and determines signaling (Idzerda *et al.,* 1990). The IL-4 receptor heterodimerizes with the γ common chain of the IL-2 receptor to form a complete receptor (Russell *et al.,* 1993). Binding of IL-4 results in the phosphorylation on tyrosine of cellular substrates. The IL-4 receptor has no intrinsic kinase activity. As is the case for the LIF receptor, Jak1 and Jak3 are tyrosine phosphorylated in response to IL-4, as is Stat 6 (Witthuhn *et al.,* 1994). Jak3 associates with the γ common chain, suggesting that the IL-4 receptor-specific chain associates with Jak1. It is assumed that the phosphorylation of cellular substrates is mediated by the Jak kinases. IL-4 binding does cause the tyrosine phosphorylation of ligand specific substrates such as insulin receptor substrates 1 and 2. Activation of these unique substrates is likely to be part of how different ligands confer specific activities to their targets.

IL-4 appears to inhibit bone remodeling. In organ culture, IL-4 antagonized the resorption inducing activity of IL-1, tumor necrosis factor, 1,25-dihydroxyvitamin D_3, and prostaglandin E2 (PGE2) without affecting basal resorption (Watanabe *et al.,* 1990). As mentioned previously, IL-4 inhibits macrophage function. Osteoclasts are in the macrophage lineage, suggesting that at least one of the targets for IL-4 are mature osteoclasts or more likely osteoclast precursors. Addition of IL-4 to cultures of bone marrow cells (source of osteoclast precursors) and stromal cells causes the inhibition of osteoclastogenesis (Shioni *et al.,* 1991). In this system, osteoclastogenesis induced by PTH, PTH-related peptide (PTHrP), 1,25-dihydroxyvitamin D_3, IL-1, and PGE2 was inhibited by the addition of IL-4 (Lacey *et al.,* 1995). Using this same system, bone marrow macrophages were substituted for unfractionated bone marrow cells with similar results, suggesting that the target for the IL-4 inhibitory activity was the osteoclast precursor. Recently, the effects of IL-4 on osteoclastogenesis were demonstrated to occur through direct actions on the peroxisome proliferator-activated receptor gamma 1 (Bendixen *et al.,* 2001).

In vivo bone resorption in mice that were transplanted with a PTHrP- and IL-1-secreting tumor was inhibited by continuous infusions of IL-4 (Nakano *et al.,* 1994). In addition, IL-4 delivery by adenovirus vector was shown to suppress RANKL and IL-17 production and prevent bone resorption in an *in vivo* collagen induced arthritis mouse model (Lubberts *et al.,* 2000). At least part of this inhibitory effect could be related to the ability of IL-4 to block local prostaglandin synthesis in bone cells (Kawaguchi *et al.,* 1996, Onoe *et al.,* 1996). Further evidence for the role that IL-4 may play in the control of bone remodeling is provided by IL-4-overexpressing transgenic mice (Lewis *et al.,* 1993). These mice have pronounced cortical thinning in the long bones and vertebrae. Although IL-4 overexpressing mice have normal numbers of osteoclasts, their function appeared altered as measured by a decrease in TRAP activity. Interestingly, these animals have

a marked reduction in bone formation. Both the number of cells lining the bone surface and the function of these cells was decreased. IL-4 treatment also decreased bone turnover in ovariectomized mice (Okada *et al.,* 1998).

Hence, osteoblasts, as well as osteoclasts, appear to be a target of IL-4. IL-4 induces the secretion of macrophage colony-stimulating factor (M-CSF) from the murine osteoblast-like cell line MC3T3-E1 as well as primary osteoblasts in a dose-dependent manner (Lacey *et al.,* 1994). MC3T3 cells express IL-4 receptors that can be upregulated by treatment with 1,25-dihydroxyvitamin D_3 (Lacey *et al.,* 1993). Primary human osteoblast-like cells and the human osteosarcoma MG-63 also express IL-4 receptors (Riancho *et al.,* 1993; Ueno *et al.,* 1992). Furthermore, IL-4 stimulated the proliferation of human osteoblast-like cells *in vitro* as well as their expression of alkaline phosphatase

Additional Cytokines

Interleukin-7 stimulates both B and T-lymphopoiesis. *In vivo* injection of IL-7 into mice leads to increased osteoclast formation and bone loss by a mechanism that requires T-lymphocyte production of resorptive cytokines (Miyaura *et al.,* 1997; Weitzmann *et al.,* 2000). In addition IL-7 receptor deficient mice have increased bone mass compared to wild-type controls (Miyaura *et al.,* 1997). Interleukin 12 is a regulator of immune responses that stimulates T-lymphocytes to produce an unknown cytokine, which inhibits osteoclast formation either alone or in synergy with interleukin 18 (Horwood *et al.,* 2001). Interleukin 15, which shares many activities with IL-2, was shown in one report to enhance the differentiation of osteoclast precursor cell toward mature osteoclasts (Ogata *et al.,* 1999). Interleukin 17 is a product of activated T-lymphocytes and has been shown to enhance osteoclastogenesis in two reports (Kotake *et al.,* 1999; Van bezooijen *et al.,* 1999). In one report IL-17-mediated increases in osteoclast numbers in bone marrow-osteoblast cocultures were dependent on prostaglandin synthesis (Kotake *et al.,* 1999) while in the other they were seen only in cultures that were also treated with TNF (Van bezooijen *et al.,* 1999). Interleukin 18 is produced by osteoblastic cells (Udagawa *et al.,* 1997) and inhibits osteoclast formation in cocultures of spleen and osteoblastic cells through its ability to stimulate granulocyte-macrophage colony-stimulating factor (GM-CSF) production in T-lymphocytes (Horwood *et al.,* 1998).

Effects of the LIF/IL-6 Subfamily of Cytokines on Bone

Abundant data have accumulated demonstrating that the LIF/IL-6 subfamily of cytokines has important effects on the regulation of the normal and pathologic remodeling cycles. These factors are related based on sequence homology, chromosome location, and receptors (Rose and Bruce 1991). This subfamily is composed of leukemia inhibitory factor (LIF), oncostatin-M (OSM), IL-6, IL-11, ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1). The data known about these cytokines as they relate to bone is variable. Information is known about which bone cells secrete LIF and IL-6. Receptor expression for all of the cytokines is better described and some of the biological consequences of that binding are known. More data is known about LIF, OSM, and IL-11, less is available on CNTF and CT-1, and substantial information is known about IL-6 and its receptors (Bellido *et al.,* 1997; Elias *et al.,* 1995; Girasole *et al.,* 1994; Horowitz *et al.,* 1994; Jay *et al.,* 1996). Although information is available about the receptors for these cytokines and some of the downstream events that occur following ligand binding in skeletal cells, it is more recently that the signal transduction pathways used by these cytokines has begun to be elucidated (Bellido *et al.,* 1994, Horowitz *et al.,* 1996; Levy *et al.,* 1996). These cytokines may interact with their receptors in two settings, both of which can result in signal transduction. The first and more conventional setting is when ligand binds to its appropriate plasma membrane expressed receptor component. This interaction initiates receptor component assembly, culminating in a functional receptor capable of transmitting a signal to the interior of the cell. This form of ligand–receptor interaction can occur for all of the cytokine subfamily members. The second setting, which involves the interaction between ligand and soluble receptor, is limited to specific members of the subfamily (IL-6, IL-11, and CNTF).

Originally known as differentiation inducing factor, LIF is a pleotropic cytokine exerting multiple effects in bone. Its effects on bone resorption *in vitro* are variable and, like other members of the group, depend on the model system that is used. LIF is secreted as a diffusible molecule and in an immobilized form associated with the extracellular matrix as a result of alternate promoter usage, again suggesting an important developmental control mechanism (Rathjen *et al.,* 1990). LIF mRNA is found in various rodent and human immortalized cell lines and primary fetal rat calvarial cells (Marusic *et al.,* 1993). It induces bone resorption in cultured mouse calvaria via a prostaglandin-mediated mechanism (Reid *et al.,* 1990). However, in fetal rat and mouse long bone cultures it inhibits resorption (Lorenzo *et al.,* 1990a; Van Beek *et al.,* 1993). Mice injected with a hematopoietic cell line overexpressing LIF developed cachexia and increased bone turnover with marked bone formation (Metcalf and Gearing, 1989). LIF can either potentate or inhibit the expression of alkaline phosphatase and type I collagen synthesis depending on the cells or assay used, suggesting a role for LIF in osteoblast development (Lorenzo, 1991). Addition of LIF to fetal rat calvarial cell cultures results in inhibition of bone nodule formation (Malaval *et al.,* 1995). LIF had variable effects on osteoblast-like cell proliferation. LIF had no effect on the proliferation of the human osteosarcoma cell line MG-63, induced a 40% increase in proliferation in murine MC3T3-E1 cells and had no effect on authentic murine calvarial osteoblasts (Bellido *et al.,* 1997). The addition of LIF to MC3T3-E1 cells inhibited apoptosis induced by either serum

starvation or addition of TNF (Jilka *et al.,* 1998). Treatment of MC3T3-E1 cells with PTH results in the increased transcription of LIF and IL-6 but not 14 other cytokines including IL-1, GM-CSF, and TGFβ-1 (Greenfield *et al.,* 1993) Similarly, treatment of a rat osteosarcoma cell line with PTH resulted in IL-6 secretion (Horowitz *et al.,* 1990). This suggests that some of the effects of PTH may be mediated by LIF and PTH (Grey *et al.,* 1999). Because murine osteoblasts respond to LIF, they must express cell surface receptors. LIF induced signal transduction in nonskeletal cells results in protein tyrosine phosphorylation.

Therefore, appropriate substrates should also be phosphorylated in bone cells. Treatment of the murine osteoblast cell line MC3T3-E1 with LIF results in tyrosine phosphorylation of Jak1, Jak2, gp130, and what appears to be the LIFR (Lowe *et al.,* 1995). The fact that osteoblasts respond to LIF and secrete LIF indicate that it is a true autocrine growth factor. However, few data are available on the effects of LIF on primary human osteoblast-like cells, effects which may be particularly important because osteoblast-like cells derived from patients 60 years and older fail to respond to LIF as measured by the induction of phosphoproteins (M.Horowitz unpublished observation). Whether this reflects an intrinsic unresponsiveness in these cells or is related to other processes is unknown. It is tantalizing to speculate that if this failure was age related, it could account for the onset of osteopenia and possibly osteoporosis. This idea is supported by the observation that mice rendered LIFR negative by homologous recombination develop severe osteopenia with increased osteoclastic activity (Ware *et al.,* 1995).

OSM, which is structurally similar to LIF, is a 28-kDa glycoprotein originally isolated from media of PMA stimulated U937 human histiocytic leukemia cells (Zarling *et al.,* 1986). OSM is secreted by phytohemagglutinin-stimulated T cells, lipopolysaccharide activated monocytes, and cell cultures from AIDS-related Kaposi's sarcoma for which OSM is a growth factor. OSM mRNA is also found in bone marrow cells. Its expression in bone, however, is currently unknown. OSM, like LIF, induces murine osteoblasts to proliferate and secrete matrix proteins and, of the family members, it is the most potent stimulator of IL-6 (Jay *et al.,* 1996, Bellido *et al.,* 1997). However, it inhibits alkaline phosphatase activity and bone resorption (Jay *et al.,* 1996). OSM has also been reported to inhibit the proliferation of MG-63 cells (Bellido *et al.,* 1997). Anti-Fas antibody induced apoptosis in MG-63 cells was inhibited by OSM (Jilka *et al.,* 1998). OSM activation of MG-63 cells leads to transcriptional activation of the p21WAF1,CIP1,SDI1 gene which has been shown to be important in protecting against apoptosis and could explain the anti-apoptotic effects of OSM (Gorospe *et al.,* 1997; Wang *et al.,* 1996). Transgenic mice that overexpress bovine OSM develop osteopetrosis (Malik *et al.,* 1995). Although the overall syndrome is similar to the picture seen in the LIF overexpressing mice, it does not have the associated increase in bone resorption. In fact, the osteopetrosis seen in both the LIF- and the OSM-overexpressing mice is just the opposite picture to that seen in the LIFR knockout mice. Human OSM induces tyrosine phosphorylation in both primary human and mouse osteoblast-like cells. Three JAK family members (Jak1, Jak2, and Tyk2) are tyrosine phosphorylated in response to OSM in calvarial osteoblastic cells, a response similar to that observed in nonskeletal cells (Levy *et al.,* 1996). However, human osteoblast-like cells express a number of additional phosphoproteins in the 150- to 200-kDa range not found in the mouse. These differences may be due to the mouse cells' use of the LIFR+gp130 and the alternate receptor, or of the OSM-specific receptor (OSMR)+gp130 complex vs the human cells' use of only the OSM-specific receptor, because human cells do not express the LIFR (Gearing and Bruce, 1992; Rose *et al.,* 1994). These data suggest that differences exist between the ability of these ligands to bind and activate osteoblasts and the complexity may be increased by differences in species and age.

The newest member of the LIF/IL-6 family is CT-1, a 21.5-kDa protein originally isolated from differentiated mouse embryonic stem (ES) cells (Pennica *et al.,* 1995a). The amino acid sequence of CT-1 is similar to LIF (24% homology) and CNTF (19% homology). mRNA for CT-1 is expressed in heart, liver, and kidney with less in brain and none in spleen. Its expression in bone is currently unknown. CT-1 is a potent inducer of myocyte hypertrophy, as are LIF, IL-11, and OSM, while IL-6 and CNTF are much less effective. CT-1 is inactive in the B9 assay (detects IL-6) and inhibits the differentiation of mouse ES cells (Pennica *et al.,* 1995b). CT-1 binds the LIFR directly and requires gp130 to effect signal transduction (Pennica *et al.,* 1995b). No α component of the receptor has been identified. Based on the receptor component composition, CT-1 should activate osteoblasts. Addition of CT-1 to calvarial osteoblastic cultures induces numerous tyrosine phosphorylated proteins in a time-dependent manner (M. Horowitz, unpublished observation). Because CT-1 uses the LIFR+gp130, it seems reasonable that the defects observed in heart development in the gp130 knock-out mouse are due, at least in part, to the failure of CT-1 to function. CT-1 must also be considered a candidate, as are LIF and OSM, for the cytokines contributing to the maintenance of normal bone remodeling which is profoundly altered in the LIFR knockout mice.

The second setting for the interaction of ligand and receptor is restricted to IL-6, CNTF, and IL-11 by the ability of their receptors to be cleaved from the membrane and function in soluble form (sIL-6R, sCNTFR, and sIL-11R). It is these α components that confer a level of specificity to the interaction of ligand and cell. Soluble receptor can bind ligand, forming a complex which can interact with the appropriate β subunits to trigger signaling. Addition of premixed sIL-6R plus IL-6 to murine calvarial osteoblasts results in their activation. This indicates that for IL-6 this interaction can occur in solution. This interaction can also occur on the cell membrane, as is the case for IL-11 and CNTF interacting with their receptor on calvarial osteoblasts. This is due to the presence of the α components for IL-11 and CNTF being expressed on the cell membrane

while the IL-6 α component is absent. This type of interaction may induce signal transduction in cells which would not normally be responsive to these cytokines due to lack of the appropriate α components on their membrane surface. It is thought that the major effect of IL-6 is its colony-stimulating activity on osteoclast precursors (Roodman, 1992). This is an important issue since estrogen inhibits cytokine induced IL-6 secretion by bone cells and osteoclastogenesis (Girasole *et al.,* 1992). Antibodies to IL-6 or estrogen blocked the increased formation of osteoclasts in ovariectomized mice (Jilka *et al.,* 1992). However, Tamura has reported that IL-6 does not directly induce osteoclast formation in coculture experiments (Tamura *et al.,* 1993). This failure can be overcome by the addition of sIL-6R. IL-11, OSM, and LIF (OSM>IL-11>LIF) all directly induce osteoclast formation in this system. Because of the design of the studies, the target of the cytokines could not be determined. However, treatment of primary mouse osteoblast cultures with IL-11 resulted in the upregulation of RANKL gene expression (Yasuda *et al.,* 1998). Increased RANKL expression would explain the increase in osteoclast formation in coculture induced by all four cytokines. We have shown that IL-6 does not directly induce tyrosine phosphorylation in primary mouse or human osteoblasts, but this failure can be overcome by the addition of sIL-6R (Horowitz *et al.,* 1994). However, it has been demonstrated that treatment of murine osteoblastic cells with dexamethasone induced a marked increase in IL-6 receptor expression (Udagawa *et al.,* 1995). This suggests that osteoblastic cells may be the target for IL-6 under the appropriate conditions. Therefore, the target cell in bone for IL-6 remains ambiguous. The ambiguity over the role of IL-6 in bone is heightened by the differences reported in the ability of IL-6 to induce bone resorption (Ishimi *et al.,* 1990, al-Humidan *et al.,* 1991). Because osteoblasts secrete IL-6, it is possible that IL-6 may function as an autocrine or paracrine factor, but this could only occur in the presence of sIL-6R or an increase in cortisol levels. The fact that other members of the subfamily also induce osteoclast formation supports our hypothesis that these factors play an important role in the regulation of bone remodeling. Although the LIFR has no α component, LIFR can be detected in the circulation (Layton *et al.,* 1992). Unlike the agonist effect of sIL-6R+IL-6, sLIFR+LIF may have an antagonistic effect (Gearing, 1993). Whether this is due to α vs β receptor component usage or downstream events is unknown. Equally unknown is the effect of sLIFR+LIF on bone cells.

IL-11 is a 23-kDa stromal cell derived cytokine which causes the maturation of hematopoietic precursor cells (Quesniaux *et al.,* 1993). The IL-11-specific receptor is 24% homologous with the IL-6R, suggesting that it also occurs in soluble form (Hilton *et al.,* 1994). IL-11, unlike IL-6, appears to function in the estrogen replete state to induce osteoclast formation and bone resorption and therefore may regulate normal remodeling (Girasole *et al.,* 1994). We have demonstrated that IL-11 is secreted by primary human osteoblasts and induces tyrosine phosphorylation in primary mouse osteoblasts (Elias *et al.,* 1995; Horowitz *et al.,* 1994). This suggests that unlike IL-6, but like LIF, IL-11 is a true autocrine factor for osteoblasts. This is supported by our demonstration that IL-11 induces IL-6 secretion by primary osteoblastic cells (Horowitz *et al.,* 1994). The IL-11 receptor, which is 32 and 30% identical by amino acid composition to the CNTF and IL-6 receptors, respectively, is expressed by day 9 in the developing embryo (Neuhaus *et al.,* 1994). The highest expression is in mesenchymal cells, particularly in regions containing chondro and osteo progenitors. However, no IL-11R mRNA was detected in mature osteoblasts, chondroblasts, odontoblasts, or osteocytes. This suggests that IL-11R is a marker of early bone development. MC3T3-E1 cells treated with IL-11 resulted in the phosphorylation of Jak1, Stat1, and Stat3, the inhibition of cellular proliferation, and stimulation of alkaline phosphatase activity (Shih *et al.,* 2000).

One way in which these ligands are related is by the use of a common receptor. This receptor is composed of multiple components and expression of different components allows the cell to discriminate between ligands. Therefore, a clear understanding of how this receptor functions cannot be separated from the significance of how multiple ligands sharing a common receptor regulate cell function. The receptor is composed of a set of proteins which are assembled in a defined order and which have specific functions. However, the members of the subfamily utilize different combinations of the receptor components. Each subfamily member appears to possess at least three receptor binding sites. The first site binds the α component of the receptor. The receptors for IL-6, IL-11, and CNTF have ligand binding α components while the receptors for OSM, LIF, and CT-1 lack this component (Davis *et al.,* 1991).

The other two binding sites on the ligand are required for interaction with the β1 and β2 components of the receptor. The β1 component is a transmembrane protein which forms a complex with the ligand and the α component, or with the ligand alone if the α component is absent. The β1 component has been found in the receptor of all subfamily members that have been examined. However, it is not necessarily the same molecule for every member. OSM, CNTF, and CT-1 use the same β1 molecule that was originally identified for LIF, known as LIFRβ or LIFR (Gearing *et al.,* 1992). LIFR is now known to be the low-affinity receptor for LIF (Fig. 1).

The β2 component has been identified as gp130. GP-130 was originally identified as a signal transducer for IL-6 and is now known to be a common and obligatory component of the receptor for all of the cytokines in the subfamily and is required for signal transduction (Ip *et al.,* 1992). GP-130 has been referred to as a high-affinity converting subunit. An example of this is cell activation with LIF, an event which requires LIF, LIFR, and gp130. LIF can bind LIFR and transmit a suboptimal signal. However, the presence of gp130 allows for high-affinity binding, optimal signal transduction, and cell activation. With the exception of

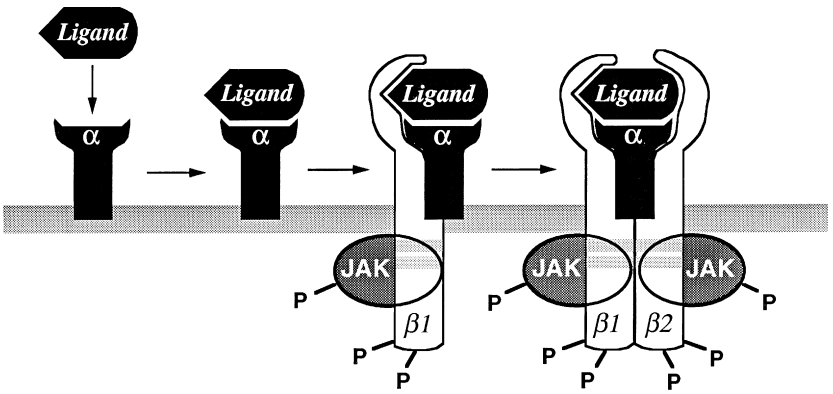

Figure 1 LIF/IL-6 cytokine receptor with component assembly including ligand, α component, β1 component, β2 component (gp130), and JAK kinase. P indicates tyrosine phosphorylation sites; cross-hatched portion in β components represents box 1 and 2 regions where JAK kinase is associated.

OSM, it appears that gp130 is the last member of the receptor components to bind the ligand and form the completed and functional receptor. As can be seen in Table 1, CNTF, LIF, OSM, and CT-1 use the LIFR subunit as the β1 component and gp130 as the β2 component. IL-6 and IL-11 use two gp130 molecules. Like LIF, OSM uses gp130 as a high-affinity converting subunit. However, unlike LIF, OSM binds gp130 preferentially. OSM may also use the OSM alternate receptor (OSMR), which is similar in structure to LIFR (32% homology) and requires gp130 for signaling (B. Mosley *et al.,* 1996). The OSMR is expressed on primary murine calvarial osteoblasts (Horowitz *et al.,* 1996). LIF does not bind the OSMR (Fig. 2).

It has been demonstrated in nonskeletal cells that signal transduction stimulated by this subfamily of cytokines is mediated by tyrosine kinases. With respect to bone cells, two major examples can be cited to demonstrate the importance of tyrosine phosphorylation in bone remodeling. Both examples involve altered bone remodeling due to genetic defects in some aspect of the process involving tyrosine phosphorylation. The first, as demonstrated by the *op/op* mouse, has a defect in the secretion of biologically active M-CSF, which can no longer signal through its receptor, c-*fms,* a receptor tyrosine kinase (Yoshida *et al.,* 1990). The second example involves c-*src,* a nonreceptor tyrosine kinase whose gene deletion by homologous recombination, like the *op/op* defect, results in osteopetrosis (Soriano *et al.,* 1991).

Table I Cytokine Receptor Composition

	Subunits		
Cytokine (ligand)	α	β1	β2
IL-6	IL-6R	None	gp130
IL-11	IL-11R	None	gp130
LIF	None	LIFR	gp130
OSM	None	LIFR	gp130
OSM	None	OSMR	gp130
CNTF	CNTFR	LIFR	gp130
CT-1	None	LIFR	gp130

The JAK family (Janus or just another kinase) are associated with the receptors for the LIF/IL-6 subfamily and other cytokines including interferon-γ, erythropoietin, growth hormone, IL-3, and GM-CSF (Silvennoninen *et al.,* 1993). The JAK family presently includes five members, Jak1–4 and Tyk2, which range in size from 130 to 137 kDa. In mammals Jak1, Jak2, Jak3, and Tyk2 are used. These kinases can be distinguished from other nonreceptor tyrosine kinases in that they have no SH2 or SH3 (*src* homology) domains and contain two catalytic domains, one being the classical kinase domain and the second a kinase-related domain which is nonfunctional (Firmbach-Kraft *et al.,* 1990; Wilks *et al.,* 1991). Different JAK kinases are activated depending on cell type and ligand bound and may phosphorylate different substrates, representing a second level of discrimination by the cell (Stahl, 1994).

The LIF/IL-6 subfamily of cytokines use Jak1, Jak2, and Tyk2. IL-12 uses a gp130-related receptor but not gp130 itself. Cytokines whose receptors are homodimers such as growth hormone, prolactin, erythropoietin, and thrombopoietin use Jak2. Signal transduction is initiated by ligand binding to its receptor which causes receptor multimerization and an apparent conformational change in the receptor complex allowing for activation of the associated JAK kinases and phosphorylation of three groups of proteins. The first group includes the β chains of the receptor (LIFR, gp130, and OSMR). Second, the kinase itself becomes phosphorylated. The third group includes a family of cytoplasmic proteins. In both skeletal and nonskeletal cells some of these cytoplasmic proteins are transcription factors belonging to the STAT family (signal transducers and activators of transcription). In addition to the STAT proteins, a group of adaptor proteins (i.e., Shc) may also become phosphorylated on tyrosine. Tyrosine phosphylation of Shc is important because it pro-

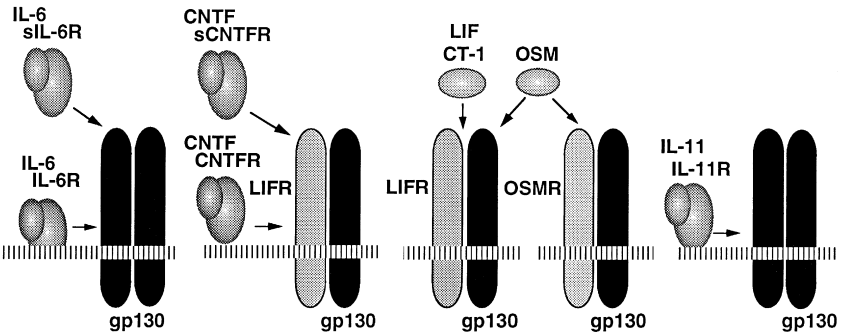

Figure 2 Receptor composition of the LIF/IL-6 family of cytokines.

vides the bridge between activated receptor chains and the mitogen activated protein kinase (MAPK) pathway. Phosphorylated tyrosine residues and their flanking amino acids on the cytoplasmic domains of the receptor chains form docking sites where proteins like the STATs, Shc, and protein tyrosine phosphatases bind and become phosphorylated (Stahl *et al.,* 1995). These proteins recognize the phosphorylated tyrosine through their SH2 domains. Because the JAKs autophosphorylate, their phosphorylated tyrosines may also serve to dock additional proteins. JAK-deficient mice have been generated with multiple phenotypes (Neubauer *et al.,* 1998; Parganas *et al.,* 1998; Rodig *et al.,* 1998). Jak1-deficient mice show perinatal lethality, are small at birth, have defects in lymphopoiesis but not myelopoiesis and fail to respond to the LIF/IL-6 subfamily of cytokines. Jak2 deficiency results in embryonic lethality due to the absence of erythropoiesis. Loss of Jak3 results in a SCID-like syndrome (Park *et al.,* 1995; Thomis *et al.,* 1995). Apparently, the skeletons of these mice have not been examined.

At the present time, six members of the STAT family have been described (Darnell *et al.,* 1994). Once phosphorylated, these proteins homo- or heterodimerize and translocate to the nucleus, by an unknown mechanism, and bind appropriate DNA sequences, resulting in gene transcription (Shuai *et al.,* 1993). A DNA promoter site in genes activated by IL-6 is similar to the IFN-γ-activated site (GAS) which mediates transcriptional induction of IFN-γ responsive genes (Pearse *et al.,* 1993). Nonskeletal cells treated with IL-6 tyrosine phosphorylate Stat1 or related proteins with subsequent binding to GAS and IL-6 sites (Bonni *et al.,* 1993; Sadowski *et al.,* 1993). CNTF induces phosphorylation of STAT proteins with subsequent translocation to the nucleus (Bonni *et al.,* 1993). Because CNTF, as well as other family members, activates early response genes, a comparison of promoter sequences was developed as a potential target sequence for STAT proteins. When nuclear extracts from CNTF-treated cells (nonskeletal) were incubated with oligonucleotides to the STAT protein recognizing sequence, binding occurred as detected by DNA–protein mobility-shift assay. This binding did not occur in the absence of CNTF and could be blocked (super shift) by the addition of anti-p91 (anti-Stat1α) (Bonni *et al.,* 1993). At present, only limited data are available relating to phosphorylation of cytosolic transcription factors and subsequent gene activation in bone cells. We have shown by immunoprecipitation that OSM induces tyrosine phosphorylation of both Stat1α and Stat3 in primary mouse osteoblastic cultures (Levy *et al.,* 1996). Stat1α and Stat3 are also phosphorylated in MC3T3 cells in response to LIF (Lowe *et al.,* 1995). Expression of a dominant negative Stat3 in a stromal/osteoblastic cell line that normally supports osteoclastogenesis results in the failure of those cells to further support osteoclast formation (O'Brien *et al.,* 1999). The inhibition of osteoclastogenesis by the dominant negative Stat3 occurs following stimulation by OSM, IL-6+sIL-6R, and IL-1 but not by parathyroid hormone or 1,25-dihydroxyvitamin D3. In the same system the dominant negative Stat3 blocked the ability of OSM or IL-6+sIL-6R to induce expression of RANKL, indicating an important role for Stat3 in gp130-mediated osteoclast formation. Stat3-deficient mice have a severe defect in development and die early in fetal life (Takeda *et al.,* 1997). A conditional knockout of Stat3 has been developed (Takeda *et al.,* 1998). These mice demonstrate the importance of Stat3 in IL-2, IL-6, and IL-10 signaling. Their bone phenotype(s), if any, have not been reported. Although the data are extremely limited at present, CNTF appears not to play a major role in bone cell activation. Of the family members, CNTF is the poorest at inducing IL-6 secretion from primary cultured osteoblastic cells (Horowitz *et al.,* 1994). However, CNTF did induce cell proliferation, increase alkaline phosphatase activity, and induce IL-6 secretion in MC3T3 cells (Bellido *et al.,* 1995). One pathway that has been shown to be activated by tyrosine phosphorylation in nonskeletal cells is Ras, a small GTP-binding protein that functions to relay signals to a group of kinases referred to as mitogen-activated protein kinases (MAPKs) or extracellular signal-regulated kinases (ERKs) (Bollag and McCormick, 1991). Activation of the MAPK cascade leads to the stimulation of transcription factors resulting in specific biological consequences. In nonskeletal cells it is known that OSM and IL-6 phosphorylate and activate MAPK (Amaral *et al.,* 1993, Kumar *et al.,* 1994). Our data show induction of a 43- to 45- kDa phosphorylated protein in human and mouse osteoblasts following OSM stimulation, which we have recently shown is MAPK (Horowitz and Levy, 1995). Tyrosine kinase pathways are linked to the Ras signaling pathway via Grb2, a small adaptor protein that contains domains referred to as src homology

(SH2 and SH3), which are involved in protein–protein interactions. Grb2 associates with the guanine nucleotide exchange factor for Ras, SOS (the mammalian homolog of the *Drosophila* son of sevenless gene product), via interactions between its SH3 domain and proline-rich sequences in SOS. These Grb2–SOS complexes then associate with tyrosine kinase receptors through binding of the SH2 domain of Grb2 to tyrosine residues in growth factor receptors that become phosphorylated following ligand binding and cellular activation. The tripartite complex of receptor/Grb2/SOS aids in translocating SOS from the cytoplasm to the plasma membrane where it is placed in close proximity to its target, Ras, converting Ras from the GDP to the biologically active, GTP-bound state. Another adaptor protein, Shc, contains an SH2 domain which has been shown to bind to tyrosine phosphorylated receptors in nonskeletal cells (Pelicci *et al.,* 1992). Shc also contains an additional phosphotyrosine binding domain (PTB) which is capable of interacting with other phosphorylated proteins (Kavanaugh and Williams, 1995). Subsequent to cytokine binding, Shc becomes tyrosine phosphorylated and can interact with phosphorylated cytokine receptors, thereby enabling its interaction with Grb2 and activation of the Ras pathway. OSM stimulation of human osteoblasts results in tyrosine phosphorylation of Shc (Horowitz and Levy, 1995). The presence of tyrosine phosphorylated MAPK supports the idea that family members may be stimulating the Ras pathway as well as the JAK/STAT signaling pathway. The tyrosine kinases that are responsible for phosphorylating Shc upon cytokine stimulation may be members of the JAK family or other cytoplasmic tyrosine kinases such as the src-family of tyrosine kinases.

As with other types of cytokine signaling, it is as important, if not more so, to turn off the signaling cascade as it is to turn it on. This can be accomplished by the activation, through phosphorylation on tyrosine residues, of protein tyrosine phosphatases. Once activated these proteins dephosphorylate other phosphorylated proteins, in particular tyrosine kinases resulting in deactivation. As mentioned previously, protein tyrosine phosphatases dock to phosphorylated tyrosine residues on the cytoplasmic domains of the cytokine receptor through their SH2 domains, resulting in phosphorylation of the phosphatase. Although there is clear evidence that protein tyrosine phosphatases are activated in response to the LIF/IL-6 family of cytokines (PTPase, Shp1) little if any direct data is available as to whether this negative regulation occurs in bone cells (Stahl *et al.,* 1995). In addition to the negative regulatory activity of phosphatases, a new family of proteins has been recently identified that can inhibit JAK-STAT signaling. The first member of this family (CIS/CIS1) inhibited the signaling of IL-3 and erythropoietin (Yoshimura *et al.,* 1995). A second member of the family was identified and is referred to as JAB (JAK binding protein), SOCS1 (suppressor of cytokine signaling), or SSI-1 (STAT-induced STAT inhibitor) (Endo *et al.,* 1997; Naka *et al.,* 1997, Starr *et al.,* 1997) and is a negative regulator of IL-6 signaling. At present seven members of this family have been identified. In some cases (JAB and CIS3) inhibit the kinase activity by directly binding to the JAKs and in others cases (CIS/CIS1) the STATs are inhibited. Thus ligand binding results not only in activation of the signaling pathway, but also in its inactivation by a negative feedback loop. Whether this family of proteins downregulates cytokine activation in bone cells remains to be determined.

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CHAPTER 54

Prostaglandins and Bone Metabolism

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Introduction

In the 30 years since prostaglandin E_2 (PGE_2) was first shown to stimulate cyclic AMP production and resorption in bone organ cultures, ample evidence has accumulated that prostaglandins (PGs) and other eicosanoids have important physiologic and pathologic roles in skeletal metabolism. However, an understanding of these roles has been complicated by the fact that PGs have dual effects in most test systems and that little is known about the skeletal production of PGs *in vivo.* This chapter will summarize current knowledge on the regulation of PG production in bone and the effects of PGs and other eicosanoids on bone resorption and formation.

PG Production

Metabolic Pathways

Eicosanoids are oxygenated 20-carbon fatty acids derived from polyunsaturated eicosatrienoic, eicosatetranoic (arachidonic), and eicosapentanoic ("fish oil") fatty acids. The predominant precursor is arachidonic acid (AA), cleaved from phospholipids in the lipid bilayer of cells. There are three major families of eicosanoids, as shown in Fig. 1: (1) prostanoids (PGs and thromboxane), synthesized via the cyclooxygenase pathway; (2) leukotrienes, lipoxins, and hydroxy-fatty acids, synthesized via the lipoxygenase pathway; and (3) epoxy and omega derivatives, synthesized via the cytochrome P-450-dependent epoxygenase pathway (Capdevila *et al.,* 2000; Smith, 1989). Eicosanoids are "local hormones" and are synthesized in most tissues. They generally circulate at low levels (less than 1 nM) in the plasma, concentrations below those at which they are normally able to elicit responses (Smith 1989). PGs have been the eicosanoids most studied in bone because they are highly expressed in osteoblastic cells and can have marked effects on both bone resorption and formation.

The production of PGs involves three major steps: (1) hormone- or stress-activated mobilization of AA; (2) conversion of AA to prostaglandin endoperoxide H_2 (PGH_2); and (3) conversion of diffusible PGH_2 by tissue-specific isomerases and reductases to PGE_2, PGD_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), and thromboxane. The committed step in the conversion of AA to PGs is catalyzed by a bifunctional enzyme which converts free AA to PGG_2 in a cyclooxgenase reaction followed by reduction of PGG_2 to PGH_2 in a peroxidase reaction (Smith *et al.,* 1996). This enzyme, formally named prostaglandin endoperoxide H synthase or prostaglandin G/H synthase (PGHS), is popularly called cyclooxgygenase (COX) in reference to its first function. At higher AA concentrations than expected to occur naturally (≤ 2 μM), COX can also exhibit some lipoxygenase activity (Thuresson *et al.,* 2000). Although synthesis of eicosanoids is predominantly dependent on enzymatic metabolism of free AA, nonenzymatic free-radical-induced peroxidation of free or esterified AA can give rise to natural products that are isomeric with PGs and leukotrienes, called isoprostanes and isoleukotrienes (Morrow and Roberts, 1997; Rokach *et al.,* 1997). These products may be important in inflammation.

The individual PG synthases are differentially distributed in tissues and have been thought to influence PG production largely by determining the predominant type of prostanoid

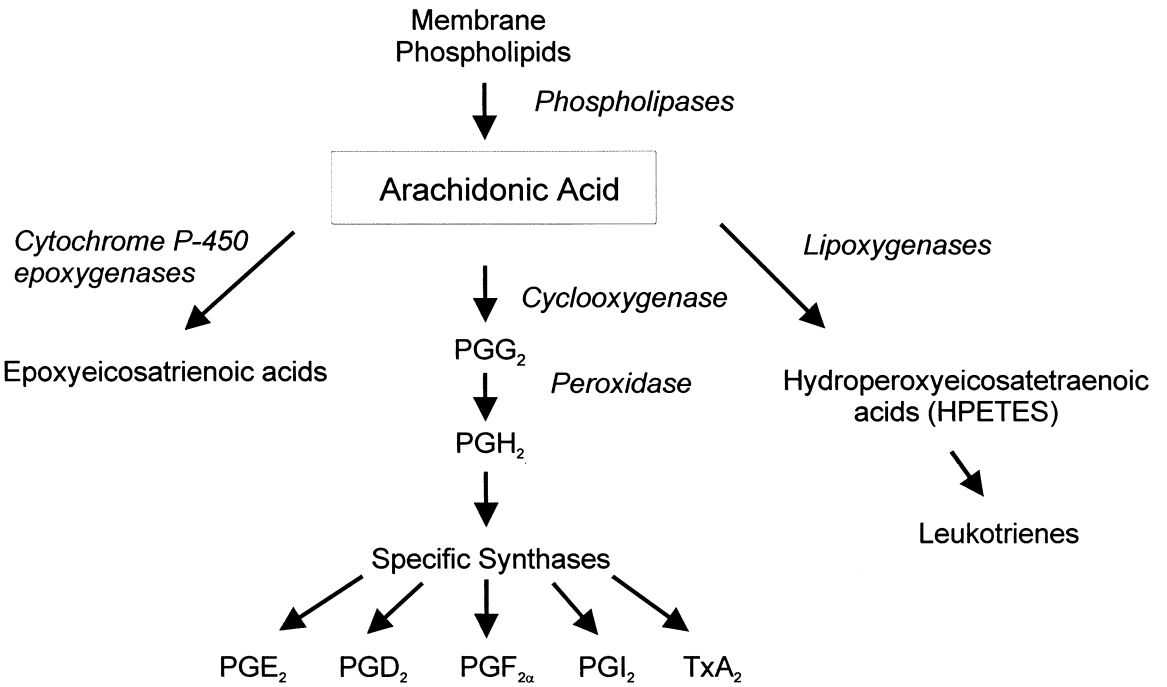

Figure 1 Diagram of the three major families of eicosanoids derived from arachidonic acid: prostanoids (PGs and thromboxane); (2) leukotrienes, lipoxins, and hydroxy-fatty acids; and (3) products of the cytochrome P-450-dependent epoxygenase pathway. The cyclooxygenase reaction that converts arachidonic acid to PGG_2 and the peroxidase reaction that subsequently converts PGG_2 to PGH_2 are both carried out by prostaglandin G/H synthase (PGHS), also called cyclooxygenase (COX).

synthesized in a particular tissue. However, PGD and PGE synthases have recently been cloned, and studies have shown that their expression in a single tissue may be regulated by a variety of factors, including fluid shear stress and interleukin-1 (IL-1) (Forsberg *et al.,* 2000; Pinzar *et al.,* 2000; Taba *et al.,* 2000). As more is learned about these synthases, they are likely to become important targets for drug therapy aimed at manipulating specific PGs.

Two Isoforms for COX

There are two enzymes for COX, COX-1 and COX-2, encoded by separate genes. COX-1 is expressed at relatively stable levels in most tissues and is considered to be "constitutive," while COX-2 is generally expressed at very low levels in most tissues but can be induced to high levels by multiple factors (Herschman 1994; Smith *et al.,* 1996; Williams and DuBois, 1996). COX-2 was initially identified as a phorbol ester-inducible primary response or immediate–early gene (TIS10) in murine 3T3 cells (Kujubu *et al.,* 1991) and a v-*src*-inducible gene product in chicken fibroblasts (Xie *et al.,* 1991). The human COX-2 gene has about 80% homology with the chicken and murine genes (Hla and Neilson 1992). The COX-1 gene is about 22 kb long with 11 exons, while the COX-2 gene is approximately 8 kb long with 10 exons (Fletcher *et al.,* 1992; Kraemer *et al.,* 1992). The gene for COX-2 lacks an intron that encodes a hydrophobic region in the amino-terminus of COX-1 protein but contains an extra sequence that encodes an 18-amino-acid insert in the carboxy-terminal region of COX-2 protein, as well as a longer 3′ untranslated region with multiple AUUUA sequences which have been associated with rapid degradation of mRNA. COX-1 and COX-2 are about 60% identical at both amino acid and nucleic acid levels. Both COX-1 and COX-2 are homodimeric (72 kDa/subunit), heme-containing glycoproteins (Smith *et al.,* 1996). Although some studies have suggested differences in cellular locations of the two enzymes, other studies find both enzymes to be located similarly on the inner and outer membranes of the nuclear envelope and on the lumenal surface of the contiguous endoplasmic reticulum (Smith *et al.,* 1996; Spencer *et al.,* 1998). Unlike many membrane proteins which have a transmembrane domain, COX-1 and COX-2 are anchored only to one leaflet of the lipid bilayer through the hydrophobic surfaces of amphipathic helices (Smith *et al.,* 1996).

Although COX-1 and COX-2 have essentially the same catalytic mechanisms, they appear to be independently functioning biosynthetic pathways. This is due in part to the differential regulation of their expression but may also involve differential substrate utilization and differential association with specific PG synthases. Because COX-1 is generally constitutively expressed while COX-2 is inducible, it was hypothesized that COX-2 is responsible for acute PG responses associated with inflammation and pain, while COX-1 produces those prostanoids needed for ongoing "housekeeping" functions, including maintenance of renal blood flow, platelet aggregation, and gastric cytoprotection. This hypothesis led to

the development of highly selective inhibitors of COX-2 activity, such as rofecoxib and celecoxib, for treatment of pain and inflammation associated with such diseases as arthritis (Golden and Abramson 1999; Marnett *et al.,* 1999). These agents cause fewer gastric ulceration and bleeding problems than nonselective NSAIDs.

The roles of COX-1 and COX-2 are probably more complex than originally conceived. Mice in which either the COX-1 or the COX-2 gene is disrupted have been engineered (Dinchuk *et al.,* 1995; Morham *et al.,* 1995; Langenbach *et al.,* 1995, 1999). COX-1-deficient mice survive normally, have no gastric pathology, show less indomethacin-induced gastric ulceration than wild-type mice, and have a reduced inflammatory response to exogenous AA but not to phorbol ester. As expected, COX-1-deficient mice have reduced platelet aggregation. COX-2-deficient mice have progressive renal disease which may limit their life span, and they have normal inflammatory reactions to both AA and phorbol ester. COX-1-deficient female mice have delayed parturition. COX-2-deficient females are infertile, with multiple failures in female reproductive processes, including ovulation (absence of corpora lutea), fertilization, implantation, and decidualization, none of which are due to deficiency of gonadotrophins or ovarian hormones (Lim *et al.,* 1997). Disruption of either COX-1 or COX-2 has been shown to be associated with reduced intestinal tumorgenesis in Min mice (Chulada *et al.,* 2000). These studies demonstrate that COX-2 can be a mediator of pathologic processes but has physiologic functions as well. To make matters more complicated, recent studies suggest that induction of COX-2 may also play an important role in the repair of damaged gastric and colonic mucosa (Colville-Nash and Gilroy 2000).

Substrate for COX

Phospholipase A_2 (PLA_2) enzymes catalyze the hydrolysis of membrane phospholipids, releasing free fatty acids and lysphospholipids. More than 10 mammalian PLA_2 enzymes have been identified. At least two classes of PLA_2 enzymes play important roles in prostanoid generation—cytosolic PLA_2 ($cPLA_2$) and the group II secretory PLA_2 ($sPLA_2$) enzymes (Balsinde *et al.,* 1999; Chaminade *et al.,* 1999; Murakami *et al.,* 1997a). $cPLA_2$, with a molecular mass of 85 kDa, is specific for AA release. $cPLA_2$ is constitutively expressed in the cytoplasm of cells, is activated at micromolar concentrations of Ca^{2+} in stimulated cells, and, after activation, translocates to the nuclear and endoplasmic recticulum membranes. $sPLA_2$ enzymes, with molecular mass of 14 kDa, nonselectively release fatty acids from the *sn*-2 position of phospholipids and require millimolar Ca^{2+} concentrations for activation. $sPLA_2$ enzymes can be induced by proinflammatory agents. After secretion, $sPLA_2$ enzymes can hydrolyze phospholipids in cell membranes or in microvesicles shed from activated cells or possibly act via specific membrane receptors (Lambeau and Lazdunski 1999). Two $sPLA_2$ enzymes, types IIA and V, have been shown to be the major contributors to PG production (Bingham *et al.,* 1999; Sawada *et al.,* 1999). These two enzymes have different cellular locations but may be able to compensate for each other since some mouse strains, such as C57Bl/6 mice (and the MC3T3-E1 osteoblastic cells derived from them), naturally lack type IIA $sPLA_2$.

COX-1 and COX-2 may be functionally coupled to the different phospholipases (Balsinde *et al.,* 1998; Naraba *et al.,* 1998; Reddy and Herschman, 1996, 1997), but the relationship is complex. Similar to the COX enzymes, phospholipases have both constitutive and inducible members. There is cross-talk between the phospholipases, with studies showing that $sPLA_2$ activation requires previous $cPLA_2$ activation (Balsinde *et al.,* 1998; Fujishima *et al.,* 1999) and vice versa (Hernandez, *et al.,* 1998). Moreover, PGs themselves can enhance COX-2 and $cPLA_2$ expression (Murakami *et al.,* 1997b; Pilbeam *et al.,* 1994). It is not surprising, therefore, that both $cPLA_2$ and $sPLA_2$ enzymes have been implicated in both the "immediate" and "delayed" phases of stimulated PG generation associated with constitutively expressed COX-1 and inducible COX-2 activities, respectively (Murakami *et al.,* 1997a).

PG Receptors

G-protein Receptors

Much of the complexity of PG effects on skeletal tissues may be attributable to the fact that there are multiple transmembrane G-protein-coupled receptors to which PGs can bind and which can produce different effects through different signaling pathways. For PGE_2 itself there are four classes of receptors. The EP_1 receptor acts largely by increasing calcium entry and phosphatidyl inositol turnover and has been implicated in mineralized nodule formation (Fujieda *et al.,* 1999) and the induction of c-*fos* and c-*jun* (Suda *et al.,* 2000), as well as IL-6 and COX-2 (Kozawa *et al.,* 1998; Suda *et al.,* 1998). The EP_2 and EP_4 receptors, both of which activate adenylyl cyclase, have been the most extensively studied. Both receptors appear to be involved in regulation of osteoblast function, including the ability of these cells to induce osteoclast formation, based on experiments with knockout animals and selective agonists, which are described below. There are limited studies of PGE_2 effects on chondrocytes, which suggest that the EP_1 and EP_2 receptor pathways may be involved in regulating proliferentiation and differentiation of these cells as well (Del Toro *et al.,* 2000; Sylvia *et al.,* 2000). The EP_3 receptor acts largely by inhibiting cyclic AMP production and has not been shown to have a specific role in bone. However, there are alternative transcripts of this receptor which can act through other signal transduction pathways (An *et al.,* 1994).

The PGI_2 or IP receptor has not been extensively studied, but PGI_2 is produced by bone cells and selective agonists for this receptor have been shown to stimulate bone resorption (Conaway *et al.,* 1986). Studies suggest the existence of both PGE_2 and PGI_2 receptors on bone cells (Khanin *et al.,*

1999; Zaman *et al.,* 1997), and one recent study demonstrated IP receptors in mouse osteoblastic MC3T3-E1 cells induced by tumor necrosis factor (TNF)-α (Wang *et al.,* 1999). Finally there is evidence for a $PGF_{2\alpha}$ receptor (FP) pathway in bone, which may act through a protein kinase C (PKC) pathway to induce COX-2 (Pilbeam *et al.,* 1994), inhibit collagen synthesis (Fall *et al.,* 1994), and activate the MAP kinase pathway (Hakeda *et al.,* 1997). Additional pathways through PGD_2 or thromboxane A receptors have not been identified.

Peroxisome Proliferator-Activated Receptors (PPARs)

PPARs are members of the nuclear receptor family of transcription factors that are important in lipid metabolism and may have a variety of effects on cellular function. Three major subtypes, α, γ, and δ, have been described, reviewed in Greene *et al.* (2000). Upon ligand binding, PPARs form heterodimeric complexes with retinoid X receptors and bind to PPAR response elements, attracting coactivators and other proteins, to mediate gene transcription. PPARγ is expressed in bone marrow stromal cells and might play a role in determining adipocyte versus osteoblastic differentiation from a common stem cell (Gimble *et al.,* 1998). PPARγ has a wide array of potential ligands, including arachidonic acid, NSAIDS at high concentrations, some lipoxygenase metabolites, and a metabolite of PGD_2, 15-deoxy-Δ^{12-14} PGJ_2. Metabolites of the lipoxygenase pathway have also been reported to bind PPARα (Greene *et al.,* 2000). PPARδ has recently been reported as the receptor mediating the effects of PGI_2 that are essential for embryo implantation in the mouse (Lim *et al.,* 1999), demonstrating the first functional role for this receptor. However, the importance of naturally occurring PGD_2 metabolites or other endogenous ligands as activators of PPARs is still unclear.

Regulation of PG Production in Osteoblasts

Stimulation of PG Production

Early studies using complement-sufficient antisera which contain antibodies to rodent cell surface antigens showed that this serum could increase resorption of fetal rat long bones by stimulating endogenous PG production (Raisz *et al.,* 1974). Subsequently, a large number of agonists were found to increase bone PG production, including many regulators of bone metabolism. Prostanoids produced by bone cells include PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$, the metabolite of PGI_2, as well as some PGD_2 and thromboxane (Feyen *et al.,* 1984; Raisz *et al.,* 1979). Among the factors which stimulate PG production in bone are mediators of inflammatory processes, such as interleukin-1 (IL-1) (Sato *et al.,* 1986) and TNF-α (Tashjian *et al.,* 1987); multifunctional regulators of cell growth and differentiation, such as transforming growth factor (TGF)-α and -β (Hurley *et al.,* 1989; Sumitani *et al.,* 1989; Tashjian *et al.,* 1985); systemic calcium-regulating hormones, such as parathyroid hormone (PTH) (Pilbeam *et al.,* 1989) and $1,25(OH)_2D_3$ (Klein-Nulend *et al.,* 1991); and mechanical loading of bone (Cheng *et al.,* 1997; Lanyon, 1992; Rawlinson *et al.,* 1991; Reich and Frangos, 1993). Thyroid hormone (Klaushofer *et al.,* 1995), platelet-derived growth factor (PDGF) (Tashjian *et al.,* 1982), bradykinin (Ljunggren *et al.,* 1991a), and thrombin (Ljunggren *et al.,* 1991b) can also stimulate PG production in osteoblasts.

Importance of COX-2 Expression for Stimulated PG Responses

Most commonly used osteoblastic cell models constitutively express COX-1 and, except for ROS 17/2.8, which expresses only COX-1 (Pilbeam *et al.,* 1997b), can be induced to express COX-2, as shown in Fig. 2. Induction of COX-2 in osteoblastic cells appears to be necessary for most stimulated PG responses (Fig. 2). Agonists shown to induce COX-2 in osteoblastic cells include cytokines—IL-1 (Harrison *et al.,* 1994; Kawaguchi *et al.,* 1994, 1996; Min *et al.,* 1998; Pilbeam *et al.,* 1997a), TNF-α (Kawaguchi *et al.,* 1996), and IL-6 (Tai *et al.,* 1997); growth factors—TGFα (Harrison *et al.,* 1994; Pilbeam *et al.,* 1997a), TGF-β (Pilbeam *et al.,* 1993, 1997a), and basic fibroblast growth factor (FGF-2) (Kawaguchi *et al.,* 1995a); systemic hormones—PTH (Kawaguchi *et al.,* 1994; Tetradis *et al.,* 1997) and $1,25(OH)_2$ vitamin D_3 (Okada *et al.,* 2000a); and fluid shear stress or mechanical loading (Forwood *et al.,* 1998; Klein-Nulend *et al.,* 1997; Pavalkon *et al.,* 1998). Serum is also a potent stimulator of COX-2 expression and PG production in cultured osteoblasts (Pilbeam, Kawaguchi *et al.,* 1993) (Fig. 2).

In many osteoblastic cell cultures (Pilbeam *et al.,* 1994), in contrast to bone organ cultures (Kawaguchi *et al.,* 1994, 1996), little PGE_2 is produced despite induction of COX-2 expression unless serum is present or AA is added, suggesting that substrate availability is limiting. Some agonists, such as TGF-β (Pilbeam *et al.,* 1997a) and PDGF (Chen *et al.,* 1997), stimulate more PGE_2 production in serum-free cultures of osteoblasts than other agonists, probably secondary to their effects on phospholipases. PDGF has been shown to activate $cPLA_2$ in osteoblasts (Chen *et al.,* 1997).

Autoamplification of PG Responses by PGs

$PGF_{2\alpha}$ was found to increase PGE_2 production in organ culture, thereby enhancing the resorptive effects of PGE_2 (Raisz *et al.,* 1990). It was subsequently shown that many PGs induce COX-2 expression and can, therefore, amplify PG responses to other agonists (Kawaguchi *et al.,* 1994; Pilbeam *et al.,* 1994). Cyclic AMP (cAMP) increases COX-2 expression and PG production, and PGs increase cAMP production which could provide the basis for a positive feedback system (Raisz *et al.,* 1990; Oshima *et al.,* 1991; Tetradis *et al.,* 1996, 1997). However, PGs also induce COX-2 expression

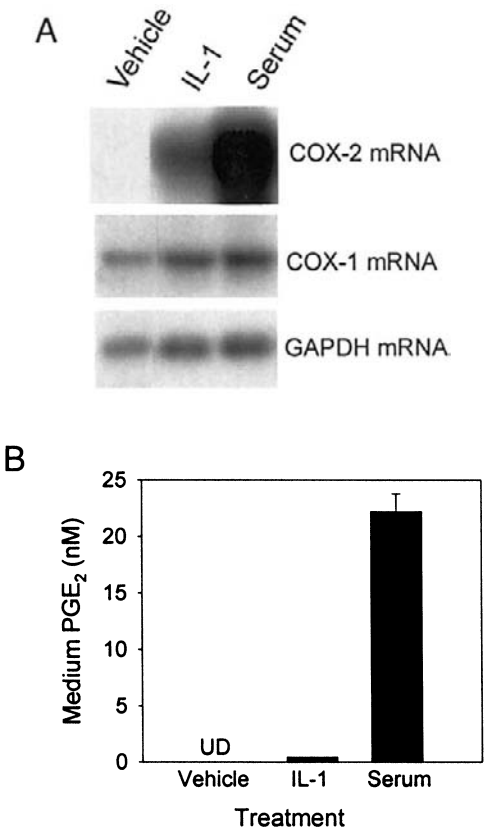

Figure 2 Induction of cyclooxygenase-2 (COX-2) and PGE_2 production by interleukin-1 (IL-1) and freshly added serum in murine osteoblastic MC3T3-E1 cells. Cells were grown to confluence and serum deprived for 24 hr before treatment for 3 hr with vehicle, IL-1 (10 ng/ml), or fetal calf serum (10%). Methods are as described in Pilbeam *et al.,* (1997a). (A) Total RNA was extracted and Northern analysis performed. COX-2 mRNA was not detectable in control cultures but was markedly induced by IL-1 and serum. There was little effect on cyclooxygenase-1 (COX-1) mRNA. mRNA levels for the housekeeping gene glyceraldehyde 3-phosphatase dehydrogenase (GAPDH) are shown to assess RNA loading. (B) PGE_2 levels were measured by radio-immunoassay ($n = 3$ wells per group). PGE_2 was undetectable (UD) in the medium of vehicle-treated cultures.

through PKC pathways (Pilbeam *et al.,* 1994; Takahashi *et al.,* 1994). This amplifying mechanism could be important in sustaining production of PGs in cell and organ cultures, in mediating the prolonged effects of short periods of impact loading or mechanical strain in skeletal tissue, and in prolonging effects of cytokines in inflammatory diseases.

Transcriptional Regulation of COX-2

In osteoblasts, COX-2 behaves like an immediate–early gene in response to most agonists, with the induction of mRNA expression being rapid, transient, and independent of new protein production (Min *et al.,* 1998; Kawaguchi *et al.,* 1995a; Pilbeam *et al.,* 1993, 1997a). In murine osteoblastic cells transfected with murine COX-2 promoter–luciferase reporter constructs, the 371 bp of the 5′-flanking COX-2 gene proximal to the transcription start site is adequate to mediate transcriptional induction by many of the agonists discussed above (Harrison *et al.,* 2000; Kawaguchi *et al.,* 1995a; Okada *et al.,* 2000b; Pilbeam *et al.,* 1997a; Tetradis *et al.,* 1997; Wadleigh and Herschman, 1999). *Cis*-acting sequences in this region shown to mediate effects on COX-2 expression in osteoblastic cells include a cyclic AMP response element (CRE) (Okada *et al.,* 2000b; Wadleigh and Herschman 1999), an activator protein-1 (AP-1 or Fos/Jun) binding site (Okada *et al.,* 2000b), and CCAAT enhancer binding protein (C/EBP) sites (Harrison *et al.,* 2000; Wadleigh and Herschman, 1999; Yamamoto *et al.,* 1995).

To study the transcriptional regulation of COX-2 *in vivo,* we generated mice transgenic for -371/+70 bp of the COX-2 5′-flanking DNA fused to a luciferase reporter (Freeman *et al.,* 1999). When these mice were injected with lipopolysaccharide (LPS), a potent inflammatory mediator, COX-2 mRNA and luciferase activity were induced in multiple tissues. The LPS induction of both luciferase activity and of endogenous COX-2 mRNA in calvarial bone was among the highest of all the tissues sampled, including brain, colon, heart, lung, and uterus. In freshly isolated tissues from mice injected with LPS, we have consistently found Northern analyses for COX-2 mRNA levels to show higher levels of expression of COX-2 in bone than in bone marrow (unpublished data). Hence, although PGs produced by other cell types interfacing with bone, such as macrophages and vascular endothelial cells, may influence bone cell differentiation and function, the converse may also be true—that osteoblasts are an important source of PGs which may influence neighboring cells in the bone marrow and in the vascular network.

Inhibition of PG Production

Glucocorticoids have been shown in many studies to be potent inhibitors of stimulated PG production, and their anti-inflammatory actions are thought to be due in part to this inhibition. Prior to the identification of COX-2, glucocorticoids were thought to work predominantly through interference with release of AA release. It is now clear that glucocorticoids have a major inhibitory effect on the inducible COX-2 mRNA and protein expression which accounts for the majority of their effects on PG production in bone and other tissues (Kawaguchi *et al.,* 1994; Pilbeam *et al.,* 1993). We have found that retinoic acid is a potent transcriptional inhibitor of COX-2 gene expression and PG production in osteoblasts (Pilbeam *et al.,* 1995). The cytokines IL-4 and IL-13 also inhibit COX-2 expression and PG production in bone organ and cell cultures (Kawaguchi *et al.,* 1996; Onoe *et al.,* 1996).

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin and aspirin, inhibit PG production by competing directly with AA for binding to the cyclooxygenase catalytic site. Hence, NSAIDs will not inhibit isoprostane or leukotriene production. NSAIDs are widely used to study the role of endogenous PGs, but it should be remembered that NSAIDs can have multiple effects in addition to inhibiting COX activity (Paik *et al.,* 2000). Until recently, most available NSAIDs inhibited both COX-1 and COX-2 activity.

NS-398 is the first selective NSAID to become widely available for *in vitro* and animal studies. In rodent osteoblastic cells, NS-398 at a concentration of 0.01 μM was selective for inhibition of COX-2 activity; however, at concentrations of 0.1 and 1 μM, NS-398 also inhibited COX-1 activity by 60 and 85%, respectively (Pilbeam *et al.,* 1997b). Hence, the selectivity of NS-398 was lost at higher doses.

As noted above, PGs themselves can induce COX-2 expression and, hence, NSAIDs can also decrease COX-2 expression by reducing PG-mediated autoamplification. Other agents reported to inhibit PG production in bone cells are vitamin K_2 (Hara *et al.,* 1993) and interferon-γ (Peterlik *et al.,* 1985).

Regulation of PG Production by Estrogen

Estradiol can decrease stimulated PG production in cultured murine calvaria (Pilbeam *et al.,* 1989). However, this effect of estrogen on PG production is not mediated through changes in COX-1 or COX-2 transcription (Pilbeam, unpublished data). Cytokine activity is hypothesized to increase with estrogen withdrawal *in vivo* (Kimble *et al.,* 1995), and cytokine-stimulated PG production by osteoblasts may be increased. PG production by calvaria cultured briefly from ovariectomized rats was increased, and this effect was reversed by estradiol administration *in vivo* (Feyen and Raisz 1987). Marrow supernatants from ovariectomized mice stimulated more bone resorption in calvarial cultures than supernatants from estrogen-replete mice, and this increase was mediated by increased PG production secondary to increased COX-2 expression (Kawaguchi *et al.,* 1995b; Miyaura *et al.,* 1995). The induction of COX-2 expression was secondary to increased IL-1α activity in the supernatants and was reversed by *in vivo* treatment of the ovariectomized mice with estradiol (Kawaguchi *et al.,* 1995b). The role of endogenous PGs in bone loss due to sex hormone deficiency is unclear. Partial reversal of bone loss by an NSAID in ovariectomized rats has been reported, but the effect was not sustained (Kimmel *et al.,* 1992). However, it may not have been possible to produce a sustained inhibition of COX-2 activity with nonselective NSAIDs at tolerated concentrations.

Role of PGs in Bone Resorption

PGs and Resorption in Organ Culture

Early work on the effects of PGs on bone resorption showed that PGs of the E series are the most potent activators, with an effective concentration range of 1 nM to 10 μM (Klein and Raisz, 1970; Raisz and Martin, 1983). $PGF_{2\alpha}$ is less effective than PGE_2, stimulating resorption at concentrations of 0.1 μM and above, and the ability of $PGF_{2\alpha}$ to stimulate resorption is partly dependent upon induction of PGE_2 (Raisz *et al.,* 1990). PGI_2 can also stimulate resorption, while PGD_2 is ineffective. Many factors that stimulate PG production also stimulate resorption in organ culture, and the resorption stimulated by such factors can be mediated in part by PG production (Akatsu *et al.,* 1991; Stern *et al.,* 1985; Tashjian *et al.,* 1982, 1987). However, the dependence of resorption in organ culture on stimulated PG production is quite variable (Pilbeam *et al.,* 1996), and some potent stimulators of PGE_2, such as PTH, do not exhibit PG-dependent resorption in these systems (Pilbeam and Raisz, unpublished data).

PG Effects on Osteoclast Formation and Differentiation *in Vitro*

PGE_1 and PGE_2, but not $PGF_{2\alpha}$, stimulate osteoclast formation in marrow cultures (Collins and Chambers, 1991; Kaji *et al.,* 1996). In contrast to organ culture resorption studies, studies in marrow culture consistently demonstrate a dependence of osteoclast formation on stimulated PG production. Agonists reported to stimulate PG-dependent osteoclast formation in marrow cultures include IL-1 (Akatsu *et al.,* 1991; Lader and Flanagan, 1998; Sato *et al.,* 1996), TNF-α (Lader and Flanagan, 1998), PTH (Inoue *et al.,* 1995; Sato *et al.,* 1997), 1,25 $(OH)_2$ D_3 (Collins and Chambers 1992), IL-11 (Girasole *et al.,* 1994; Morinaga *et al.,* 1998), IL-6 (Tai *et al.,* 1997), IL-17 (Kotake *et al.,* 1999), phorbol ester (Amano *et al.,* 1994), and FGF-2 (Hurley *et al.,* 1998). TGF-β enhances 1,25 $(OH)_2D_3$-stimulated osteoclastogenesis at low concentrations by a PG-dependent mechanism, but inhibits at higher concentrations (Shinar and Rodan, 1990). The PG enhancement of stimulated osteoclast formation in marrow culture may reflect the increased formation of new osteoclastic precursors, while stimulated resorption in organ culture may be more dependent on activation of a pool of available osteoclastic precursors.

The formation of mature bone-resorbing osteoclasts requires a contact-dependent interaction with cells of the osteoblastic lineage (Suda *et al.,* 1999). The molecule mediating this interaction, originally cloned as receptor activator of NF-κB (RANK) ligand or RANKL (Anderson *et al.,* 1997), is identical to TNF-related activation-induced cytokine (TRANCE) (Wong *et al.,* 1997). Subsequently, RANKL was also found to be identical to osteoclast differentiating factor (ODF) and to be a ligand for osteoprotegerin (OPG), a decoy receptor for RANKL, and is, therefore, also called OPGL (Yasuda *et al.,* 1998). Osteoblastic cells produce both RANKL and OPG, as well as macrophage colony-stimulating factor (M-CSF), also required for osteoclastogenesis, while osteoclastic cells express RANK. Induction of RANKL has been shown to be essential for resorption by PGE_2 (Tsukii *et al.,* 1998).

In Vitro Studies Using COX-2 Null Mice

COX-2 is the isoenzyme responsible for the PG enhancement of stimulated osteoclastogenesis. In marrow cultures from mice in which both alleles for COX-2 have been disrupted (COX-2 $-/-$ mice), osteoclast formation stimu-

lated by $1,25(OH)_2D_3$ or PTH was reduced 60–70% compared to wild-type (+/+) cultures (Okada *et al.,* 2000a). PGE_2 production was markedly reduced in marrow cultures from COX-2 −/− mice compared to cultures from wild-type mice, and decreased osteoclastogenesis in COX-2 −/− cultures was completely reversed by addition of PGE_2 to the cultures. Heterozygous COX-2 +/− marrow cultures had levels of osteoclast formation and PGE_2 production intermediate to those in COX-2 +/+ and COX-2 −/− cultures. Treatment of normal cultures with COX inhibitors (indomethacin and NS-398) mimicked the results observed in cultures from COX-2 −/− mice. The reduced response to $1,25\ (OH)_2D_3$ and PTH in COX-2 −/− cultures was associated with reduced expression of RANKL by osteoblasts, and this reduction could also be reversed by addition of PGE_2. There was no consistent difference in the expression of OPG, which inhibits osteoclastogenesis, in COX-2 −/− cultures compared to COX-2 +/+ cultures. There was no effect of disruption of both COX-1 alleles on stimulated PG production or osteoclastogenesis. We have found similar results for osteoclastogenesis stimulated by FGF-2 and IL-1 (Pilbeam, unpublished data), suggesting that PGs produced by COX-2 are necessary for *in vitro* osteoclastogenesis in response to multiple agents.

In addition to acting on osteoblastic cells to increase RANKL, PGs may also act on osteoclastic progenitors among the hematopoietic cells to influence the formation and activity of osteoclasts. Osteoclasts can be formed in spleen cultures, which contain osteoclast precursors but no osteoblasts, if the cultures are treated with M-CSF and soluble RANKL to replace the need for supporting osteoblastic cells. PGE_2 has been shown to enhance the combined effects of RANKL and M-CSF to stimulate osteoclast formation in spleen culture systems (Wani *et al.,* 1999). In such a system, we found that osteoclast formation was reduced 50% when spleen cells came from COX-2 −/− mice compared to cells from COX-2 +/+ mice (Okada *et al.,* 2000a). This reduction was due to increased expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), an inhibitor of osteoclast formation in these cultures. GM-CSF may act by diverting progenitor cells into the macrophage pathway that might otherwise differentiate into osteoclasts. Hence, PGs can enhance osteoclastogenesis by acting on osteoclast supporting cells to increase RANKL and on hematopoietic cells to decrease GM-CSF. Putative effects of PGE_2 on osteoclast formation are schematically summarized in Fig. 3.

PG Effects on Isolated Osteoclast Activity

Paradoxically, when added to isolated osteoclasts *in vitro,* PGE_2 transiently inhibits bone resorption (Fuller and Chambers 1989). A similar transient inhibition of bone resorption and lysosomal enzyme release in mouse calvarial bone cultures by PGs has also been observed (Lerner *et al.,* 1987).

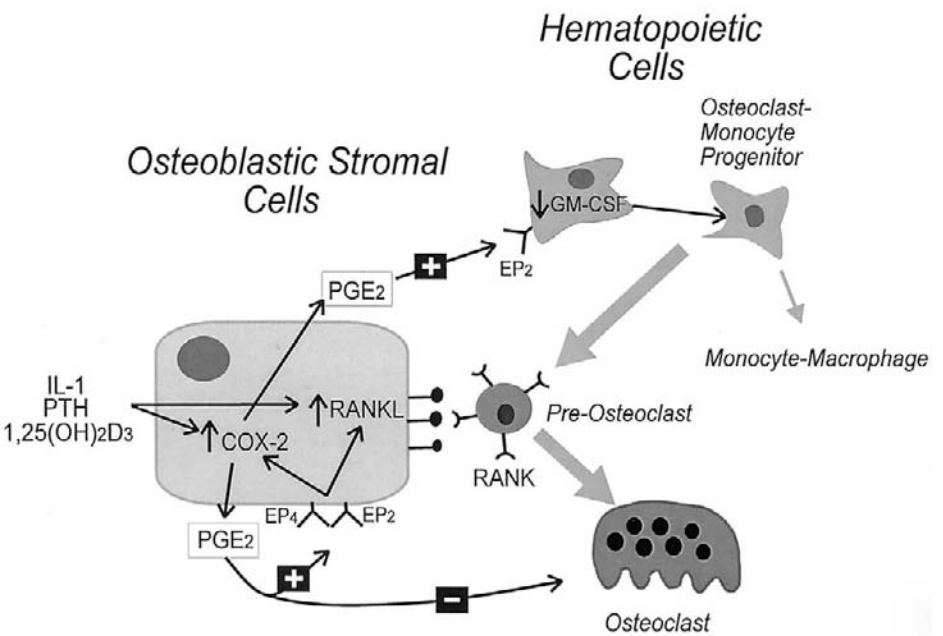

Figure 3 Schematic diagram of the putative roles for PGs in bone resorption. PGs have positive effects on formation of osteoclasts but may inhibit osteoclastic activity. Osteoblastic stromal cells express both COX-2 and RANKL. Interaction of RANKL with RANK, which is expressed by osteoclastic precursor cells, is required for precursor cells to differentiate into mature osteoclasts. COX-2-derived PGs stimulate the expression of RANKL and enhance the stimulation of RANKL by other agonists. PGs may also act on hematopoietic cells to inhibit GM-CSF expression, resulting in increased entrance of progenitor cells into the osteoclastic differentiation pathway. PGs can have a transitory inhibitory effect on the activity of isolated mature osteoclasts.

This inhibitory effect may be due to a direct activation of adenylate cyclase in osteoclasts, thereby mimicking the effect of calcitonin to inhibit osteoclast function. The physiologic relevance of this transient inhibition is unknown.

In Vivo Studies

Little is known about the role of PGs in bone resorption *in vivo.* A role for PGs in bone resorption might be most evident when resorption is stimulated, and the ability to generate new osteoclasts from osteoclastic precursors becomes the factor limiting the rate of resorption. In this regard, the prolonged *in vivo* resorption response to IL-1 was found to depend on PGs (Boyce *et al.,* 1989). *In vivo* injection of IL-1 above the calvaria in mice for 3 days was shown to stimulate PG-independent resorption when mice were sacrificed 24 hr after the last injection. However, resorption continued for 3–4 weeks after the IL-1 injections were stopped, and this resorption was PG-dependent.

Clearly, COX-2 is not required for basal levels of bone resorption since COX-2 −/− mice do not develop osteopetrosis (Okada *et al.,* 2000a). To study the role of COX-2 in acute resorption *in vivo,* we injected PTH at high dose above the calvaria of COX-2 −/− mice and their wild type +/+ littermates (Okada *et al.,* 2000a). This protocol has been shown to result in localized resorption at the site of injection which is reflected by systemic hypercalcemia (Zhao *et al.,* 1999). After 3 days of injection, PTH caused hypercalcemia in the COX-2 +/+ mice but not in the COX-2 −/− mice. Hence, the absence of COX-2 limits the maximal resorption response to PTH *in vivo* as well as *in vitro.*

PG Receptors and Bone Resorption

Recent studies of cells and tissues from EP receptor knockout animals and studies using selective EP receptor agonists have added further complexity to our understanding of PGE_2 stimulation and inhibition of bone resorption. Osteoclast formation in bone marrow cultures and mixed cultures of osteoblasts and spleen cells was found to be impaired when cells from either EP_2 or EP_4 knockout animals were used (Li *et al.,* 2000; Sakuma *et al.,* 2000). These studies confirmed earlier results using selective EP agonists and antagonists (Ono *et al.,* 1998). While both EP_2 and EP_4 are involved in the PGE_2 effects on the osteoblastic support cells for osteoclastogenesis, EP_2 is also involved in the effects of PGE_2 on hematopoietic precursors (Fig. 3). Spleen cells from EP_2 knockout animals showed an impaired osteoclastogenic response when treated with M-CSF, RANKL, and PGE_2 (Li *et al.,* 2000). Resorption studies using cultured calvaria from EP_4 knockout animals showed an impaired resorptive response to PGE_2 (Miyaura *et al.,* 2000). The inhibitory effect of PGE_2 on osteoclast activity may also involve both the EP_4 and EP_2 receptors (Mano *et al.,* 2000).

Role of PGs in Bone Formation

Studies in cell and organ culture have indicated that PGs can have both stimulatory and inhibitory effects on bone formation. On the other hand, *in vivo* studies in rats have constantly demonstrated a potent anabolic effect. PGs can increase both periosteal and endosteal bone formation in the rat and produce substantial increases in bone mass, similar to the effects of PTH (Jee and Ma 1997; Lin *et al.,* 1994; Suponitzky and Weinreb, 1998). Similar anabolic effects can be seen in fetal rat calvarial organ cultures, in which PGE_2 can stimulate both cell replication and differentiation (Woodiel *et al.,* 1996). The most consistent anabolic effect is seen in cultures designed to study osteoblastic differentiation. In marrow stromal cell and primary calvarial cell cultures, PGE_2 stimulates the formation and differentiation of osteoblastic colonies (Flanagan and Chambers 1992; Scutt and Bertram 1995). In addition, PGE_2 given *in vivo* enhances osteoblastic differentiation in explanted marrow stromal cells (Weinreb *et al.,* 1997). Moreover, marrow stromal cells or primary calvarial cells cultured from COX-2 −/− mice have markedly delayed osteoblastic differentiation compared to cells from COX-2 +/+ mice (Okada *et al.,* 2000c). The receptor pathway for this anabolic effect is not yet fully elucidated. Based on early studies with selective agonists in rat calvarial organ culture and marrow cell culture, a role for the EP_2 receptor was suggested, but subsequent studies in marrow cultures support the possibility of mediation by the EP_4 receptor (Weinreb *et al.,* 1999).

At high concentrations, PGs can inhibit collagen synthesis in cell and organ culture (Fall *et al.,* 1994). This inhibitory effect appears to occur largely via transcriptional inhibition of collagen and to be mediated by the FP receptor rather than an EP receptor. Hence, similar to their effects on the osteoclast lineage, PGs can enhance the differentiation of osteoblastic progenitors but can inhibit the function of mature osteoblasts.

Little is known about the role of endogenous PGs *in vivo.* We have done preliminary studies on mice with one (+/−) or both (−/−) alleles for the COX-2 gene disrupted and their wild-type littermates (+/+) (Okada *et al.,* 2000c). Comparing 3-month-old female COX-2 −/− and +/+ mice, with no differences in body weight or serum creatinine, there was a 9.0% ($P < 0.05$) decrease in tibial bone mineral content (BMC) and a 10% ($P = 0.05$) decrease in femoral BMC of −/− mice compared to +/+ mice ($n = 4$). Histomorphometric analyses of the proximal tibiae of 2-month-old mice ($n = 4$) showed decreases of 40 and 15% ($P < 0.05$) in trabecular bone volume (TBV) and mineral apposition rate (MAR), respectively, in heterozygous COX-2 +/− mice compared to COX-2 +/+ mice. There were small decreases in percentage osteoclast surface and osteoclast number in COX-2+/− mice which were not significant. In femurs from 6-month-old female mice ($n = 3–4$), there was no significant difference in TBV between COX-2 +/− and +/+ mice, but there was a 34% decrease in MAR and 42% decrease in osteoclast number per bone surface

($P < 0.01$) in COX-2 +/− mice compared to +/+ mice. These studies suggest that bone formation may be reduced more than bone resorption as a result of COX-2 deficiency.

Role of PGs in the Effects of Mechanical Loading on Bone

Many studies have suggested that PGs may mediate some of the effects of mechanical loading on bone. Orthodontic movement of teeth stimulates PG production, and endogenous PGs enhance the rate of tooth movement, presumably as the result of PG-stimulated bone turnover (Giunta *et al.,* 1995; Grieve *et al.,* 1994; Kehoe *et al.,* 1996; Saito *et al.,* 1991). Mechanical loading of bone explants can increase PG production and result in new bone formation (Cheng *et al.,* 1997; Lanyon, 1992; Rawlinson, *et al.,* 1991). Loading induced new bone formation *in vitro,* in an isolated avian ulna preparation (Pead and Lanyon 1989), and *in vivo,* in externally loaded tail vertebrae of rats (Chow and Chambers 1994), was blocked with inhibitors of PG production. In humans, *in situ* microdialysis showed a 2.5–3.5-fold increase in the release of PGE_2 in the tibial metaphysis after loading (Thorsen *et al.,* 1997).

How mechanical loading is coupled to cellular responses in bone is unknown, but fluid flow is thought to play a role in the response to physiologic loading (Hillsley and Frangos, 1993; Piekarski and Munro, 1977; Turner *et al.,* 1994). Small displacements in the mineralized matrix of bone are hypothesized to generate interstitial fluid flow in the lacunar–canalicular network. Fluid shear stress (force per unit area) is exerted in the direction of the flow on membranes of cells lining the surfaces. Osteocytes which contact each other and the osteoblasts lining the mineralized matrix via gap junctions are thought to be the main strain sensing network (Kufahl and Saha, 1990).

Osteoblasts and osteocytes subjected to fluid shear stress produce PGs (Klein-Nulend *et al.,* 1997; Pavalko *et al.,* 1998; Reich and Frangos 1993). When osteoblastic cells are subjected to fluid flow, there is an early release of PGE_2 followed by a more sustained production. The early PG production is likely to be due to release of arachidonic acid converted to PGs by the constitutively expressed COX-1, while the sustained PG production is due to induction of COX-2 (Klein-Nulend, *et al.,* 1997; Pavalko *et al.,* 1998). Using MC3T3-E1 cells stably transfected with a COX-2 promoter–luciferase reporter construct and calvarial cells from mice transgenic for this construct, we have shown that fluid shear stresses as low as 0.1 dynes/cm^2 rapidly induce new COX-2 gene transcription and that the induction is dependent on the mitogen-activated protein signaling pathway that activates ERK 1 and 2 (Wadhwa *et al.,* 2000). This transcriptional activation may be mediated by a combination of C/EBP-β, CRE, and AP-1 *trans*-acting sites (Ogasawara *et al.,* 2000).

Less is known about the role of COX-2 *in vivo* in the response to loading. The increase in endosteal bone formation following a single short period of bending applied to rat tibiae was prevented by a selective COX-2 inhibitor (NS-398) (Forwood 1996). However, an immunohistochemistry study by the same group reported increases in COX-1 as well as COX-2 protein in osteocytes in the tibial bone of rats following 4-point bending, with sparse expression of COX-2 relative to COX-1 (Forwood *et al.,* 1998).

Role of PGs in Inflammatory Bone Loss

PG production is frequently found to be increased in inflammatory processes, such as rheumatoid arthritis (Crofford *et al.,* 1994). In a study of acute inflammation in the rat footpad induced by carrageenan, COX-2 expression was elevated, and selective inhibition of COX-2 activity inhibited edema and hyperalgesia (Seibert *et al.,* 1994). Because of the association of COX-2 derived PG production with inflammation and because PGs are potent stimulators of bone resorption, it is often assumed that COX-2 derived PGs contribute to the bone loss and cartilage destruction associated with inflammatory diseases. There are some studies showing that NSAIDs decrease alveolar bone loss in peridontitis to support this conclusion (Howell *et al.,* 1991; Jeffcoat *et al.,* 1993). However, other agents which are elevated in inflammatory diseases, such as cytokines, are themselves potent inducers of bone resorption independent of their effects on PG production. In addition, cytokines can inhibit bone matrix proteins independently of cytokine-induced PG production (Rosenquist *et al.,* 1996). As discussed above (under Role of PGS

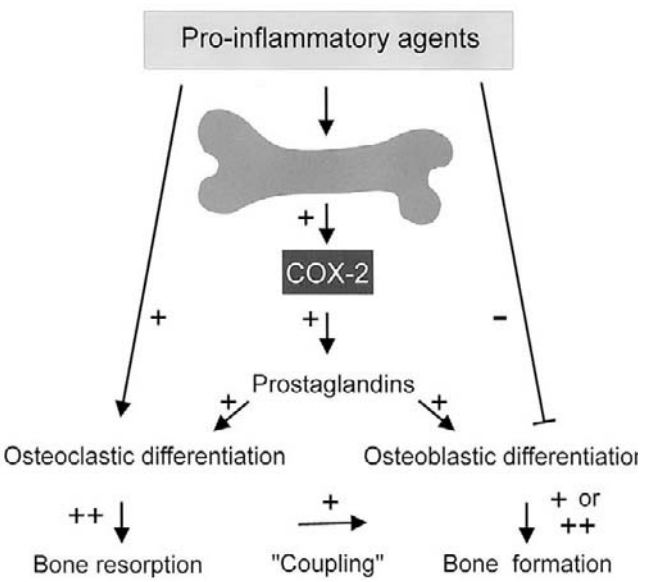

Figure 4 Diagram of the potential effects of PGs on bone turnover in inflammation. Inflammatory mediators, such as IL-1 and TNF, induce COX-2 expression and PG production. These inflammatory mediators also have direct effects on bone to stimulate resorption and inhibit formation. The induced PGs might enhance or prolong the stimulatory effects on resorption but might counter the inhibitory effects on osteoblastic formation.

in Bone Resorption, subsection titled *In Vivo* Studies), PGs produced by proinflammatory agents may prolong the resorption responses to these agents, particularly if PGs play an important role in maintaining or replenishing the pool of differentiated osteoclasts. Nevertheless, it is possible that the ability of PGs to stimulate osteoblastic differentiation might have a compensatory effect (Fig. 4).

It is interesting to note that some studies have suggested that COX-2 metabolites are proinflammatory only during the early stages of an inflammatory response; and, at later times, COX-2 generates a different set of PGs, the cyclopentenone PGs, which have anti-inflammatory effects (Gilroy *et al.,* 1999). Although these PGs include putative ligands for the PPARs, such as 15-deoxy-Δ^{12-14} PGJ_2, it is thought that the anti-inflammatory effects are more likely to occur through a PPAR-independent pathway (Gilroy *et al.,* 1999).

Effects of NSAIDS on Bone in Humans

Effects of NSAIDs on Bone Mineral Density (BMD) in Humans

Only a few studies have examined the effects of NSAIDs on BMD in animals or humans. In a study of postmenopausal women 65 years of age or older, there was no significant difference found in N-telopeptide cross-link excretion, a marker of bone resorption, between self-reported users of NSAID or aspirin compared to nonusers (Lane *et al.,* 1997). In a similar cohort, the use of aspirin or NSAID was found to be associated with small but significant increase in hip and spine BMD, but there was no clinically significant protective effect on risk for fracture (Bauer *et al.,* 1996). In a study of older women from Rancho Bernardo, California, the regular use of propionic acid NSAIDs (ibuprofen, naproxen, ketoprofen), but not acetic acid NSAIDs (indomethacin, diclofenac, sluindac, tolmetin), was associated with higher BMD at multiple skeletal sites (Morton *et al.,* 1998). When women with self-reported osteoarthritis were excluded, significantly higher BMD was observed in the hip in propionic acid NSAID users. Those who concurrently used estrogen and priopionic acid NSAIDs had the highest BMD at all sites.

There are no published studies examining the effects of selective COX-2 inhibitors on BMD. It seems likely that continuous inhibition of COX-2 activity might not have been attained in studies with nonselective NSAIDs because the frequent administration and high doses necessary to inhibit COX-2 activity might produce toxic side effects. Moreover, nonselective NSAIDs *in vitro* can have biphasic effects — low concentrations can increase, rather than decrease, PG production (Lindsley and Smith, 1990; Raisz *et al.,* 1989). Hence, studies with the new selective inhibitors of COX-2, which have fewer limiting gastrointestinal or bleeding side-effects at doses which effectively inhibit COX-2, may help to clarify the role of PGs in bone metabolism.

Other Effects of NSAIDs on Bone in Humans

Nonselective NSAIDs have been used to examine effects of PGs after orthopedic surgical procedures. Heterotropic ossification is a complication of hip arthroplasty that can adversely affect the outcome. Several studies have shown that perioperative treatment with aspirin or other NSAIDs can prevent this complication (Kienapfel *et al.,* 1999; Neal *et al.,* 2000; Nilsson and Persson, 1999), suggesting that the problem is caused by PG-mediated bone formation. Because PGs may enhance the bone remodeling needed for fracture healing, there is concern that NSAIDs might adversely affect fracture healing. Studies suggest that NSAIDs can inhibit repair of fractured femurs (Altman *et al.,* 1995; Reikeraas and Engebretsen, 1998) and spinal fusions (Dimar *et al.,* 1996) in rats and the fixation of implants in femora of rabbits (Jacobsson *et al.,* 1994), while exogenously applied PGs can stimulate callus formation in rabbits (Keller *et al.,* 1993). Ultrasound stimulation can enhance bone healing, and studies have suggested both that ultrasound acts by decreasing PG production (Sun *et al.,* 1999) and by stimulating COX-2 induction and associated PG production (Kokubu *et al.,* 1999).

Leukotrienes and Bone

Compared with the products of the COX pathway, little is known about the products of 5-lipoxygenase pathway in bone. Most studies have examined effects on bone resorption. 5-Lipoxygenase products have been shown to stimulate bone resorption in neonatal mouse organ cultures. Leukotrienes and hydroxyeicosatetraenoic acids (HETEs) can affect resorption in a biphasic manner, with low doses stimulating and high doses inhibiting resorption (Meghji *et al.,* 1988). Maximal stimulation of resorption by leukotriene B_4 (LTB_4), LTC_4, LTE_4, and 5-HETE occurred at 10 pM, while 12-HETE and LTD_4 required 100 pM and 10 nM, respectively. The effects of LTB_4, C_4, and 12-HETE were partially inhibited by indomethacin, suggesting some mediation by PGs. A cell line established from giant cell tumors of bone produced an activity which stimulated bone resorption in organ culture and stimulated isolated osteoclasts to form resorption lacunae on dentine (Gallwitz *et al.,* 1993). This activity was identified as 5-HETE and a peptidoleukotriene, either LTC_4, $-D_4$, or $-E_4$. Commercially available LTC_4/LTD_4 and 5-HETE stimulated bone resorption in neonatal mouse calvariae and pit formation by isolated rat osteoclasts (Gallwitz *et al.,* 1993). Peptidoleukotrienes were also shown to stimulate isolated avian osteoclasts to form resorption lacunae (Garcia *et al.,* 1996a). These authors reported that LTD_4 receptors are present on avian osteoclasts. LTB_4, a more stable lipoxygenase product, increased bone resorption and osteoclast numbers in mice when injected locally above the calvariae and stimulated resorption in mouse calvarial organ cultures (Garcia *et al.,* 1996b). LTB_4 also stimulated isolated rat osteoclasts to form resorption lacunae with greater potency than

the peptidoleukotrienes, LTD_4 and LTE_4. Isolated avian osteoclasts also express both high and low-affinity LTB_4 receptors and LTB_4 can increase TRAP activity and pit formation by these osteoclasts (Flynn *et al.,* 1999).

Much less is known about the effects of leukotrienes on osteoblasts, but recent evidence suggests that leukotrienes may inhibit bone formation. 5-HETE and LTB_4 inhibited BMP-2-induced bone formation in mouse calvarial organ culture and blocked the formation of mineralized nodules in response to BMP-2 and dexamethasone in fetal rat calvarial cell culture (Traianedes *et al.,* 1998). Consistent with an inhibitory role of leukotrienes on bone formation, 5-lipoxygenase null mice were reported to have increased cortical bone thickness (Bonewald *et al.,* 1997).

Summary

PGs are abundant in bone and are potent regulators of bone cell function. Osteoblasts produce PGs, and this production is highly regulated by local and systemic factors. Stimulated production of PGs by osteoblasts requires both the induction of COX-2 expression and the availability of arachidonic acid substrate. PGs are complex, potent regulators of bone cell function *in vitro.* Recent studies indicate that COX-2 overexpression is associated with tumorigenesis (Williams *et al.,* 1999), suggesting that PGs can function as general regulators of cell replication and/or apoptosis. PGE_2, which may be the most important local eicosanoid in skeletal regulation, can stimulate or inhibit both bone resorption and formation. *In vitro,* PGE_2 can stimulate the differentiation of both osteoblasts and osteoclasts, and the net balance of these two effects under physiologic or pathologic conditions *in vivo* is not yet clear. The physiologic importance of the inhibitory effects of PGs on collagen production by osteoblasts and on the activity of mature osteoclasts is unknown. Some of the complexity of PG actions on bone can be explained by the multiplicity of receptors for PGs. There are at least four distinct receptors for PGE_2 with differential signaling pathways that have not yet been fully elucidated. Further studies are needed to clarify the specific pathways of PGs action in bone. Once this is accomplished, it may be possible to identify therapeutic applications of manipulating PGs in skeletal disorders.

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CHAPTER 55

Nitric Oxide and Other Vasoactive Agents

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Nitric Oxide

General

Nitric oxide (NO) is a gas radical that is enzymatically produced by the oxidation and cleavage of one of the terminal nitrogen atoms of the amino acid L-arginine. The reaction is dependent on electrons donated by the cofactor NADPH, requires oxygen, and yields L-citrulline as a coproduct. Nitric oxide is involved in a variety of physiological and pathophysiological processes including vasodilatation, neurotransmission, and the killing of various pathogens and in the genesis of inflammatory diseases. These broad-ranging actions of NO are determined largely by the site of NO synthesis, the rate of NO synthesis, and how much is generated and by the nature of the environment into which it is released. The reactivity of NO is influenced in particular by the presence of reactive oxygen intermediates and the activity of anti-oxidant defense systems (Moncada and Higgs, 1993; Knowles and Moncada, 1994; Gross and Wolin, 1995).

A family of three related enzymes, the NO synthases (NOS), regulates the synthesis of NO. These are characterized as a neuronal form (type 1; nNOS) originally isolated from brain (Bredt and Snyder, 1990), an endothelial form (type 3; eNOS) originally isolated from bovine aortic endothelial cells (Pollock *et al.,* 1991), and an inducible form (type 2; iNOS) originally isolated from murine macrophages (Xie *et al.,* 1992). Both eNOS and nNOS are expressed constitutively and are characterized by highly regulated, rapid but low-output NO production that has a tonic, physiological function (Moncada and Higgs, 1993; Knowles and Moncada, 1994). Conversely, iNOS is generally not present or active under normal physiological conditions but can be induced in a number of different cell types in response to infection, inflammation, or traumatic injury (Moncada and Higgs, 1993; Knowles and Moncada, 1994; Gross and Wolin, 1995). In contrast to the constitutive enzymes, iNOS expression is induced over several hours requiring transcription of mRNA and *de novo* protein synthesis. Once activated iNOS is capable of sustained generation of NO over many hours, which in some circumstances can lead to localized production of high levels of NO (Gross and Wolin, 1995). In these circumstances NO can exacerbate the inflammatory response as seen in septic shock and can also contribute to localized cell and tissue damage (Gross and Wolin, 1995).

Although nNOS, eNOS, and iNOS catalyze the same reaction, generating NO from L-arginine, they are distinct enzymes, each the product of separate genes, that share only 50–60% homology and each subject to widely differing mechanisms of regulation. Some of the general characteristics of NOS isoenzymes are presented in Table I. All three isoforms exhibit a bidomain structure containing consensus sequences for binding several cofactors along the C-terminal domain, including NADPH, FAD, FMN, and calmodulin. The arginine-binding site is retained within the N-terminal domain, which also contains a heme group and a consensus site for binding tetrahydrobiopterin. All cofactors are required for full activity and in their native, catalytically competent state, all three isoforms are homodimers (Griffith and Stuehr, 1995). The constitutive NOS enzymes are also

Table I General Molecular and Biochemical Characteristics of NOS Enzymes and their Association with Bone

	nNOS, NOS I	iNOS, NOS II	eNOS, NOS III
Molecular weight (kDa)	160 ~260 Homodimer (active form)	130 ~260 Homodimer (active form)	135 ~260 Homodimer (active form)
Gene size/structure	160 kb, 29 exons, 28 introns	21 kb, 26 exons, 25 introns	37 kb, 26 exons, 25 introns
Chromosome	12	17	7
Cell pro-type	Neurons	Macrophages	Endothelial
Subcellular localization	Cytosolic/membrane	Cytosolic	Membrane
Calcium/calmodulin dependent	✔	✔/✖	✔
Typical characteristics	Low output, regulated by protein activation	High-output, transcription regulation	Low output, regulated by protein activation
Bone expression (mRNA/protein)	Equivocal	1. Osteoblasts (Ralston *et al.,* 1994; Hukkanen *et al.,* 1995; Helfrich *et al.,* 1997) 2. Osteoclasts (Fox and Chow, 1998); Brandi *et al.,* 1995; Helfrich *et al.,* 1997)	1. Osteoblasts (Raincho *et al.,* 1995; Fox and Chow 1998; Armour and Ralston 1998; O'Shaughnessy *et al.,* 2000) 2. Osteoclasts (Brandi *et al.,* 1995; Helfrich *et al.,* 1997) 3. Osteocytes (Fox and Chow, 1998; Zaman *et al.,* 1999)

dependent on mobilization and intracellular Ca^{2+} availability. Comparisons of sequence homologies reveal that NOS enzymes bear resemblance to just one other known mammalian enzyme, cytochrome P450 reductase (CPR), sharing around 60% sequence similarity along their C-terminal domain (Bredt *et al.,* 1991; Griffith and Stuehr, 1995). This has been instrumental in elucidating the catalytic mechanism of NOS showing that the C-terminal domain, like CPR, is essentially an electron transporter. Bound calmodulin then acts as gate to regulate flow of electrons from the C-terminal domain to the N-terminal domain where they are donated to the heme group and their energy is used to drive the generation of NO (Griffith and Stuehr, 1995). While the C-terminal domains of NO synthase isoenzymes are very similar the N-terminal domains demonstrate some variations. Most notably, eNOS possesses consensus sequences for myristoylation/palmitoylation at its N-terminus and is important in determining its subcellular trafficking, locating the enzyme to membranes and in particular the caveolae (Garcia-Cardena *et al.,* 1996).

Other important aspects of NO biology include the molecular regulation of NO synthases. For the purposes of this review we need only consider mechanisms of eNOS and iNOS regulation. The molecular regulation of iNOS is particularly complex and occurs at several sites in the gene expression pathway with both transcriptional and posttranscriptional mechanisms (Ganster and Geller, 2000). Expression of iNOS can be induced by various "proinflammatory" cytokines including tumour necrosis factor alpha (TNF-α), interleukin 1-beta (IL-1β), and interferon-gamma (IFN-γ) and the effects of these inductive stimuli are mediated by the NF-κB and Jak-STAT signaling pathways (Ganster and Geller, 2000). Downregulators of iNOS expression include glucocorticoids such as dexamethasone which inhibits cytokine-induced iNOS mRNA at the transcriptional level (Ganster and Geller, 2000). The cytokine TGF-β has been shown to downregulate iNOS expression at the posttranscriptional level by inducing instability in iNOS mRNA. There is also evidence for a negative feedback loop whereby NO downregulates iNOS gene expression as well as inhibits the activity of iNOS protein (Ganster and Geller, 2000).

While the eNOS gene is considered to be constitutively expressed there are number of factors that exert positive or negative effects on the level of expression. The activity of eNOS is stimulated by a number of agonists including estrogen, vascular endothelial growth factor (VEGF), and shear stress and involves activation of various signaling pathways particularly those resulting in mobilization of intracellular Ca^{2+} and phosphorylation of eNOS (Gratton *et al.,* 2000). It is also clear that in addition to stimulating the activity of eNOS protein, shear stress, estrogen, VEGF, as well as TGF-β and fibroblast growth factor (FGF), upregulate eNOS expression at the level of transcription. Conversely, TNF-α and hypoxia downregulate eNOS gene expression by inducing instability in eNOS mRNA (Kleinert *et al.,* 2000).

NO Signaling

Biological Chemistry of NO

Although NO might be regarded as chemically very simple, its biochemistry in biological systems is often complex and this accounts in part for the wide range of biological

processes that are influenced by NO. Nitric oxide is probably the representation of several related species including its higher oxides and derived oxidants. For the purpose of this review it is necessary to consider only the NO-derived oxidant peroxynitrite with all other actions generalized as NO.

Despite its varied reactivities, NO is eventually oxidized to nitrite and nitrate in biological systems. In aqueous buffers and culture conditions nitrite is the principal oxidation product of NO (Ignarro *et al.,* 1993), whereas *in vivo* NO is in general almost completely oxidized to nitrate and excreted. These "end products" have proved to be useful markers for evidence of NO biosynthesis within biological samples. Nitrate and nitrite can be detected by the Greiss reaction, which relies on the reduction of nitrate to nitrite after the addition of the enzyme nitrite reductase or a metallic catalyst to the sample, the resultant color changes being then detectable spectroscopically (Green *et al.,* 1982, Stuehr and Marletta, 1987). This technique, however, is quite insensitive requiring the accumulation of micromolar amounts of nitrite. For greater sensitivity a chemiluminescence analyzer is often used to measure authentic NO. A strong reducing agent is used to reduce the nitrite or nitrate present in the sample of interest back to NO. The NO is then reacted with ozone, which results in the generation of light or chemiluminescence, with the amount of light being directly proportional to NO levels (Archer, 1993).

Perhaps one of the better-understood reactions of NO in biological systems is its combination with superoxide anion (O_2^-) to form peroxynitrite anion ($ONOO^-$) (Beckman *et al.,* 1990). Both molecules show a high affinity for each other, the reaction proceeding several-fold more quickly than catalysis of superoxide anion dismutation by superoxide dismutase (SOD). Peroxynitrite is a powerful oxidant and can either augment or abrogate some of the biological actions associated with NO synthesis.

CELLULAR TARGETS OF NO

NO does not require specific receptors to communicate its physiological actions; its small size and lipophilic nature allow it to traverse plasma membranes without the need for a recognized transporter. In biological systems, the principal cellular targets for NO are transition metal ion (containing) proteins. Soluble guanylate cyclase is perhaps the most significant of these metal-ion proteins, with NO demonstrating high affinity for the heme moiety of the enzyme, forming a transient and reversible nitrosyl-heme group. Binding of NO induces a conformational change in the enzyme stimulating the catalysis of cGMP formation. Also well characterized is the binding of NO to hemoglobin, resulting in the formation of met-hemoglobin and nitrate and termination of the activity of NO. It has been proposed that hemoglobin acts as a biological "sink" for NO (Gross and Wolin, 1995), but Jia *et al.* (1996) reported that NO can *s*-nitrosylate hemoglobin to form *s*-nitrosohaemoglobin and this may be important for preserving or potentiating the vascular actions of NO. *s*-Nitrosylation of proteins by NO is also an important intracellular signaling mechanism activating for example the small G-protein $p21^{ras}$ and the ensuing MAP kinase cascade (Lander, 1997).

NO will also bind to and inhibit various iron–sulfur (Fe–S) cluster proteins (Hibbs *et al.,* 1990). Many Fe–S proteins play a significant role in energy metabolism and growth, and NO therefore is able to effect cellular cytotoxicity and cytostasis. In particular, NO inhibits key enzymes involved in mitochondrial respiration, including mitochondrial aconitase, NADH–ubiquone oxidoreductase, and NADH–succinate oxidoreductase (Hibbs *et al.,* 1990; Stuehr and Nathan, 1989), as well as ribonucleotide reductase (Kwon, 1991), which is the rate-limiting enzyme in DNA synthesis. Very recently, a further very important action of NO has been described (Beltrán *et al.,* 2000; Orsi *et al.,* 2000; Fig. 1). Following prolonged exposure to NO, cells evidence a decrease in oxygen utilization as a consequence of two synergistic actions: persistent inhibition of mitochondrial oxygen consumption and production of a hypoxic microenvironment following extraction of oxygen from extracellular medium. It is uncertain whether or not these striking effects occur *in vivo* but should they do so these are likely to be associated with pathological effects.

As mentioned previously, NO and superoxide anion demonstrate a strong affinity, reacting to form peroxynitrite (Beckman *et al.,* 1990). Peroxynitrite is a powerful oxidant with the potential to cause tissue damage; its decomposition products are potent nitrating agents with a particular affinity for protein–tyrosine residues (Beckman *et al.,* 1994; Freeman, 1994) and capable of interfering with tyrosine kinase signaling pathways. Evidence for endogenous peroxynitrite activity has been provided through the immunochemical (Beckman *et al.,* 1994) and biochemical detection of nitrotyrosine species (Kaur and Halliwell, 1994) in tissues and fluids.

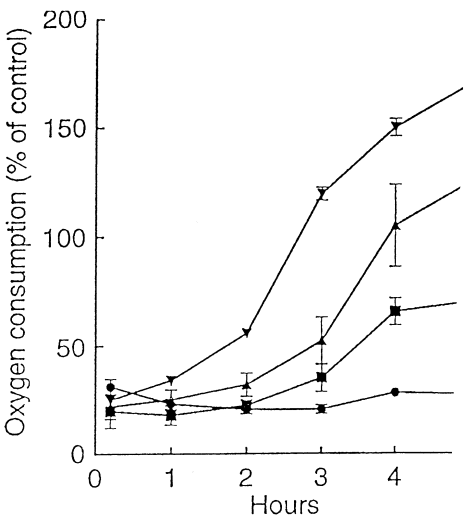

Figure 1 Effects of exposure to the NO donor (DETA-NO) on oxygen consumption by J774 cells. Reproduced from Orsi *et al.* (2000), with permission.

NO or NO species, in particular peroxynitrite, can damage DNA bases, either by oxidation or by base deamination. This damage to DNA can directly stimulate the activation of the enzyme poly-ADP ribose polymerase (PARP), which facilitates DNA repair, by a process that utilises both NADH and ATP (Zhang, 1994). However, prolonged activation of PARP and in particular the continued consumption of NADH and ATP can lead to rapid energy depletion and result in cell death. This may be one mechanism through which NO is able induce cell death by apoptosis. Increased production of NO also correlates with increased expression of the proapoptotic proteins P53 and caspases and downregulation of bcl2.

Nitric Oxide in Bone

Occurrence and Identification of NOS Isoforms

There are now a large number of antibodies and probes available to investigate the expression and localization of NOS enzymes. On the whole, many of the studies performed on bone tissues and cultured cells have resulted in similar descriptions of NOS expression and localization. However, there have been some "controversies" with some groups failing to find expression or localization where others had. In part, such discrepancies can be reconciled by the lack of standardization between studies. Many of the antibodies raised against different NOS enzymes may be species- or even tissue-specific and some may be cross-reactive (Coers *et al.,* 1998). This is also compounded by variations in cell lines studied, differences in culture conditions, or differences in the differentiation status of the cultures. Nonetheless, provided that rigorous controls are implemented, expression and localization studies can yield important information on the role of NO in bone biology.

On the basis of a number of RT-PCR and immunocytochemical investigations performed on whole bone preparations and cultured bone-derived cells from both human and rodents, eNOS and iNOS are the principal NOS isoforms expressed in bone (Helfrich *et al.,* 1997; Fox and Chow, 1998; Macpherson *et al.,* 1999). Although, some investigators have shown expression of nNOS mRNA in bone tissues by RT-PCR (Pitsillides *et al.,* 1995; Helfrich *et al.,* 1997) there is scant evidence for the presence of nNOS protein in bone. That is not to say, however, that nNOS does not have a role in bone. As discussed below, nNOS knockout mice have altered responses to osteogenic hormones like estrogen. It is also likely that nNOS-containing nerves will be present within the bone and may be relevant in regulating bone blood flow.

From the studies performed to date there is consensus that under normal, physiological conditions eNOS is the predominant NOS enzyme expressed by bone and is present in bone marrow stromal cells, osteoblasts, osteocytes, and osteoclasts (Brandi *et al.,* 1995; Helfrich *et al.,* 1997, Fox and Chow, 1998; Macpherson *et al.,* 1999). Studies in our laboratories on rat long bones have shown that expression of eNOS varies according to developmental stage with eNOS mRNA and protein being most abundant in the bones of neonates and subsequently declining in 4-, 8-, and 12-week-old animals (Hukkanen *et al.,* 1999). This age-related regulation of eNOS expression was reflected in the cellular localization. Robust eNOS immunostaining was seen particularly in cuboidal columnar osteoblasts lining trabecular and endosteal bone from neonates and to a lesser extent in osteocytes and multinuclear osteoclasts located in resorption lacunae. In older, 4- to 12-week-old rats the incidence of eNOS positive osteoblasts, osteocytes and osteoclasts became more patchy although stained cells of all three types were still evident and particularly so around remodeling sites. This enzyme was also seen in hypertrophic and degenerating chondrocytes of the epiphyseal growth plate of all ages but particularly in neonatal–4 weeks period. Chondrocytes within the proliferation zone were consistently negative for eNOS expression/staining.

More recent work in our laboratories on NOS expression during fetal development in murine and human fetal long bones (mouse embryonic day 14 and human 8- to 12-week-old abortuses) have shown robust eNOS immunostaining, principally in hypertrophic/degenerating chondrocytes but not in the proliferation zone (Bielby, Buttery, Nohadani, and Polak, unpublished observations).

As discussed above, iNOS is generally absent from untreated cells and tissues and associated with pathophysiology. However, studies by Hukkanen *et al.* (1995), Riancho *et al.* (1995), and Helfrich *et al.* (1997) have detected low levels of iNOS transcripts in unstimulated whole bone and cultured osteoblast-like cells by RT-PCR. Brandi *et al.* (1995) have also described low-level iNOS expression in untreated cultures of a human preosteoclastic cell line. There is also evidence to indicate that in some cells and tissues including bone, iNOS is expressed transiently and has a physiological function. Kasten *et al.* (1994) initially suggested a role for iNOS in regulating osteoclast activity on the basis of *in vivo* administration of NOS inhibitors and this was subsequently supported in studies by Tsukahara *et al.* (1996) and Turner *et al.* (1997). We have shown iNOS expression in whole bones of the developing rat (neonate to 12 weeks old) by RT-PCR being particularly prominent in neonates (Hukkanen *et al.,* 1999). In addition, immunostaining for iNOS protein was seen in sections of neonatal bone, but not older animals, and localized to both osteoblasts and osteoclasts where it was compartmentalized toward ruffled border.

On the whole, it is fair to say that iNOS expression and localization seen in bone preparations or isolated cells is mostly associated with responses mounted to challenge with an inflammatory stimulus. Several groups have demonstrated the expression of iNOS mRNA and protein in cultured rodent and human osteoblast like cells after cytokine stimulation (Ralston *et al.,* 1994; Hukkanen *et al.,* 1995). The level of expression of iNOS by osteoblasts is also quantitative and correlates with the type and combination of cytokine stimuli. In general, combinations of two or more cytokines are more effective than single cytokines. The synergism between IFN-γ, TNF-α, and IL-1β is particularly effective for iNOS induction in osteoblasts.

Moreover, rodent osteoblasts are more responsive to cytokine-stimulated iNOS induction than human osteoblasts.

The evidence for cytokine-stimulated iNOS expression by osteoclasts is less definitive. Brandi *et al.* (1995) have shown marked iNOS expression in rodent and avian osteoclastic cell lines and comparatively "weaker" expression in a human preosteoclastic cell line after cytokine stimulation *in vitro.* Similarly, Sunyer *et al.* (1996) have described marked cytokine-induced iNOS expression in an avian osteoclastic cell line. Conversely, Helfrich *et al.* (1997) failed to detect iNOS by *in situ* hybridization or immunocytochemistry in osteoclast cultures even after cytokine stimulation. The reasons for such differences are unclear but there are corollaries with other cells of leukocyte lineage. Induction of iNOS expression can be readily achieved in rodent monocytes/macrophage after cytokine challenge and the response is often exaggerated, whereas induction of iNOS in human monocytes/macrophages is much more tempered. Human cells are also responsive to combinations of cytokines that are distinct from those described for rodent cells. One possible explanation for these idiosyncracies, other than differences between cell lines or differentiation status, might lie with the "distinctness" of the human iNOS gene sharing only 80% homology with the rodent gene. This has added relevance considering that the constitutive NOS isoforms (eNOS and nNOS) are very highly conserved, with >93% homology between rodent and human.

Other important sites of iNOS expression include the articular joint where iNOS is expressed and localized to chondrocytes and the synovium of arthritic but not normal subjects (Sakurai *et al.,* 1995).

Collectively, these observations demonstrate that NOS isoforms, in particular eNOS and iNOS, are widely expressed in bone tissue and while it is difficult to assign function on expression and localization data alone it can at least be inferred. On this basis these observations are consistent with NO being involved in many aspects of bone growth, modeling, and remodeling under both physiological and pathophysiological conditions.

Osteoclast Effects

As discussed above, osteoclasts express both eNOS and iNOS (Brandi *et al.,* 1995; Sunyer *et al.,* 1996; Helfrich *et al.,* 1997, Fox and Chow, 1998; Macpherson *et al.,* 1999) and accordingly these cells demonstrate sensitivity to both low, basal NO synthesis and higher levels of NO synthesis stimulated by cytokines. The effect of NO on osteoclasts is quite varied and can either suppress or stimulate bone resorption. One of the very first observations on the effects of NO on osteoclasts came from our own laboratories (MacIntyre *et al.,* 1991) showing that exposure of cultured osteoclasts to NO–donor compounds inhibited cell spreading and caused profound inhibition of bone resorption measured by the bone slice assay. Although the concentrations of NO used were supraphysiological, these initial findings have subsequently been confirmed by numerous *in vitro* and *in vivo* studies. Kasten *et al.* (1994), Tsukahara *et al.* (1996), and Turner *et al.* (1997) examined the effects of chronic *in vivo* administration of the NOS inhibitor compounds in rats, in particular aminoguanidine, and demonstrated marked reductions in bone mass. This would seem to indicate that NO is an endogenous inhibitor of osteoclast activity and is consistent with the profound inhibitory effect of NO donors on osteoclastic bone resorption (MacIntrye *et al.,*1991; Kasten *et al.,* 1994). Moreover, on the basis of the reported selectivity of aminoguanidine for iNOS and in the absence of any significant effects following administration of other, "nonselective" NOS inhibitors this has led to the suggestion that iNOS is the principal source of NO in the regulation of osteoclast activity. While there are some concerns relating to NOS inhibitor studies (see below) these observations have important implications for the control of iNOS expression in bone cells and suggest that iNOS might be expressed "consistutively." This would be consistent with studies demonstrating low-level expression of iNOS transcripts in unstimulated whole bone preparations and isolated bone cell cultures (Brandi *et al.,* 1995; Helfrich *et al.,* 1997). Whether or not there is transient localized production of iNOS protein with subsequent activation of the enzyme and, perhaps more importantly, in which cell types this occurs remains to be determined.

It is well established that cytokines including IL-1β, TNF-α, and IFN-γ are powerful regulators of bone resorption (Mundy, 1993). These cytokines are also potent stimulators of iNOS (Ganster and Geller, 2000) and it has been convincingly shown that cytokine-induced iNOS activity exerts marked effects on osteoclastic bone resorption (Löwik *et al.,* 1994; Brandi *et al.,* 1995; Ralston *et al.,* 1995; Ralston and Grabowski *et al.,* 1996). Cytokine-induced NO synthesis can either stimulate or inhibit osteoclastic bone resorption and correlates to the apparent "level of iNOS induction/ activation." The synergetic effects of IL-1β, TNF-α and IFN-γ induce the highest levels of iNOS expression and activity in bone, resulting in marked inhibition of resorption. However, when added singly these cytokines produce very different results. Of the three cytokines IFN-γ is probably the most potent stimulator of iNOS and accordingly IFN-γ stimulated NO production is thought to account for the selective inhibitory effects of this cytokine on bone resorption (Mundy, 1993; Löwik *et al.,* 1994; Ralston *et al.,* 1995). Conversely, IL-1β and TNF-α exert a more modest inductive effect on iNOS expression and activity and this is manifest by inhibition of the proresorptive effects of these cytokines by NOS inhibitors (Löwik *et al.,* 1994; Ralston *et al.,* 1995). These studies also demonstrated synergism between NO and prostaglandin E2, another important regulator of bone resorption (Ralston *et al.,* 1995). This is consistent with recent observations on bone marrow cocultures from iNOS-gene-deficient mice, revealing that iNOS is essential for mediating the bone-resorptive effects of IL-1β (Van't Hof *et al.,* 2000).

In addition to cytokine-induced (iNOS) NO synthesis, osteoclasts are known to express eNOS and the activity of

this constitutive enzyme seems to be important in stimulating bone resorption (Brandi *et al.,* 1995). Indeed, administration of a general NOS inhibitor to untreated neonatal rodent osteoclasts blocks resorption, demonstrating that transient, tonic release of NO or, as discussed above, moderate induction of NO synthesis is an important facet of osteoclast resorptive function.

While it is clear that NO is significant in the regulation of osteoclastic bone resorption, the cellular source(s) that contribute to this process are more controversial. There is evidence to show that preosteoclastic and osteoclast cell lines have a low basal NO synthesis (Brandi *et al.,* 1995), which can be further stimulated by fluid shear-stress (McAllister *et al.,* 2000). In response to cytokine stimulation iNOS expression is induced in avian, rodent, and human osteoclastic cell lines (Brandi *et al.,* 1995; Sunyer *et al.,* 1996) and is accompanied by marked elevation in NO synthesis, as suggested by nitrite accumulation (Sunyer *et al.,* 1996). Such data would be consistent with an autocrine role for NO in osteoclast function. However, other investigators have argued against this after failing to detect appreciable levels of iNOS expression in osteoclasts even after cytokine stimulation (Helfrich *et al.,* 1997). Moreover, on the basis of bone organ culture or osteoblast–bone marrow coculture experiments, this same group of investigators have implicated the osteoblast as being the predominant source of inducible NO affecting osteoclast activity (van't Hof and Ralston, 1997; Van't Hof *et al.,* 2000).

The mechanisms of action of NO on osteoclasts are also quite varied and dependent on the stage of cell development. Exposure to high levels of NO associated with iNOS expression and activation is known to induce damage and death in a number of cell types. Consistent with this, cytokine-stimulated NO synthesis diminishes proliferation of preosteoclasts (Brandi *et al.,* 1995) and may involve induction of apoptotic cell death (van't Hof and Ralston, 1997). What is particularly remarkable about the latter observation is the relative sensitivity of osteoclast progenitors to NO-induced apoptosis with mature osteoclasts and osteoblasts apparently being more resistant (Van't Hof and Ralston, 1997). The upshot of this is a reduction in the formation of mature osteoclasts and suppression of bone resorption.

In mature osteoclasts NO has been shown to both stimulate and inhibit activity and as discussed above this bidirectional effect (Brandi *et al.,* 1995) is related to changes in the local concentration of NO. Exposure of mature osteoclasts to NO stimulates contraction and causes the cells to become detached from the underlying bone surface (MacIntyre *et al.,* 1991; Brandi *et al.,* 1995). This effect is illustrated in isolated rat osteoclasts (Fig. 2). The contraction of isolated osteoclasts is known to be induced by fluxes in calcium ion concentration and appears to be mediated by a calcium-sensitive isoform (eNOS) since NOS inhibitors largely prevent the calcium-induced osteoclastic contraction (Brandi *et al.,* 1995). It is now known that this action of nitric oxide is largely due to cGMP, although this was not apparent in initial studies using slowly permeant cGMP analogs such as 8-bromo- or di-butyryl-cGMP (MacIntyre *et al.,* 1991). However, more recently we demonstrated that rapidly permeant cGMP analogs (8-pCPT-cGMP) produce a dramatic osteoclast contraction. This is also seen after exposure to NO donors and is largely prevented, although not entirely, by guanylate cyclase inhibitors (Mancini *et al.,* 1998). The calcium-stimulated effect of nitric oxide may well be physiological and during osteoclastic bone resorption in which periodic calcium-induced detachment occurs this contracting and detaching effect likely forms an essential part of osteoclastic bone resorption involving osteoclast

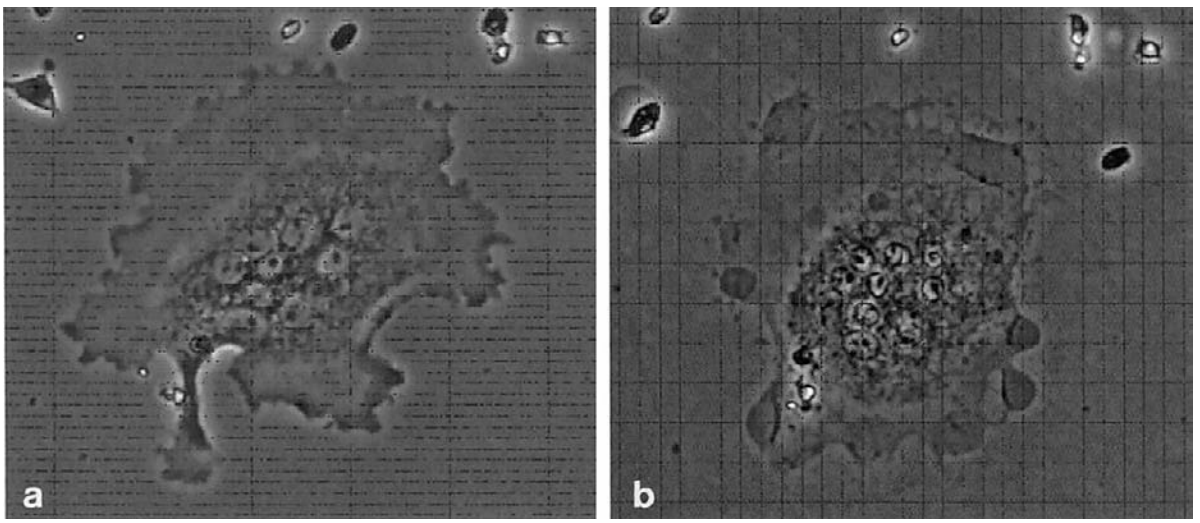

Figure 2 Videomicroscope image of a primary rat osteoclast. (a) A typical rat osteoclast showing large cytoplasmic area and numerous nucleii. (b) The same cell after addition of a nitric oxide donor showing reduced cell area.

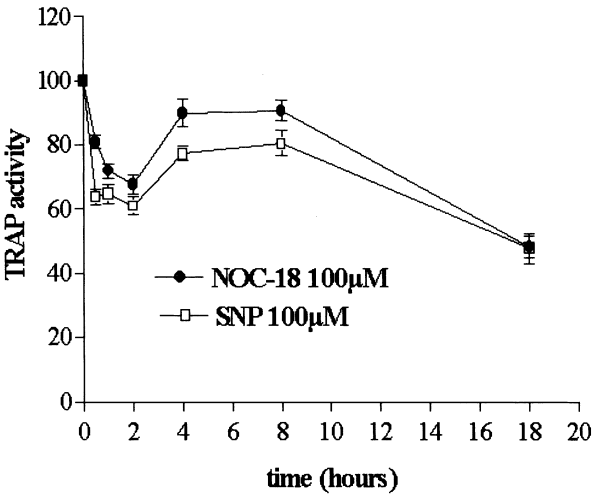

Figure 3 Effect of NO-donors on human type 5 acid phosphatase activity. The two NO donors, SIN-1 and NOC-18, are both able to decrease TRAP activity over time with a maximun reduction observed after 18 hr. Values are the mean of three different experiments and are expressed as percentage of control.

movement. With more prolonged activation this is likely to become inhibitory, increasing the frequency of contraction and detachment and suppressing resorption activity.

Very recently we have shown that NO inhibits the activity of recombinant tartate-resistant acid phosphatase (TRAP) (L. Mancini, T. Cox and I. MacIntyre, unpublished observations; Fig. 3) and this effect might contribute to the inhibitory action of NO on osteoclastic bone resorption. TRAP is mainly localized in resorption vacuoles, suggesting that it is secreted by the osteoclasts at the site of active resorption, although the mechanism by which it promotes bone resorption is still unclear. However, it is clearly established that inhibition of acid phosphatase by chemical and immunological means can abolish osteoclastic bone resorption (Zaidi *et al.,* 1989). Further, acid phosphatase knockout mice have mild osteopetrosis while genetically engineered mice overexpressing acid phosphatase have an increased bone turnover (Hayman *et al.,* 1996, Angel *et al.,* 2000). Thus, acid phosphatase seems to be essential for bone resorption and osteoclast activity and the finding that NO can inhibit TRAP activity, possibly by binding to the iron moiety of the protein, could account for some of the inhibitory actions of NO on osteoclastic bone resorption.

OSTEOBLAST/OSTEOCYTE EFFECTS

The first definitive evidence for synthesis of NO by cells of the osteoblast lineage came from studies on responses of these cells to cytokine stimulation. Several groups independently demonstrated induction of iNOS mRNA and protein expression in cultures of both rodent and human primary osteoblasts and osteoblast cell lines after cytokine challenge (Damoulis and Hauschka, 1994; Löwik *et al.,* 1994; Ralston *et al.,* 1994; Hukkanen *et al.,*1995). Activity of iNOS and synthesis of NO by these cells was confirmed by marked increases in the accumulation of nitrite, one of the oxidation products of NO biosynthesis, in the culture medium. Furthermore, these studies revealed a rank order of potency for induction of iNOS by cytokines. When added to the cultures singly, IFN-γ was found to induce the highest levels of NO synthesis, as assessed by nitrite accumulation, followed by TNF-α and finally IL-1β, which had only mild to inductive effect. Combinations of two or more cytokines and in particular the combination IFN-γ, TNF-α, and IL-1β significantly augmented NO synthesis over levels achieved by single cytokines, suggesting synergistic effects on induction of iNOS.

By including inhibitors of NOS activity in the culture medium during or after cytokine-induced NO production it has been possible to examine the effects of NO on osteoblast function. Under these conditions NO causes profound inhibition of cell proliferation and DNA synthesis (Ralston *et al.,* 1995; Hukkanen *et al.,* 1995) and also induces apoptotic cell death (Damoulis and Hauschka 1997; Jilka *et al.,* 1998). In addition, cytokine induced NO synthesis causes reductions in the expression and activity of markers of osteoblast differentiation including alkaline phosphatase and osteocalcin (Ralston *et al.,* 1995; Hukkanen *et al.,*1995) and formation of mineralized bone nodules (Buttery, Hughes and Hukkanen, unpublished observations; Fig. 4). More recently, Hughes *et al.* (1999) demonstrated that NO generated by iNOS or supplied exogenously by an NO donor was important in stimulating expression of cyclooxygenase 2 and prostaglandin (E2) synthesis and might be relevant to osteoblastic control of bone resorption.

There are also some physiological processes that are stimulated by osteoblast/osteocyte constitutive (eNOS) NO synthesis. Riancho *et al.* (1995) demonstrated basal, constitutive NO synthesis by osteoblasts which promoted cell proliferation and could be augmented by 1,25-dihydroxy vitamin D_3. This basal NO synthesis also stimulated release of IL-6, suggesting a possible role for osteoblast-derived NO stimulating osteoclastic bone resorption. It has been shown in rodent models that the new bone formation induced by mechanical strain is blocked by NOS inhibitors (Fox *et al.,* 1996; Turner *et al.,* 1996). Furthermore, osteocytes and osteoblasts rapidly produce NO when exposed to mechanical strain or pulsatile fluid flow *in vitro* (Pitsillides *et al.,* 1995; Klein-Nulend *et al.,* 1995; Turner *et al.,* 1996) and this is now known to be dependent on the expression and activation of eNOS (Klein-Nulend *et al.,* 1998; Zaman *et al.,* 1999). Such a role for eNOS in sensing and responding to mechanical loading or shear flow is consistent with the molecular regulation of this enzyme (Kleinert *et al.,* 2000). More recently McAllister and Frangos (1999) have examined the kinetics of NO release from osteoblasts exposed to both steady and transient fluid shear stress. The results demonstrated two distinct biochemical pathways with transient flow stimulating a transient "burst" of NO that was dependent on G-protein activation and calcium mobilization, while steady flow produced sustained NO release that was G-protein- and calcium-independent. Such studies are important since they not only confirm

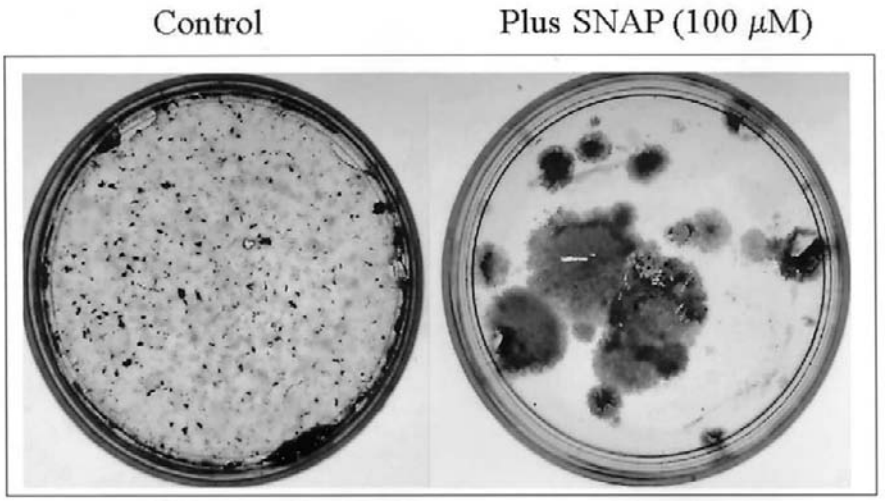

Figure 4 Effects of exogenous NO on rat osteoblast differentiation and formation of mineralized bone nodules. Exposure to SNAP (12 hr) at the time of cell seeding severely inhibited subsequent cell growth and differentiation.

the involvement NO in transducing osteogenic signals but also provide an insight into how perturbations in this signaling pathway might contribute to bone pathology.

In addition to conducting osteogenic effects induced by mechanical loading and fluid flow, NO generated by cells of the osteoblast lineage is important in mediating the anabolic effects of estrogen on bone. While the major effect of estrogen has been thought to be its ability to inhibit osteoclastic bone resorption, estrogen has also been shown to have a direct effect on osteoblast-like cells (Ernst *et al.,* 1989). The presence of functional estrogen receptors in cells of the osteoblast lineage (Eriksen *et al.,* 1988; Komm *et al.,* 1988) suggests estrogen compounds such as 17β-estradiol stimulate bone formation *in vivo* by a direct action on osteoblasts (Chow *et al.* 1992a,b; Tanko-Yamamato and Rodan, 1990; Samuels *et al.,* 1999). Studies *in vitro* have shown 17β-E_2 to enhance both proliferation and differentiation of cultured osteoblasts (Qu *et al.,* 1998; Ernst *et al.,* 1988; Scheven *et al.,* 1992). Although a link between estrogen and stimulation of eNOS had been established in the vascular system (Hayashi *et al.,*1995; Weiner *et al.,*1994) the first studies to demonstrate a link between the osteogenic effects of estrogen and NO in bone were perhaps those of Wimalawansa *et al.* (1996). They demonstrated that the NO donor compound nitroglycerin, long used in the treatment of ischemic heart disease, could prevent or reverse the bone loss and onset of osteopenia induced by ovariectomy in a rat model. Subsequently, Armour and Ralston (1998) demonstrated upregulation of eNOS expression and activity in a human osteoblast cell line after stimulated by 17β-estradiol. Studies in our laboratory (O'Shaughnessy *et al.,* 2000) on primary human and rat osteoblast cultures showed that 17β-estradiol dose-dependently stimulated osteoblast proliferation and differentiation as assessed by alkaline phosphatase activity and bone nodule formation (Fig. 5) and this could be abolished by inhibitors of NOS activity. Additionally, 17β-estradiol increased total eNOS enzyme expression in rat osteoblasts and stimulated increases in NO metabolite levels (Fig. 6), which once again could be abolished NOS inhibitors. A final and important finding from these studies is the lack of response to 17β-estradiol of primary osteoblasts cultures from eNOS gene knockout mice (studies on NOS-gene-deficient mice are discussed in more detail below). Collectively these observations suggest that the stimulatory effect of estrogen on osteoblast proliferation and differentiation rely on local production of NO by bone cells via the eNOS isoform.

Similar results to those described above are also seen after exogenous administration of NO donor compounds to osteoblast cultures. NO donors increase cGMP production, alkaline phosphatase activity, osteocalcin expression, and

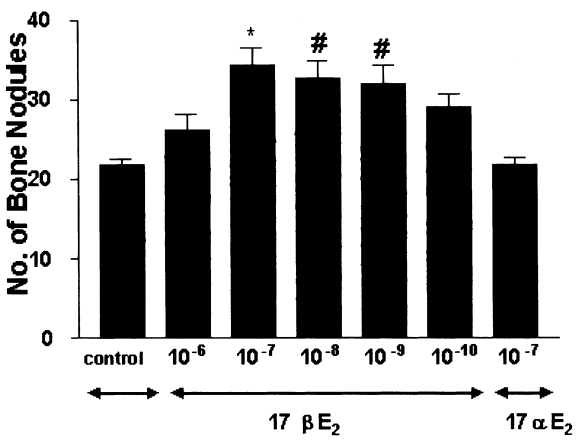

Figure 5 Quantitative assessment of rat osteoblast differentiation by *in vitro* formation of mineralized bone nodules. Estradiol has a dose-dependent effect on rat osteoblast differentiation. Note α-estadiol does not have any effects on osteoblast differentiation. Values are means $\pm$ SEM of six different experiments. $^{*}P < 0.001$; $^{\#}P < 0.01$.

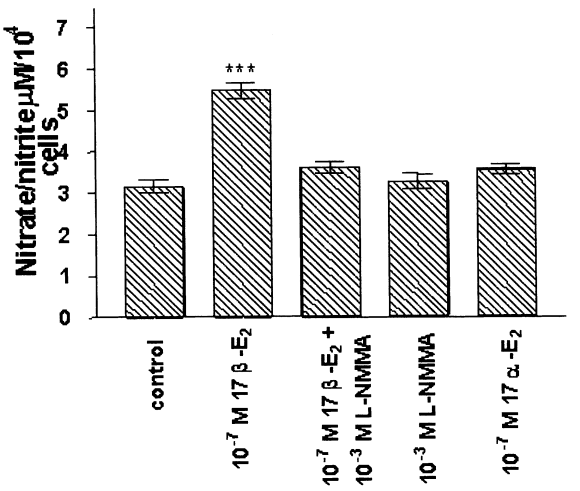

Figure 6 Analysis of NOS activity in osteoblasts by nitrite accumulation. Estradiol stimulates NOS (eNOS) activity and blocked by the addition of the NOS inhibitor L-NMMA. Values are means ± SEM of six different experiments. *** $P<0.01$ versus control.

in vitro bone nodules formation (Chae *et al.,* 1997; Hikiji *et al.,* 1997; Otsuka *et al.,* 1998). Studies by our group (Mancini *et al.,* 2000) found that while slow release and generation of low concentrations of NO stimulated osteoblast replication and alkaline phosphatase activity, rapid release, and high concentrations of NO inhibited proliferation and induced apoptosis. These observations help to emphasize a fundamental aspect of the biological chemistry of NO, showing that alterations in the kinetics of NO synthesis (loosely equating to eNOS versus iNOS activity) can evoke profoundly different biological responses. Importantly these studies also demonstrated that the biphasic effects of NO on osteoblasts are mediated, in part, by the second messenger cGMP (Mancini *et al.,* 2000).

As such, these results confirm that NO is generated by osteoblasts via eNOS and iNOS and has an important role in osteoblast function. As a general rule of thumb transient low-level production of NO regulated by eNOS is associated with stimulation of osteoblast activity and bone formation. Whereas, more sustained high output NO synthesis associated with iNOS expression and activity inhibits osteoblast function.

NO DONORS

NO donors can be used in the characterization of NO-mediated effects and provide a means of supplying "controlled" levels of NO from an exogenous source to mimic endogenous NO synthesis in biological systems. However, in practice it is often difficult to exert adequate control over NO release and as such studies on NO donors, although useful, should be interpreted with some caution. The kinetics of NO release varies from compound to compound. Some release NO spontaneously in aqueous buffers, whereas others require active metabolic uptake and degradation. Compound-specific susceptibility to changes in pH, oxygen, light, and temperature and formation of different by-products and metabolites during decomposition or metabolism also vary. Studies on NO donors are also complicated by the vast choice of compounds. In terms of bone research the following lists of some of the compounds used and their characteristics:

Nitroglycerin (glyceryl trinitrate, GTN) is an organic nitrate with over a 100 years of therapeutic use particular in cardiovascular disease. It appears to require active metabolic uptake and degradation by cells to be effective and therein lies one the drawbacks of this cell, with cells becoming tolerant after repeated exposure, requiring consistently higher dosage. It does, however, have the advantage that it can readily be used *in vivo* and has been shown to be effective in the prevention or reversal of estrogen-depleted osteoporosis in rodent models and human subjects (Wimalawansa *et al.,* 1996, 2000).

S-Nitroso-*N*-acetyl-penicillamine (SNAP), *S*-nitrosoglutathione (SNOG) 3−morpholinosydnonomine (SIN-1), and sodium nitroprusside (SNP) are NO donor compounds that have been used in a vast number of *in vitro* (and some *in vivo*) experiments including cultured bone cells. All seem to liberate NO or NO adducts more or less spontaneously on contact with aqueous buffers. This can potentially "flood" the culture medium with NO making difficult to quantify cellular responses. However, careful adjustment of donor concentration can be used to create NO gradients that more faithfully mimic both physiological and pathophysiological responses *in vitro* (Damoulis and Hauschka, 1997; Otsuka *et al.,* 1998; Hughes *et al.,* 1999; O'Shaughnessy *et al.,* 2000; Aguirre *et al.,* 2001)

The final class of compounds, the diazeniumdiolates or NONOates, are perhaps the most useful for most closely mimicking endogenous NO synthesis. Although the NONOates release NO spontaneously, their rate of decomposition can be precisely controlled. This enables the kinetics and concentrations of NO liberated into the culture medium to be more accurately predicated and adjusted to correlate more closely with the activity of eNOS or iNOS (Otsuka *et al.,* 1998; Mancini *et al.,* 2000).

INHIBITION OF NO SYNTHESIS

Research directed toward the control of NO synthesis has flourished in the past few years, particularly in view of the roles that NO production may play in various pathophysiological states. Following the initial discovery that N^G-monomethyl-L-arginine (L-NMMA) could inhibit the synthesis of NO, a variety of N^G-substituted L-arginine derivatives and related compounds have been investigated as inhibitors of NO synthesis.

L-NMMA and N^G-N^G-dimethyl-L-arginine (ADMA) are naturally occurring inhibitors of NO synthesis with an equal affinity for the constitutive and inducible isoforms of NO synthase (Vallance *et al.,* 1992). Other commonly used inhibitors of NO synthesis include N^G-nitro-L-arginine (L-NNA), N^G-L-argininemethylester (L-NAME), N^G-iminoethyl-L-ornithine (L-NIO), and L-canavine. Some of these are reported to demonstrate rank-order specificity for particular isoforms

(Knowles and Moncada, 1994). For example, L-NNA, L-NIO, and L-canavine are more selective for iNOS than for either nNOS or eNOS. Aminoguanidine (AG), has been much used as a selective inhibitor of iNOS including studies on bone (Kasten *et al.,*1994; Tsukahara *et al.,*1996) and is reported to be 20- to 30-fold more selective for iNOS than the constitutive isoforms. However, none of these inhibitors is sufficiently effective in their selectivity (and may have effects independent of NOS inhibition) (Peterson *et al.,* 1992) to be able to assign, unequivocally, a specific cellular function to a particular NOS isoform.

NOS GENE-DEFICIENT MICE

Gene knockout mice represent powerful tools for studying possible functions and targets of regulatory factors involved in physiological and pathological processes. Murine gene knockouts have been created for all three isoforms of NOS (Huang *et al.,* 1993, 1995; MacMicking *et al.,* 1995; Wei *et al.,*1995). In all cases the mutant mice were found to be viable, fertile, and identical in general appearance and behavior to their wild-type counterparts.

Mice deficient in nNOS were the first knockouts to be created and revealed that the nNOS gene-deficient mice are largely resistant to brain damage induced by vascular stokes (Huang *et al.,* 1993), confirming the importance of nNOS-derived NO in mediating stroke damage. It is also apparent that nNOS knockout male mice are highly aggressive (Nelson *et al.,* 1995). The observations of iNOS gene-deficient mice were also in keeping with what might have been expected, corroborating the participation of iNOS in mediating inflammatory responses with iNOS gene-deficient mice demonstrating attenuated microbicidal and tumouricidal responses (MacMicking *et al.,*1995; Wei *et al.,* 1995). The findings from mice deficient in eNOS are again in accord with what might have been expected, based on prior observations of its actions. These mice had an elevated blood pressure and isolated aortic rings did not display a relaxant response when challenged with acetylcholine. This demonstrated the important role of eNOS-derived NO in mediating vascular homeostasis (Huang *et al.,* 1995). However, an unexpected finding was the induction of a hypotensive effect following administration of a NOS inhibitor, which could be reversed by infusion of excess L-arginine (Huang *et al.,* 1995). This suggests that blockers of NO synthesis might have independent effects on the vasculature or that other isoforms of NO synthase contribute to the maintenance of blood pressure. The prime candidate for this is nNOS, which is known to be present in the vasomotor centers of the central nervous system and in nerves supplying blood vessels.

In the context of this review NOS gene-deficient mice represent powerful models for studying the role of NO in bone metabolism, circumventing the lack of isoform specificity of the current generation of NOS inhibitors providing a rigorous test of previous proposed actions of NO on bone. Observations by our group (Moradi-Bidhendi, Mancini, Forte, Cafferkey, Benjami, MacIntyre, unpublished observations) show that male nNOS knockout mice have a marked

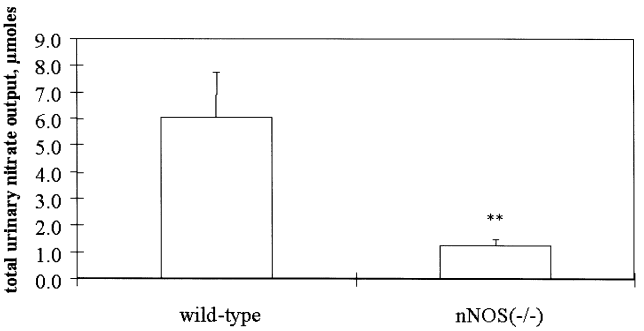

Figure 7 The mean total urinary nitrate output of c57/bl/6 wild-type and nNOS(−/−) knockout mice. nNOS(−/−) knockout mice show a significantly lower nitrate output than wild-type controls. All animals were between 8 and 12 weeks old and maintained on a low nitrate diet throughout experiments. Values mean ± SEM, $n = 6$. ** $P < 0.01$ using Student's *t* test.

reduction in urinary nitrate (Fig. 7). In addition, female nNOS knockout mice fail to respond to the stimulatory effect of estrogen based on urinary nitrate measurements (Fig. 8). Interestingly, male eNOS knockouts have urinary nitrate outputs similar to wild-type controls and females respond normally to estrogen with a cyclic increase in urinary nitrate excretion. These findings imply that nNOS is mainly responsible for urinary nitrate and that nNOS is stimulated by estrogen, thus explaining the normal increase in nitrate after administration of estrogen to eNOS knockouts. Further, it is conceivable that the aggressive behavior of nNOS knockouts resembles the effect of estrogen deficiency on cerebral nNOS.

More detailed investigations by our group and another group on eNOS knockout mice have now demonstrated the considerable role of eNOS in the maintenance of physiological bone mass (Aguirre *et al.,* 2001; Armour *et al.,* 2001). As mentioned earlier, eNOS knockout mice are viable and fertile, and in terms of gross physical appearance (stature, gait, and anatomy) are unremarkable. However, on closer examination of the long bones we found that both the femur and the tibia of eNOS knockouts were significantly shorter (1–2 mm) than the corresponding wild-type. Histomorphometry analysis of the same bones showed a marked reduction in both bone formation rate and bone volume (Fig. 9).

Interestingly, the consequences of eNOS gene deficiency were most pronounced in young (6–9 weeks old) adults and by 12–18 weeks bone phenotype was restored toward wild-type. Regardless of eNOS expression bone turnover was significantly higher in young mice than old mice and similar observations have been made on rats and humans demonstrating that bone modeling/remodeling activity is related to sexual maturity. Moreover, we have shown that eNOS expression correlates with skeletal development and is most abundant in the bones of neonates and young adults, decreasing markedly in older animals (Hukkanen *et al.,* 1999). These data suggest that eNOS expression and activity is important during phases of rapid bone growth or turnover such as in the period from neonate through to

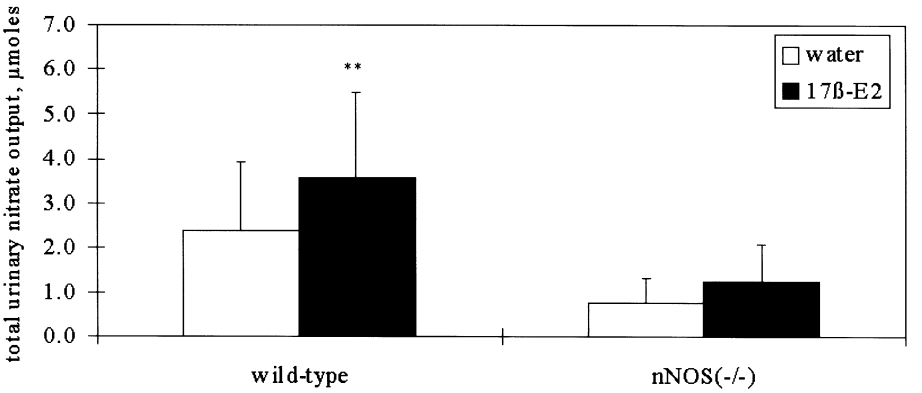

Figure 8 The mean total urinary nitrate output of c57/bl/6 and nNOS(−/−) with estrogen administration. Control c57/bl/6 mice showed a significant increase in urinary nitrate with administration of 10 μg 17β-E_2. There was no significant increase in the urinary nitrate output of nNOS(−/−) mice. All animals were between 8 and 12 weeks old and maintained on a low-nitrate diet throughout experiments. Values mean ± SEM, $n = 6$. $^{**}P < 0.01$ using Student's t test.

sexually mature adult and the attainment of peak bone mass. Clearly, eNOS gene deficiency does not represent a severe or irreversible impediment to skeletal growth as exemplified by our studies, where despite the initial expression of a juvenile osteoporosis-like phenotype these knockouts are still able to attain peak bone mass. The mechanism of recovery is unknown. However, it is possible to suggest that these mice will exhibit altered responses to physiological and, in particular, pathophysiological demands placed upon the skeleton such as the effects of endocrine imbalance (i.e., estrogen deficiency; Armour *et al.,* 2001). Furthermore, upregulation of eNOS activity is important in mechanical load-induced bone growth (Pitsillides *et al.,* 1995; Turner *et al.,* 1996; Klein-Nulend *et al.,* 1998; Zaman *et al.,* 1999) and fracture healing (Corbett *et al.,* 1999a,b; Diwan *et al.,* 2000). Thus, eNOS knockout mice are likely to exhibit defective or protracted bone repair mechanisms following traumatic injury.

In the same study (Aquirre *et al.,* 2001) we found that the reduction in bone formation and volume was not related to increased osteoclast numbers or activity but rather to dysfunctional osteoblasts. Osteoblast number and synthetic and mineralizing activity were all reduced *in vivo. In vitro,* cultures of primary osteoblasts from fetal calvarial explants showed retarded growth and differentiation as assessed by alkaline phosphatase activity and formation of mineralized bone nodules and could be reversed by exposure to low concentrations of the NO donor SNAP. As presented in studies by O'Shaughnessy *et al.* (2000), osteoblasts from eNOS knockouts, unlike wild-type cultures, were unresponsive to the mitogenic effects of estradiol over a range of concentrations, providing further evidence that the eNOS–NO signaling pathway is important in mediating the osteogenic actions of estrogen. Moreover, these cells demonstrated an attenuated chemotaxis response and failed to migrate along a TGF-β gradient (Aquirre *et al.,* 2001), which is known to be a potent cytokine in recruiting osteoblasts to remodeling sites (Pfeilschifter *et al.,* 1990). Taken together these data illustrate that distinct components of the osteoblast phase of the bone remodeling cycle are altered in eNOS knockout mice, strongly implicating NO-dependent signaling via eNOS in the regulation of osteoblast growth, differentiation, recruitment, and extracellular matrix synthesis. Recent studies by Armour *et al.* (2001) show that ovariectomized eNOS knock-

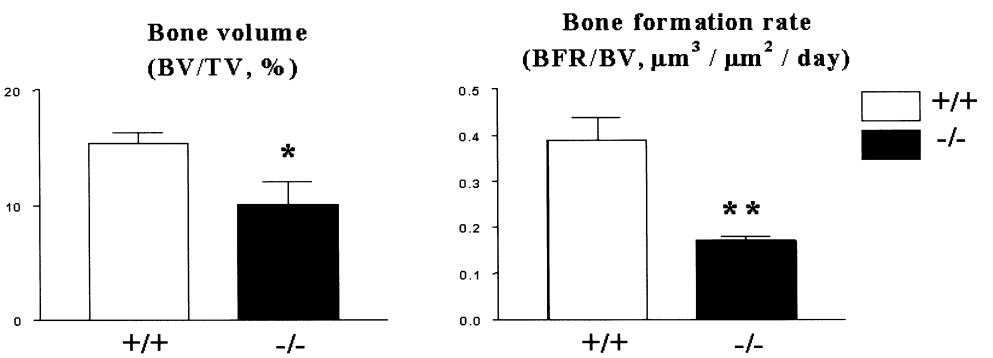

Figure 9 Histomorphemtry analysis of femurs from 6-week-old eNOS gene knockout mice and age-matched wild-types. Both bone formation rate and bone volume are significant reduced in eNOS knockouts. Values mean ± SEM $n = 7$. $^{*}P < 0.05$; $^{**}P < 0.01$.

out mice have a significantly blocked anabolic response to high doses of exogeneous estrogen.

It is important to note that at the time of completing this chapter several issues relating to the effects of eNOS gene deficiency on bone biology remained unresolved. In particular, studies to test the role of eNOS in sensing/transducing signals associated with mechanical loading and shear flow had not be reported, although it is likely that these responses will be blunted. The potential impact of the persistent hypertension associated with eNOS gene deficiency (Huang *et al.,* 1995) on bone turnover needs to be thoroughly evaluated. There is evidence that bone vasculature can modify bone formation through intraosseous pressure (Kelly and Bronk, 1990) and factors released from endothelial cells (including NO) can modulate bone cell function (Collin-Osdoby, 1994). Finally, in addition to the osteoblast we found that osteoclast activity was diminished in eNOS knockouts, based on histomorphometry, with a reduction in eroded surface. This again was prevalent in young (6 weeks old) mice and reversed in older (9–18 weeks) mice. This is consistent with studies by Brandi *et al.* (1995), suggesting that eNOS activity might be required to stimulate osteoclast although more detailed investigation is required to confirm these observations.

With regard to iNOS gene knockouts, studies *in vitro* and *in vivo* by van't Hof *et al.* (2000) demonstrated that IL-1-induced (iNOS) NO production was essential for promoting osteoclast formation and activity. Moreover, osteoblast-bone marrow cocultures revealed that osteoblasts were the principal source of NO regulating osteoclast activity, particularly in osteoclast precursors, and was dependent on NFκB translocation and DNA binding. Importantly, the defects in IL-1-induced bone resorption could be reversed by exogenous supply of the NO donor SNAP (van't Hof *et al.,* 2000). Initial studies on iNOS knockouts by our group (Aguirre, Buttery, Hukkanen, MacIntyre, and Polak, unpublished observations) have revealed marked reductions in bone formation and volume similar to the abnormalities described in eNOS knockouts (Aguirre *et al.,* 2001). However, the underlying mechanism is distinct and appears to relate to increased osteoclast number and activity and would support the NOS inhibitor (iNOS) studies of Kasten *et al.* (1994), Tsukahara *et al.* (1996) and Turner *et al.* (1997). Another interesting observation from iNOS knockouts is a three- to fourfold increase in eNOS mRNA expression in whole bone preparations. The significance of this remains to be determined but could represent a compensatory mechanism to counter the loss of iNOS. Such a phenomenon is not unusual in NOS gene knockouts (Huang and Fishman, 1996).

Potential Molecular Targets for the Action of NO in Bone Cells

As discussed in the section on NO signalling is able to activate various signal transduction pathways in a number of different cell types. Despite this, there is a surprising paucity of information of the detailed signaling mechanisms of NO in bone cells. The guanylyl cyclase-cGMP pathway is a classical target for NO and there is a substantial body of evidence to show that the cGMP-dependent signaling is crucial to normal bone formation. Early studies demonstrated that cGMP was important in transducing the anabolic effects of mechanical loading (Rodan *et al.,* 1975) and more recently those of naturetic peptides on bone formation (Suda *et al.,* 1996; Mericq *et al.,* 2000). cGMP analogs increase the expression of markers of osteoblast differentiation alkaline phosphatase and osteocalcin and augment formation of mineralized bone nodule in rat calvaria cell cultures (Inoue *et al.,* 1995). The marked bone abnormalities and dwarfism seen in mice lacking protein G kinase type II is particularly compelling evidence for the role of cGMP in endochondral ossification (Pfeifer *et al.,* 1996). Evidence for a direct association between NO and cGMP has come from observations on cultured osteoblasts using NO donors. These studies showed that increased alkaline phosphatase activity, osteocalcin expression, and *in vitro* bone nodule formation was accompanied or dependent, at least in part, on cGMP synthesis (Chae *et al.,* 1997; Hikiji *et al.,* 1997; Otsuka *et al.,* 1998; Mancini *et al.,* 2000).

The role of cGMP in mediating osteoclast function is less conclusive. Initial studies suggested cGMP production is not required for the functional modulation of osteoclasts by NO (MacIntyre *et al.,* 1991; Ralson and Grabowski, 1996). However, more recent studies using newer generation cGMP analogs and guanlyly cyclase inhibitors have demonstrated stimulation of cGMP by low concentrations of NO does regulate osteoclast function increasing cell contraction and detachment from bone surfaces (Mancini *et al.,* 1998). In addition, Dong *et al.* (1999) showed that NO-dependent cGMP production reduced transport of HCl osteoclast membranes and inhibited activity.

In addition to the cGMP pathway NO is a potent stimulator of $p21^{ras}$ activity and MAP kinase signaling (Lander, 1997). Although there is no direct evidence for NO-dependent activation of this pathway in bone cells it has been shown that the MAP kinases are involved in stimulating osteoblast proliferation and differentiation (Matsuda *et al.,* 1998) and in regulating expression of the osteoblast transcription factor Cbfa-1 (Xiao *et al.,* 2000). MAP kinase activation is also involved in RANK ligand-induced osteoclast differentiation (Matsumoto *et al.,* 2000).

Expression and activity of VEGF is fundamental to the process of endochondral ossification stimulating chondrocyte cell death, cartilage remodeling, and angiogenesis (Gerber *et al.,* 1999). Again there is no direct evidence to support the involvement of NO in mediating the actions of VEGF in bone but there is certainly evidence to show that NO contibutes to the angiogenic effects of VEGF in other cell types (Papapetropoulos *et al.,* 1997; Frank *et al.,* 1999; Ziche, 2000). As such, NO-VEGF signaling might also be important in the angiogenic response that is crucial to fracture healing.

NO AND BONE PATHOLOGY

Alterations or disruption to NO synthesis within the bone environment has been implicated in the genesis of a number of diseases. Inflammatory diseases such as rheumatoid arthritis, which is characterized by articular cartilage erosion together with juxta articular bone loss is associated with activation of iNOS and production of NO and NO related species including peroxynitrite (Farrell *et al.,* 1992; Stefanovic-Racic *et al.,* 1993; Kaur and Halliwell, 1994; Grabowski *et al.,* 1996). On the basis of studies using NOS inhibitors and iNOS knockouts, activation of iNOS and increased levels of NO production contribute directly to the tissue damage associated with rheumatoid arthritis (McCartney-Francis *et al.,* 1993; Stefanovic-Racic *et al.,* 1994).

There are several other inflammatory conditions associated with activation of iNOS and in which increased production of NO or NO derived oxidants (peroxynitrite) are implicated in pathological bone loss. For example, Hukkanen *et al.* (1997) showed increased staining for iNOS and formation of peroxynitrite in macrophages associated with bone erosions adjacent to prosthetic hip implants, suggesting the involvement of iNOS in the process of loosening of the implant. Such subjects require reoperation to stabilize or replace the loosened prosthesis. Armour *et al.* (1999) presented evidence for iNOS activation and increased NO production as a contributory factor to the extensive bone loss seen in a model of inflammation-induced osteoporosis. Importantly, the deleterious effects of iNOS activation in this model could be largely prevented by administration of a NOS inhibitor. Recent observations by our group (Calder *et al.,* 2000a) have demonstrated robust iNOS expression and staining in osteoblasts, osteoclasts, and in particular osteocytes in bone samples from osteonecrotic subjects. Interestingly, iNOS-positive cells correlated closely with the incidence of apoptotic cells and has lead to the somewhat controversial suggestion that osteocyte apoptosis (induced by NO), might in fact be the underlying mechanism of the extensive bone destruction seen under this condition (Calder *et al.,* 2000a).

The most common bone lesion is fracture and there is evidence to show that NO is involved in various stages of fracture healing. Consistent with the initial inflammatory phase of fracture healing Corbett *et al.* (1999a) demonstrated marked iNOS expression and activity within 24 hr after fracture in rat model where it was localized principally to endosteal osteoblasts. This expression was transient and after 24 hr iNOS was expression/activity was scarcely detectable. During this same period and during the first week after fracture there was a marked increase in expression and activity of (calcium-dependent) eNOS, which was localized, in particular, to cortical blood vessels and osteocytes (Corbett *et al.* 1999a). This is consistent with eNOS being involved in the vascular response and neovascularization that is crucial to successful fracture repair. Indeed, in a further study on the same model Corbett *et al.* (1999b) demonstrated enhanced NO-dependent vasoreactivity about the fracture site supporting a role for NO in the restoration of blood flow to the fractured bone. Subsequently, Diwan *et al.* (2000) have provided similar evidence for the involvement of NO in fracture healing. Of particular interest in that study was the finding that NOS inhibitors significantly impaired fracture healing, which could be reversed by local delivery of a NO donor.

Osteoporosis is an important abnormality because it predisposes to fracture. Although clearly a multifactorial disorder it is becoming increasingly apparent that disrupted NO synthesis is a significant factor in the bone loss associated with osteopenia. Estrogen-deplete osteoporosis induced by ovariectiomy in animal models can be largely reversed by administration of NO donor compounds, notably nitroglycerin (Wimalawansa *et al.,* 1996). This has now been extended to human clinical trial showing that nitroglycerin is equally as effective as hormone replacement therapy (HRT) in preventing or restoring postmenopausal bone loss (Wimalawansa *et al.,* 2000). Additional evidence for NO mediating the osteogenic effects of estrogen is provided by cyclic increases in plasma nitrite and nitrate levels (the oxidation production of NO synthesis) which are highest at the mid-phase of the menstrual cycle, closely following estrogen levels (Cicinelli *et al.,* 1996). Postmenopausal subjects and amenorrheic athletes have reduced serum NO metabolites, which can be elevated by HRT (Rosselli *et al.,* 1995; Cicinelli *et al.,* 1998; Stacey *et al.,* 1998). Finally, *in vitro* studies on rodents, including eNOS knockouts and human osteoblasts demonstrate that eNOS expression and activity is elevated by estrogen and correlates with increased osteoblast proliferation and differentiation (Armour and Ralston, 1998; O'Shaughnessy *et al.,* 2000; Aguirre *et al.,* 2001; Armour *et al.,* 2001).

THERAPEUTIC IMPLICATIONS

It has been clearly shown that NO has an estrogen-like effect and that nitric oxide can in part replace the effect of oestrogen (Wimalawansa *et al.,* 1996; Armour and Ralston, 1998; O'Shaughnessy *et al.,* 2000; Aguirre *et al.,* 2001; Armour *et al.,* 2001). Wimalawansa (2000) has also recently shown that a NO donor in man is as effective as estrogen in inhibiting postmenopausal bone loss. Clearly this has important therapeutic implications in the treatment and perhaps prevention of osteoporosis. Further, it strongly suggests that a NO donor in combination with an osteoclast inhibitor (e.g., calcitonin or bisphosponates) should be useful in the treatment of osteoporosis. Administration of NO donor compounds could also prove to be efficacious in augmenting fracture healing (Corbett *et al.,* 1999a,b; Diwan *et al.,* 2000). There are, however, potential limitations to the use of NO donors. Cellular desensitization following prolonged use of organo-nitrate compounds like nitroglycerin is a particular problem that has been encountered in the treatment of ischaemic heart disease and requires increased dosages to reproduce the beneficial effects.

Alternatives to NO donors include delivering compounds that preserve the signaling actions of messenger molecules downstream of NO synthesis such as cGMP. Indeed, phosphodiesterase (PDE) inhibitors are able to

increase bone mass by accelerating bone formation in mice (Kinoshita *et al.,* 2000). Consequently, PDE inhibitors may have some value in the treatment of osteoporosis. Other approaches for eliciting anabolic effects in the skeleton include the lipid lowering drugs statins, which have recently been found to stimulate new bone formation (Mundy *et al.,* 1999) and reduce the risk of hip fracture in elderly human subjects (Wang *et al.,* 2000). Interestingly, statins upregulate eNOS expression and downregulate iNOS expression (Vaughn and Delany, 1999) at least in brain tissue and may also account for some the osteoprotective effects of these compounds.

Elevated production of NO following iNOS activation is a significant factor in the progression of various inflammatory conditions including rheumatoid arthritis. Consequently, inhibition of iNOS activity appears to be an attractive target in tempering inflammation-induced tissue damage. Although there are several reports to show that when administered prophylactically NOS inhibitors (L-NAME, L-NMMA) were able to suppress the onset of disease in animal models (McCartney-Fancis *et al.,* 1993; Stefanovic-Racic *et al.,* 1994), not all iNOS activity is detrimental. Indeed iNOS does have protective effects such that NOS inhibitors can exacerbate tissue injury. In part this also relates to the fact that in virtually all the studies that have been performed using NOS inhibitors none of the compounds used are sufficiently selective in their action and can often block the beneficial effects of constitutive NO activity. Moreover, there is also evidence to suggest that some iNOS activity is important in normal bone physiology. Thus, while modulation of NOS activity has important therapeutic implication in bone biology and pathology there is still much research required before this becomes commonplace. Continued investigation of NOS gene knockouts, together with developments in the generation of more selective NOS inhibitors and NO donor compounds should provide more definitive information on the cellular targets and mechanisms of action of NO.

Conclusions

The predominant NOS isoforms expressed by bone tissues are eNOS and iNOS and both are present in osteoblasts, osteocytes, and osteoclasts. Synthesis of NO by these cells is stimulated by a variety of signals including hormones, cytokines, and mechanical loading resulting in number of distinct physiological and pathophysiogical responses (Figs. 10 and 11).

PHYSIOLOGY

1. At low concentrations possibly associated with eNOS activity NO stimulates osteoclast contraction and detachment from bone surfaces, and may promote bone resorption by regulating osteoclast movement
2. At higher concentrations, possibly associated with "constututive" iNOS activity, NO inhibits osteoclastic resorption possibly by inducing apoptotic cell death in osteoclast precursors and inhibiting acid phosphatase activity in mature cells.
3. eNOS-dependent NO generation stimulates osteoblast replication and differentiation experimentally. Similar effects are evident *in vivo* where NO facilitates fracture healing and is essential for stress-induced bone formation.
4. Perhaps most importantly NO is significant in mediating the osteogenic effects of estradiol.

PATHOPHYSIOLOGY

1. Disruption of eNOS activity, secondary to estrogen deficiency can contribute to reduction in bone formation and the development of osteoporosis.

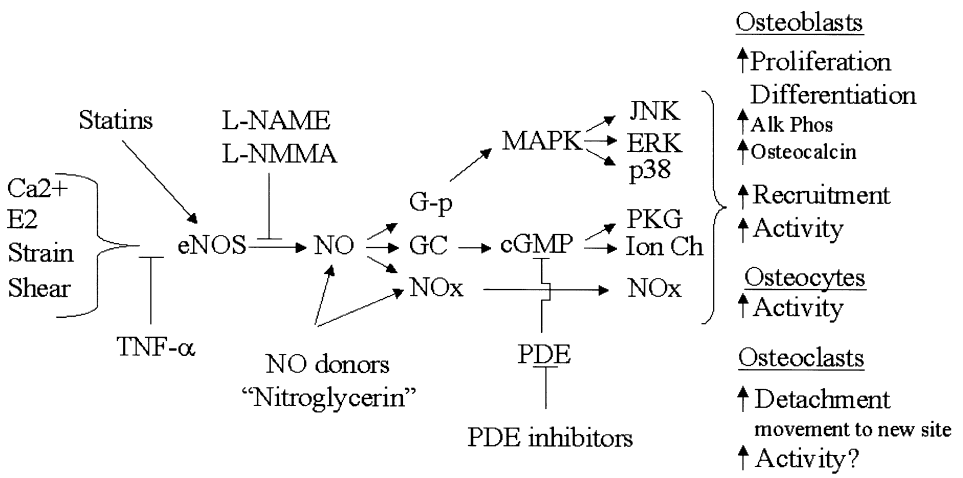

Figure 10 Diagrammatic overview of mechanism of eNOS activation and NO-mediated signaling pathways in bone cells. For detailed discussion refer to main body text. ERK, extracellular signal-regulated kinase; GC, guanylyl cyclase; G-p, G-proteins; Ion Ch, ion channel proteins; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NOx, nitric oxide related species; PKG, protein kinase G.

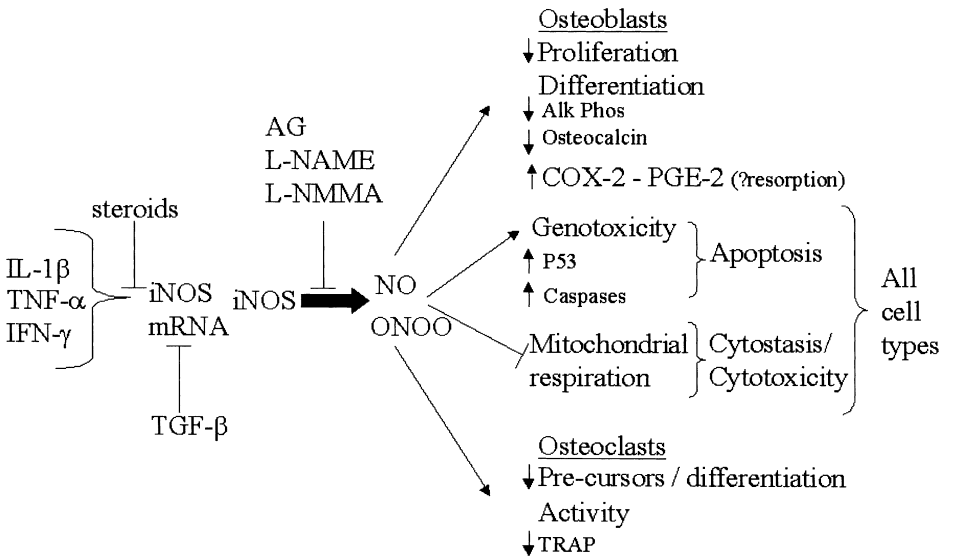

Figure 11 Diagrammatic overview of mechanism of iNOS activation and NO-mediated signaling pathways in bone cells. For detailed discussion refer to main body text. ERK, extracellular signal-regulated kinase; GC, guanylyl cyclase; G-p, G-proteins; Ion Ch, ion channel proteins; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PKG, protein kinase G.

2. Inflammation-induced activation of iNOS expression and activity is strongly associated with the development and progression of cartilage and bone lesions that characterize arthritis. *In vitro,* osteoblasts appear to be the predominant source of iNOS-derived NO inducing cell death in osteoblasts themselves, (osteocytes) and osteoclasts.

In summary, NO changes the balance of bone metabolism in favor of formation both by stimulating osteoblast replication differentiation and inhibiting osteoclast action. High concentrations of NO are likely to be damaging in diseases such as rheumatoid arthritis where iNOS is induced. Obviously with this wide range of functions there are possibilities of therapeutic intervention. However, this has so far not been adequately investigated. Thus, although a role for modulation of NO synthesis in the prevention and treatment of diseases like osteoporosis and arthritis or in the process of fracture healing seems feasible this and the other possibilities of therapeutic intervention remain to be exploited.

Other Vasoactive Agents

These include the members of the calcitonin family, the prostaglandins, endothelin, natriuretic peptides and VEGF. The calcitonin family all act as osteoclast inhibitors. Calcitonin is much the most potent while the other members of the group all share vasoactive actions and osteoclast inhibitory effects. Only calcitonin has been thoroughly studied for its action on bone and this is well described elsewhere. Briefly, calcitonin acts directly on the osteoclast to produce a marked inhibition primarily via activation of adenylate cyclase but also involving an increase in intracellular calcium. The prostaglandins have a complex but very important set of actions of bone and these are described elsewhere in this volume. Interestingly, NO is know to activate COX and increase prostaglandin synthesis (Salvemini *et al.,*1993) and may be important in bone cell function (Hughes *et al.,* 1999). Endothelin has not been studied extensively but has been reported to have an osteoclast inhibitory effect. (Alam *et al.,* 1992; Zaidi *et al.,*1993). The effects of endothelins on osteoblast function appear to be contradictory and species-specific. In rodent osteoblast cultures endothelins inhibit osteoblast differentiation (Hiruma *et al.,* 1998; Inoue *et al.,* 2000), whereas in human cultures endothelins promote osteoblast differentiation and mineralization (Kasperk *et al.,* 1996; Nelson *et al.,* 1999).

C-type natriuretic peptide is significant in the physiological control of endochindral ossification and in osteoblast proliferation differentiation (Suda *et al.,* 1996; Yasoda *et al.,* 1998; Mericq *et al.,* 2000; Inoue *et al.,* 2000). In some respects the actions of natriuretic peptide is not unlike those of NO. Finally, VEGF is an important vasoactive factor that plays a fundamental role in endochondral ossification (Gerber *et al.,* 1999).

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PART II

Molecular Mechanisms of Metabolic Bone Diseases

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CHAPTER 56

Molecular Basis of PTH Overexpression

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Introduction

Over the past several years application of powerful molecular biology techniques has provided a wealth of new information and insights into the development of tumors. The purpose of this chapter is to review this new information as it relates to our understanding of parathyroid tumorigenesis. Parathyroid hyperfunction is found in several disease states including sporadic primary and secondary hyperparathyroidism and familial disorders such as the multiple endocrine neoplasia (MEN) syndromes.

Primary hyperparathyroidism is a common disorder characterized by hypercalcemia caused by an excessive secretion of parathyroid hormone (PTH). This is due to both an increased parathyroid gland mass and a resetting of the control of PTH secretion from the parathyroid cell by the ambient calcium concentration. Patients with primary hyperparathyroidism have one or more enlarged parathyroid glands with a single, benign adenoma occurring in almost 85% of cases, while multiple hypercellular glands are present in about 15% of patients (Black and Utley, 1968; Castlemen and Roth, 1978). In modern series, parathyroid carcinoma occurs in less than 1% of cases, and the ectopic secretion of PTH from nonparathyroid tumors is extremely rare (Powell *et al.,* 1973; Simpson *et al.,* 1983).

The refractory state of secondary hyperparathyroidism, as seen, for example, in patients with uremia, and tertiary hyperparathyroidism (Galbraith and Quarles, 1994) are characterized by hyperfunctioning parathyroid tissue that no longer responds appropriately to physiological regulators such as ambient calcium and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$).

Hyperparathyroidism may also occur as part of familial syndromes, such as multiple endocrine neoplasia types 1 and 2 (MEN 1 and 2), the hereditary hyperparathyroidism and jaw tumor (HPT-JT) syndrome, and familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT).

Molecular Oncology

Cancer cells contain genetic damage to key growth-regulating genes that directly contributes to the abnormal neoplastic phenotype. A cardinal feature of cancers is their clonal, or monoclonal, nature. They arise from a single precursor cell which has a selective growth advantage over normal cells and whose progeny outgrow them and ultimately make up the tumor (Fig. 1, see also color plate). Typically, certain identical patterns of DNA damage are seen in each cell of such a tumor, indicating that important underlying genetic events occurred early before major proliferation or clonal expansion took place. The clonality of tumors also implies that these

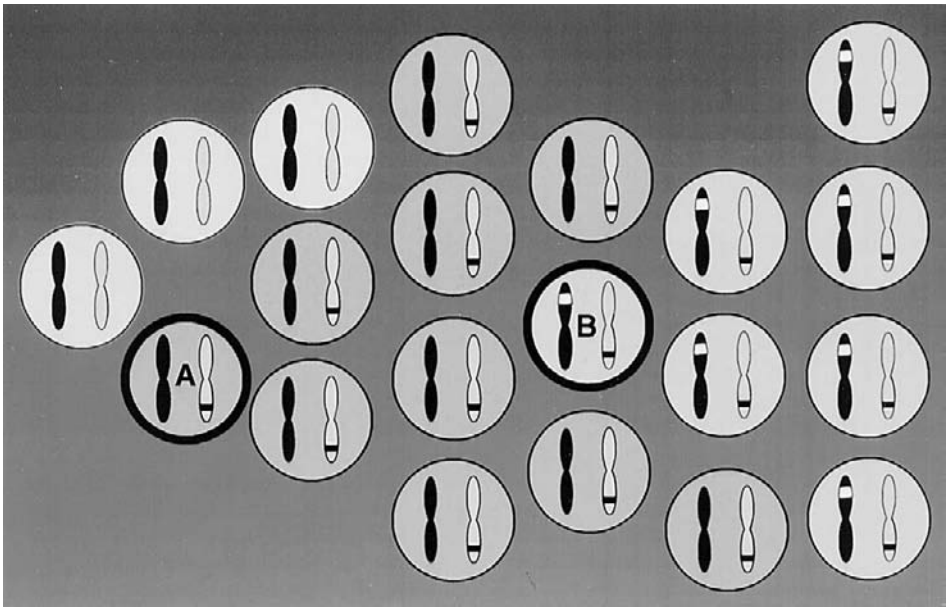

Figure 1 Schematic representation of early genetic events occurring in the development of a clonal tumor. In cell A, DNA damage has occurred to a set of key regulatory genes (one is shown here), which together confer a selective growth advantage to its progeny of identical daughter cells. In clonal precursor cell B, an additional oncogenic "hit" occurs, affecting a different gene, conferring a further growth advantage on its progeny which then overgrow all other cells to form the eventual clonal tumor; this represents the process of clonal evolution. (See also color plate)

events occur only rarely in the large population of cells making up a tissue.

It is important to note the molecular heterogeneity underlying the development of neoplasia (Vogelstein and Kinzler, 1993). Generally, accumulating damage to several distinct genes, within the same cell, is required for the expression of the complete neoplastic phenotype. While certain genes are implicated in tumors of only one or a few cell types, other genes may be involved in many different types of tumor. However, in most cases, no single gene will be both a necessary and a sufficient neoplastic agent. More commonly, the emergence of a particular tumor type may relate to disruption of a specific biochemical pathway, which can be achieved by different combinations of mutated genes resulting in similar cellular and clinical consequences.

Clonal DNA damage in two groups of normal cellular genes contributes to the development of neoplasia. These are protooncogenes and tumor suppressor genes. Protooncogenes are often involved in the physiological control of cellular growth, proliferation, or differentiation. Conversion of a protooncogene to an "oncogene" is caused by a deregulation of the expression of its protein product or by formation of an intrinsically abnormal product. The products of tumor-suppressor genes normally restrain cellular proliferation and their gene inactivation contributes to neoplasia. Protooncogenes can be activated by a variety of mechanisms, including chromosome translocations or inversions, point mutations, proviral insertions, or gene amplification. Inactivation of tumor-suppressor genes can occur by point mutation or deletion, for example.

Clonality of Parathyroid Tumors

Early studies of the clonal status of parathyroid tumors assessed X-chromosome inactivation patterns by measuring isoforms of glucose-6-phosphate dehydrogenase in parathyroid adenomas of women heterozygous for the polymorphism. These studies produced the surprising finding that parathyroid adenomas were polyclonal as opposed to monoclonal growths (Fialkow *et al.,* 1977; Jackson *et al.,* 1982). However, several years later the issue of the clonal status of parathyroid tumors was reexamined using an X-chromosome inactivation method, but this time evaluating DNA polymorphisms, which avoided potential pitfalls in the protein approach, and by the direct demonstration of monoclonal DNA alterations in parathyroid adenomas (Arnold *et al.,* 1988). It was determined that most, if not all parathyroid adenomas are in fact monoclonal (Arnold *et al.,* 1988; Arnold and Kim, 1989; Friedman *et al.,* 1989; Bystrom *et al.,* 1990; Orndal *et al.,* 1990), emphasizing that they are true neoplastic outgrowths of a single abnormal cell. This is consistent with the general experience that surgical removal of such tumors is curative of the disease. As would be expected, parathyroid carcinomas are also monoclonal (Cryns *et al.,* 1994b). In addition, monoclonal parathyroid tumors are seen in familial MEN 1 (Friedman *et al.,* 1989; Thakker *et al.,* 1989; Bystrom *et al.,* 1990), in nonfamilial, sporadic, primary parathyroid hyperplasia (Arnold *et al.,* 1995), and in the refractory secondary or tertiary parathyroid hyperplasia of uremia (Arnold *et al.,* 1995; Falchetti *et al.,* 1993). Therefore, even in parathyroid hyperplasia, which begins with a stimulus for

generalized, polyclonal, parathyroid cell proliferation affecting all of a patient's glands, monoclonal tumors can arise, at least in some of the glands. Such tumors may be more autonomous, and exhibit a more marked dysregulation of PTH secretory control than the hyperplastic, polyclonal glands within the same patient.

An important goal is to identify the specific protooncogenes and tumor suppressor genes that are clonally activated or inactivated, respectively, in parathyroid tumors. Several successes in approaching this aim have already been achieved.

Genetic Derangements in Benign Parathyroid Tumors

DNA rearrangements are some of the best characterized clonal oncogenic abnormalities, and they frequently involve juxtaposition of cellular protooncogenes with regulatory sequences of other genes. This then results in overexpression or deregulated expression of the protooncogene, converting it to an oncogene. To date the only oncogene implicated in parathyroid neoplasia is cyclin D1/*PRAD1*.

Cyclin D1/*PRAD1*

The critical observation in the identification of *PRAD1* was of an abnormally sized band on a Southern blot of DNA from a parathyroid adenoma probed with a PTH genomic fragment (Arnold *et al.,* 1988). A tumor-specific DNA rearrangement separated the 5′ regulatory region and noncoding exon 1 of the *PTH* gene from its protein coding exons with the tumor cells possessing one intact *PTH* gene which accounted for expression of PTH by the tumor. The non-*PTH* gene sequences adjacent to the breakpoint were cloned from a genomic DNA library made from this particular adenoma (Arnold *et al.,* 1989). This sequence was mapped by somatic cell genetics and *in situ* hybridization using normal genomic DNA to chromosome band 11*q*13, the *PTH* gene being located at 11*p*15. Thus, the most likely explanation for the observed tumor-specific DNA rearrangement was a pericentromeric inversion of chromosome 11 (Fig. 2). By Northern blot analysis, the novel DNA hybridized to a distinct 4.5-kb transcript which was present in RNA from a variety of tissues—it was not specific for the parathyroid—however, it was markedly overexpressed in the original parathyroid adenoma from which the DNA had been cloned, relative to other parathyroid tissue (Motokura *et al.,* 1991; Rosenberg *et al.,* 1991). This sequence, which was highly conserved among species, was considered as a putative oncogene, and designated *PRAD1,* for parathyroid adenomatosis 1.

The normal chromosomal *PRAD1* gene contains five exons and four introns spanning approximately 15 kb and is transcribed in a centromeric to telomeric direction (Motokura and Arnold, 1993). In the cases of PTH/*PRAD1* rearrangements characterized to date, the 11*q*13 breakpoints have occurred from 1 to 15 kb upstream of *PRAD1* exon 1 with the *PTH* gene 5′ regulatory region and noncoding exon 1 placed upstream of the breakpoint (Friedman *et al.,* 1990; Rosenberg *et al.,* 1991) (Fig. 3). The overexpression of the *PRAD1* gene is thought to be caused by its aberrant placement in close proximity to the strong tissue-specific enhancer elements of the *PTH* gene (Rosenberg *et al.,* 1993).

At least 5% of parathyroid adenomas contain an activated form of the *PRAD1* oncogene. However, rearrangement breakpoints on 11*q*13 associated with overexpression

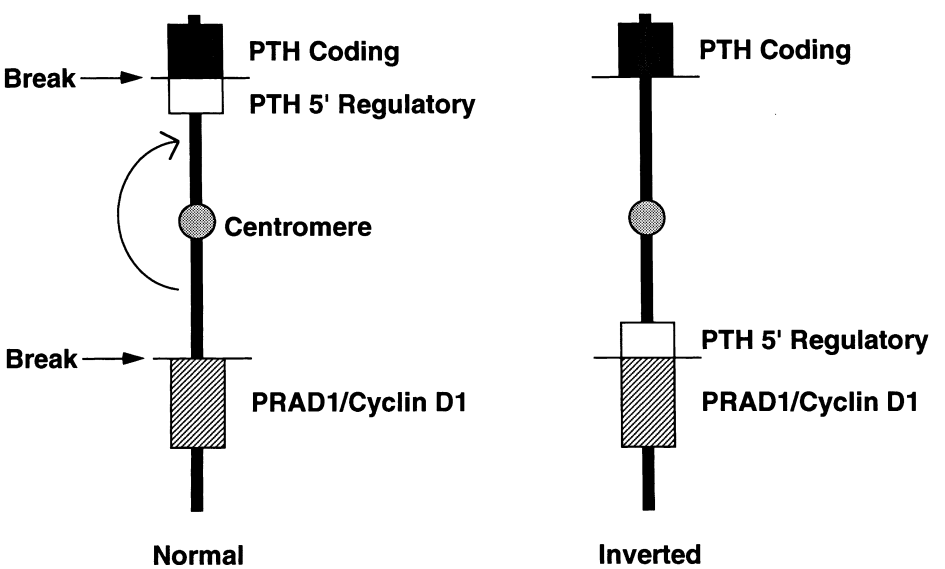

Figure 2 Schematic diagram of rearrangement of chromosome 11 in parathyroid tumors. In a subset of parathyroid adenomas a pericentrometric inversion of chromosome 11 is the most likely cause of the observed rearrangement involving the *PTH* gene and the *PRAD1* gene. Each tumor has another copy of chromosome 11 which bears a normal *PTH* gene. Reproduced with permission from Arnold (1993).

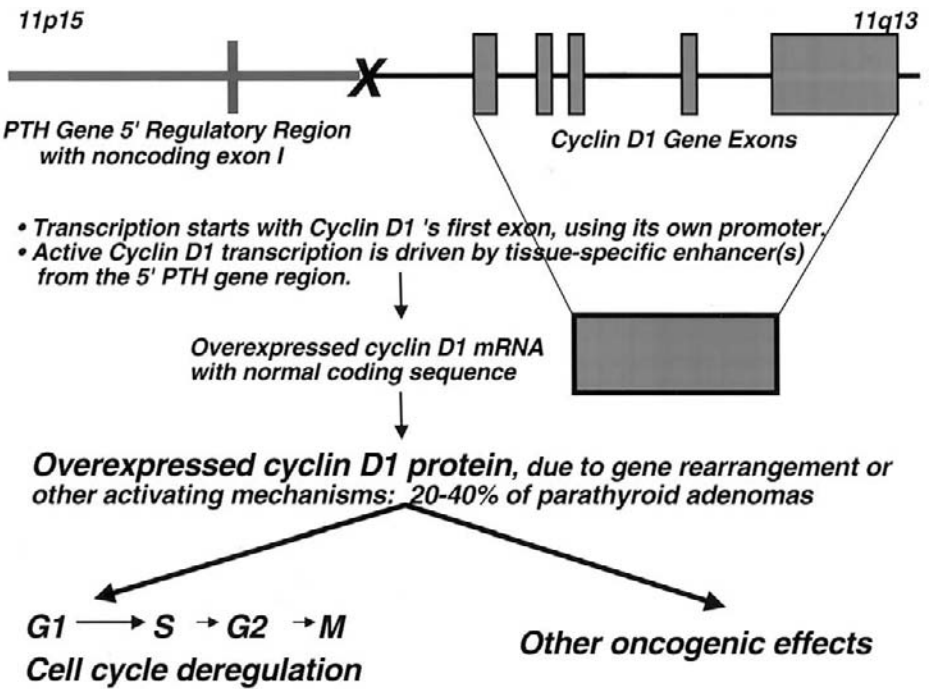

Figure 3 Diagram of directly observed molecular structure of the *PTH/cyclin D1* (*PRAD1*) DNA rearrangement and its functional consequences. Modified with permission from Arnold (1993).

of the *PRAD1* gene in other tumors can occur more than 120 kb upstream of the *PRAD1* gene (Williams *et al.,* 1993; Vaandrager *et al.,* 1996, 1997). These types of rearrangement would have been missed by the Southern blot approach used in initial studies of parathyroid tumors. In addition, PRAD1 expression could be deregulated in some parathyroid tumors by rearrangement with genes other than the *PTH* gene. Therefore, previous studies may not have revealed the full frequency with which *PRAD1* deregulation occurs in parathyroid neoplasia. Recent studies assessing the PRAD1 protein product have indicated that *PRAD1* is in fact overexpressed in 20–40% of parathyroid adenomas (Hsi *et al.,* 1995; Vasef *et al.,* 1999; Tominaga *et al.,* 1999). This may occur more commonly therefore by a *trans*-acting regulatory disturbance like that observed for *PRAD1* overexpression in a variety of human cancer cells (Hosokawa and Arnold, 1998) rather than a clonal mutation in one gene allele.

When the normal cDNA for *PRAD1* was cloned from a human placental cDNA library (Motokura *et al.,* 1991), it was revealed to encode a 295-amino-acid protein which was homologous to various members of the cyclin class of proteins. Cyclins play important roles in regulation of cell cycle progression, and human cyclins have been grouped into several types based upon sequence similarities (Pestell *et al.,* 1999). Each cyclin appears to regulate the cell cycle at a specific time point by binding to and activating cyclin-dependent kinases (CDKs) (Sherr, 1996). Expression of these cyclins is cell cycle phase-dependent and controlled by both transcriptional and posttranscriptional mechanisms.

The *PRAD1* product was designated as cyclin D1, and a large body of evidence is consistent with its role as a G1 cyclin (Sherr, 1996). As such, cyclin D1 can function as a key regulator of the critical G1–S phase transition in the cell cycle. In so doing, its major cdk partners are cdk4 or cdk6, depending upon the tissue type examined. It may also be that cyclin D1 has other cellular functions, mediated through non-cdk-dependent pathways (Bernards, 1999).

An important biochemical pathway in which D-type cyclins participate involves the protein product of the retinoblastoma tumor suppressor gene *RB*. The RB protein (pRB) is a key regulator of the G1–S transition in the cell cycle and is phosphorylated in a cell-cycle-dependent fashion (Weinberg, 1995). In early G1, pRB is hypophosphorylated and in this state is able to bind and inhibit the activity of transcription factors such as E2F. Later in G1, pRB becomes hyperphosphorylated and releases these factors which then drive the cell into S phase. Cyclin D1/cdk4 appears to be an important contributor to the early phosphorylation of pRB. For parathyroid tissue in particular, however, the major cdk (or other) partner and preferred substrates for cyclin D1/cdk activity remain to be determined. In benign tumors such as parathyroid adenomas, overexpression or deregulated expression of a G1 cyclin such as cyclin D1 could accelerate the cell's progress through G1 into S phase or move cells from quiescence (G0) into cycling mode, tilting the balance toward increased cellular proliferation (Fig. 3) without necessarily conferring a full malignant phenotype.

Animal modeling has provided further evidence that cyclin D1 overexpression is capable of driving parathyroid tumorigenesis. A transgenic mouse model for parathyroid

neoplasia has been generated (Imanishi *et al.,* 2001). The mice harbor a transgene in which the cyclin D1 gene is under the control of the regulatory region of the *PTH* gene, mimicking the rearrangement and resultant overexpression observed in the human tumors. These mice develop hyperparathyroidism as evidenced by parathyroid enlargement and increased serum calcium and PTH levels and constitute a potentially useful model of human mild chronic hyperparathyroidism.

Tumor Suppressor Genes: *MEN1*

Inactivation of both alleles of a tumor suppressor gene, often by mutation or deletion, is required to completely deplete the gene's antineoplastic product. A common inactivation mechanism is somatic deletion of a substantial portion of chromosomal DNA that includes the relevant gene. This is revealed by a loss of heterozygosity of DNA markers in tumor DNA relative to normal DNA of the same individual. Identification of regions of chromosomes that are clonally and nonrandomly lost in parathyroid adenomas can indicate the general locations of tumor suppressor genes active in parathyroid cells. Our present state of knowledge in this regard shows that over 70% of parathyroid adenomas (and possibly all) have at least one such clonal defect.

The MEN1 syndrome, which is inherited in an autosomal dominant fashion, is characterized classically by tumors of the parathyroids, pancreatic islets and anterior pituitary. Several years ago, it was established by genetic mapping studies in families affected by MEN1, that the gene responsible is on chromosome 11*q*13 (Larsson *et al.,* 1988; Friedman *et al.,* 1989; Thakker *et al.,* 1989). By analogy with familial retinoblastoma and Li-Fraumeni syndromes, which involve inheritance of mutations in the *RB* and *p53* genes, it was suggested that the *MEN1* gene was a tumor suppressor gene. Early evidence for this was provided by the demonstration of somatic genetic alterations in MEN1 tumors which inactivate one allele of a gene region at 11*q*13 and so reveal the inherited MEN1 mutation on the other allele (Fig. 4). In fact, allelic loss of polymorphic marker DNAs from this region of chromosome 11 has been found in the majority of MEN1-associated tumors including those of the parathyroid (Larsson *et al.,* 1988; Friedman *et al.,* 1989, 1992; Bystrom *et al.,* 1990; Thakker *et al.,* 1989; Radford *et al.,* 1990).

Allelic loss of chromosome 11 markers also occurs in about 25–35% of the large number of sporadic parathyroid adenomas that have been examined (Bystrom *et al.,* 1990; Friedman *et al.,* 1992; Tahara *et al.,* 1996a). This allelic loss, usually but not invariably, involves the region to which the *MEN1* gene has been mapped. By analogy to the *RB* tumor suppressor gene model in which the sporadic, nonfamilial, counterpart of the retinoblastoma tumor results from somatic genetic events, leading to loss or inactivation of both normal *RB* gene alleles (see Fig. 4), these findings suggested that some sporadic parathyroid adenomas may evolve from a clonal precursor cell in which both copies of

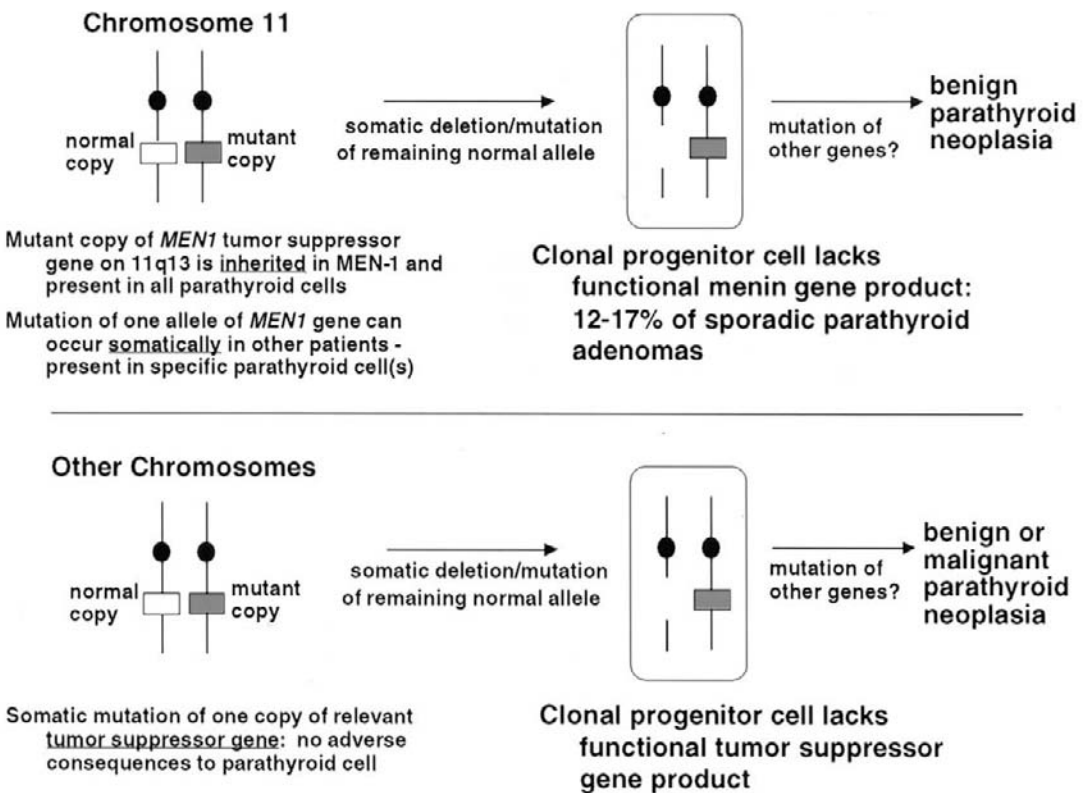

Figure 4 Schematic diagram illustrating the established (for *MEN1*) and hypothesized roles of inactivation of classic tumor suppressor genes as contributory mechanisms in parathyroid neoplasia. Modified with permission from Arnold (1993).

the *MEN1* gene have been inactivated. The recent identification of the *MEN1* gene (see below) has permitted a more direct examination of this hypothesis. Somatic mutation and/or deletion of *MEN1,* resulting in its complete inactivation, has been documented in 12–17% of sporadic parathyroid adenomas (Heppner *et al.,* 1997; Carling *et al.,* 1998; Farnebo *et al.,* 1998a). Interestingly, this 12–17% constitutes only about half of the parathyroid adenomas with allelic losses of 11*q*13. While noncoding mutations in *MEN1,* which would have been missed in these analyses, might be the explanation for some or all of this gap, the magnitude of the discrepancy when compared with other tumor suppressors raises the possibility that a different tumor suppressor gene on chromosome 11 might be the relevant target of mutation in many of the parathyroid adenomas with 11*q* allelic losses.

The *MEN1* gene was identified by positional cloning (Chadrasekharappa *et al.,* 1997; European Consortium on MEN1, 1997). Over 250 independent germ-line and somatic mutations scattered throughout the protein coding region have been identified (see Marx *et al.,* 1999; Pannett and Thakker, 1999, and references therein). There is no correlation between genotype, meaning the specific location of mutation within the gene, and phenotype. Somatic mutations have been found to a variable extent in parathyroid adenoma as well gastrinoma, insulinoma, lung carcinoid, and anterior pituitary tumors. Many of the mutations are clearly inactivating, leading to a truncated product. This would be consistent with *MEN1* acting as a tumor suppressor gene and a lack of the menin protein caused by the loss of both alleles leading to tumor development. The human gene encodes a 610-amino-acid protein with homology to no known protein nor any obvious conserved motifs that would provide clues to its function. Two nuclear localization signal sequences have now been identified at the COOH-terminal portion of the menin protein, which is predominantly located in the nucleus (Guru *et al.,* 1998; Kaji *et al.,* 1999). Menin interacts with the activator protein 1 factor, JunD, and represses JunD-activated transcription (Agarwal *et al.,* 1999). JunD can be considered to be antimitogenic, in contrast to other Jun and Fos family members. Therefore, the repressive effect of menin on JunD-mediated transcriptional activation is paradoxical, and the physiologic or pathophysiologic significance of menin and JunD binding has not been established. The level of menin changes throughout the cell cycle. Pituitary cells synchronized at the G1-S-phase boundary express menin at a lower level than G0–G1-synchronized cells (Kaji *et al.,* 1999). The expression of menin increases as the cell enters S phase, at which time JunD expression also increases. Cells synchronized at the G2–M phase express lower levels of menin. Thus, the emerging knowledge of menin suggests that it functions in transcriptional regulation, DNA replication, or cell cycle control. Stable overexpression of menin in *ras*-transformed NIH3T3 cells inhibits cell growth and tumor formation in nude mice, providing some direct evidence of menin acting as a tumor suppressor (Kim *et al.,* 1999).

A mechanism of tumorigenesis by menin inactivation is suggested by the finding that menin antisense RNA antagonizes TGF-β-mediated cell growth inhibition. Menin interacts with Smad3 and its inactivation suppresses TGF-β- and Smad3-induced transcriptional activity (Kaji *et al.,* 2001).

Putative Oncogenes and Tumor Suppressor Genes

Comparative genomic hybridization—a molecular cytogenetic technique in which the entire tumor genome is screened for chromosomal gains and/or losses—has identified amplified regions on several chromosomes (most consistently 7, 16, and 19) (Fig. 5). These observations suggest the presence of novel parathyroid oncogenes (Palanisamy *et al.,* 1998; Agarwal *et al.,* 1998; Farnebo *et al.,* 1999) in these locations, which remain to be identified.

Besides loci on chromosome 11, several other regions of nonrandom clonal allelic loss have been documented in parathyroid adenomas, pointing to the location of novel tumor suppressor genes. This finding of the involvement of multiple chromosomal regions, which include 1*p* (Cryns *et al.,* 1995a; Williamson *et al.,* 1997), 1*q* (Cryns *et al.,* 1995b), 6*q,* 9*p,* and 15*q* (Tahara *et al.,* 1996a,b), emphasize the molecular heterogeneity of parathyroid adenomatosis.

Comparative genomic hybridization has confirmed the chromosomal losses identified by loss of heterozygosity analysis (allelotyping) (Palanisamy *et al.,* 1998; Agarwal *et al.,* 1998; Farnebo *et al.,* 1999) (Fig. 5, see also color plate). One common defect uncovered to date involves allelic loss on chromosome 1*p* (Cryns *et al.,* 1995a). Interestingly, chromosome 1*p* loss occurs in several other neoplasms including medullary thyroid carcinomas and pheochromocytomas (Mathew *et al.,* 1987; Khosla *et al.,* 1991; Moley *et al.,* 1992; Mulligan *et al.,* 1993a), which occur in association with the MEN2A inherited cancer syndrome, of which parathyroid tumors are also a part. Benign parathyroid tumors are found in 10–20% of MEN2A patients (Schimke, 1984; Gagel, 1998). This pattern of allelic loss is consistent with the existence of a tumor suppressor gene(s) on 1*p,* whose loss or inactivation is pathogenetically important. Numerous candidate genes are present in this region, the involvement of any of them in the development of parathyroid or other types of tumors remains to be established. Some candidates on 1*p,* such as genes for the *p*18 cyclin-dependent kinase inhibitor, and *RAD54* have been excluded as parathyroid tumor suppressors (Tahara *et al.,* 1997; Carling *et al.,* 1999a). Candidate genes within the 9*p* region for the *p*16 and *p*15 cyclin-dependent kinase inhibitors do not appear to be involved in parathyroid tumorigenesis (Tahara *et al.,* 1996b). A major goal for future work is the identification of the entire constellation of oncogenes and tumor suppressor genes that contribute to the development of parathyroid adenomatosis.

It had been assumed that primary parathyroid hyperplasia and uremic refractory secondary hyperparathyroidism involved polyclonal, nonneoplastic cellular proliferation. However, the monoclonal nature of a substantial number of

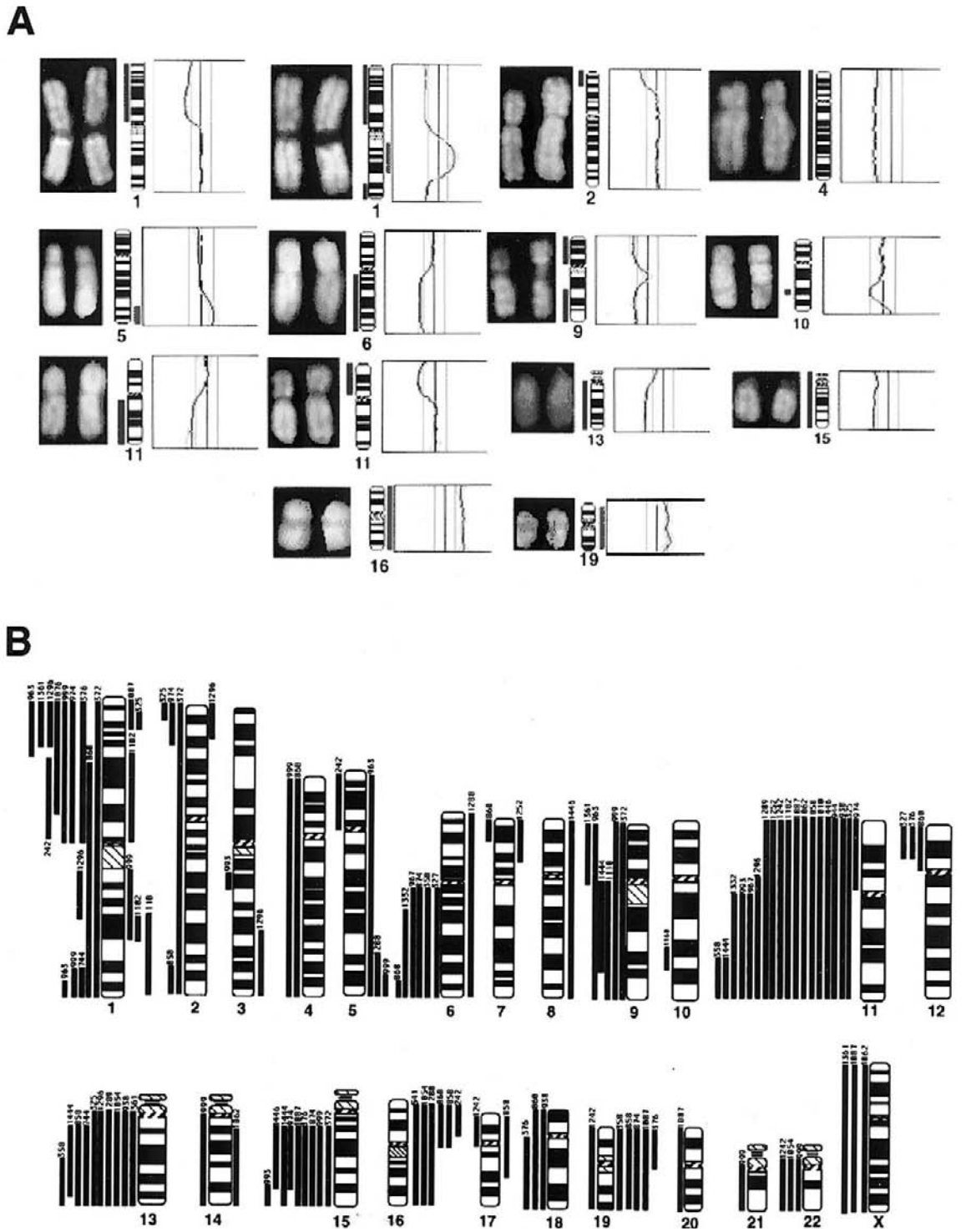

Figure 5 Novel chromosomal abnormalities identified by comparative genomic hybridization (CGH) in parathyroid adenomas. (A) Representative CGH results in parathyroid adenomas. Individual examples of fluorescent ratio profiles (right) and digital images (left) of chromosomes with recurrent gains or losses. The red vertical bar on the left side of a chromosome ideogram (middle) indicates the region of loss and the green vertical bar on the right side of an ideogram indicates the region of gain. (B) DNA copy number changes in 53 parathyroid adenomas. Summary of all gains and losses detected by CGH. The vertical bars on the left side of the chromosome ideograms indicate losses and those on the right side indicate gains of the corresponding chromosomal region for each individual tumor, as numbered. Adapted with permission from Palanisamy *et al.* (1998). (See also color plate.)

the "hyperplastic" tumors from such patients has recently been demonstrated to be monoclonal by X-chromosome inactivation analysis (Arnold *et al.,* 1995). In addition, allelic loss of chromosome 11 markers and/or *MEN1* gene inactivation has been demonstrated in only a few of these types of tumor (Falchetti *et al.,* 1993; Arnold *et al.,* 1995; Farnebo *et al.,* 1997a; Inagaki *et al.,* 1998; Imanishi *et al.,* 1997, 1999a). Furthermore, cyclin D1 overexpression does not appear to be common in these uremia-associated tumors (Vasef *et al.,* 1999; Tominaga *et al.,* 1999), nor is mutation of the vitamin D receptor (VDR) gene, despite the reportedly low expression of this parathyroid cell antiproliferative factor in such tumors (Brown *et al.,* 2000). Neoplastic transformation of preexisting polyclonal hyperplasia involving genes not yet implicated in parathyroid tumorigenesis is thus likely to play an important role in these disorders.

Further Genetic Aspects

Some genes which play an important role in the pathogenesis of other tumor types might have been expected to be involved in the pathogenesis of benign parathyroid adenomas. However, candidate genes such as *ras* (Friedman *et al.,* 1990), *p53* (Yoshimoto *et al.,* 1992; Cryns *et al.,* 1994a; Hakim and Levine, 1994), and *RB* (Cryns *et al.,* 1994b) seem rarely, or not at all, to contribute to the development of benign adenomas. In a single parathyroid adenoma, a cytogenetic translocation between chromosomes 1 and 5 has been reported (Orndal *et al.,* 1990).

The development of primary hyperparathyroidism occurs with increased frequency in individuals exposed to ionizing radiation of the neck. Thus mutations in genes involved in DNA repair and recombination may contribute to parathyroid tumorigenesis irrespective of the actual involvement of irradiation. Such candidates include the *RAD51* and *RAD54* genes on chromosomes 15*q* and 1*p,* respectively, within regions that demonstrate allelic loss in parathyroid adenomas. However, no evidence for a role of somatic inactivation of these particular genes in parathyroid neoplasia has been found (Carling *et al.,* 1999a,b). In some cases, those genes responsible for the rare inherited predisposition to a particular tumor also play a role in the development of the more common sporadic type. The *MEN1* gene, as detailed earlier, has proved to be such an example. Germline gain-of-function mutations in the *RET* protooncogene cause MEN2 (Mulligan *et al.,* 1993b, 1994; Donis-Keller *et al.,* 1993; Carlson *et al.,* 1993; Eng *et al.,* 1996). This made the *RET* gene, which encodes a tyrosine kinase receptor, a candidate for involvement in nonfamilial hyperparathyroidism. However, although MEN2-type *RET* mutations have been implicated in the pathogenesis of some sporadic medullary thyroid carcinomas and pheochromocytomas (Eng *et al.,* 1994, 1995a, 1996; Hofstra *et al.,* 1994), there is no evidence of these *RET* mutations in sporadic parathyroid adenomas (Padberg *et al.,* 1995; Pausova *et al.,* 1996; Williams *et al.,* 1996; Komminoth *et al.,* 1996; Romei *et al.,* 1996; Kimura *et al.,* 1996). It was demonstrated that *RET* is expressed in both MEN2A parathyroid tumors and in sporadic adenomas (Pausova *et al.,* 1995, 1996; Kimura *et al.,* 1996). This suggests that parathyroid disease is an integral part of the MEN2A syndrome, but that MEN2 mutations in *RET* rarely, if ever, play a role in the pathogenesis of sporadic parathyroid tumors. Mutations within the coding region of glial cell-derived neurotrophic factor (GDNF), one of the RET ligands, do not appear to play a role in the genesis of MEN2 neoplasms or in sporadic neuroendocrine tumors such as parathyroid adenomas (Marsh *et al.,* 1997). An interesting candidate parathyroid tumor suppressor, the VDR, is also lacking in evidence for a direct pathogenetic role by specific clonal mutation (Wu *et al.,* 1996; Brown *et al.,* 2000).

Familial hypocalciuric hypercalcemia (FHH), also known as familial benign hypercalcemia, is an autosomal dominant disorder which is characterized by enhanced parathyroid function due to reduced sensitivity to extracellular calcium (Marx *et al.,* 1981; Law *et al.,* 1985). Mutations in the parathyroid calcium-sensing receptor (*CASR*) gene, located on chromosome 3*q*13.3–*q*21 (Janicic *et al.,* 1995a), are a primary cause of this disorder (Pollak *et al.,* 1993; Heath *et al.,* 1996; Janicic *et al.,* 1995b, Pearce *et al.,* 1995; see Hendy *et al.,* 2000 for review). Moreover individuals homozygous for such mutations present with neonatal severe hyperparathyroidism (NSHPT) with marked parathyroid hypercellularity (Pollak *et al.,* 1994) and members of some FHH kindreds atypically manifest hyperparathyroidism and surgical removal of the adenoma or hyperplastic glands is generally curative (Soie *et al.,* 1999; Carling *et al.,* 2000). The apparent link between parathyroid calcium-sensing and proliferative pathways suggested that somatic alterations in the *CASR* gene could be tumorigenic in sporadic parathyroid tumors. However, several studies have failed to document somatic mutation of the *CASR* gene as a significant factor in parathyroid tumorigenesis (Hosokawa *et al.,* 1995; Thompson *et al.,* 1995; Degenhardt *et al.,* 1998; Cetani *et al.,* 1999). It has been reported that more than half of the parathyroid glands of patients with primary and severe uremic secondary hyperparathyroidism show reduced CASR expression (Kifor *et al.,* 1995; Farnebo *et al.,* 1997b, 1998b; Gogusev *et al.,* 1997; Chikatsu *et al.,* 2000). Thus, despite somatic mutation of the *CASR* gene rarely if ever contributing to the pathogenesis of sporadic parathyroid tumors, mutations in growth deregulating genes may secondarily alter the calcium set-point, perhaps in part by decreasing expression of the CASR. A mutation in a gene involved in calcium set-point control might secondarily stimulate proliferation until the serum calcium concentration surpasses the abnormal set-point (Parfitt *et al.,* 1994). Data indicating that the growth rate of parathyroid tumors is generally low, but must have been higher earlier in their development (Parfitt *et al.,* 1994), are consistent with this hypothesis. Interestingly, calcium regulatory abnormalities found in transgenic mice with cyclin D1-driven hyperparathyroidism show that mutations in "set-point" genes need not be the primary instigators in this disease (Imanishi *et al.,* 2001).

The syndrome of hereditary hyperparathyroidism and jaw tumors (HPT-JT) is an uncommon autosomal dominant disorder characterized by recurrent parathyroid tumors and mandibular or maxillary tumors (and occasionally by renal lesions such as Wilms tumor, polycystic kidney disease, and renal hamartomas). Parathyroid carcinoma occurs at increased frequency in this syndrome. The gene responsible for this predisposition has been mapped to chromosome 1*q*21–*q*31 (Szabo *et al.,* 1995) and in more recent linkage studies the interval has been narrowed further (Hobbs *et al.,* 1999). Ultimate identification of this gene may possibly provide insight into the etiology of the more common forms of hyperparathyroidism. Familial hyperparathyroidism also occurs as an isolated endocrinopathy and in some cases could represent a variant of HPT-JT. The distinction between HPT-JT and familial isolated hyperparathyroidism (FIHP) may be difficult (Wassif *et al.,* 1999). In a subset of kindreds with apparent FIHP the disease mapped to the HPT-JT locus (Teh *et al.,* 1998a; Williamson *et al.,* 1999). The parathyroid tumors were single cystic adenomas with allelic loss of chromosome 1*q* markers. These cases were therefore suggested to be variants of HPT-JT with expression of hyperparathyroidism only. Loss of heterozygosity for markers on chromosome 13*q* has also been documented for some parathyroid tumors (adenomas and carcinomas) in FIHP (Yoshimoto *et al.,* 1998; Williamson *et al.,* 1999). Thus a tumor suppressor gene on 13*q* may be involved in the development of these tumors. Comparative genomic hybridization has been applied to a limited number of tumors from familial hyperparathyroid patients (Farnebo *et al.,* 1999). The tumors showed few alterations, but in one HPT-JT case loss of chromosome 13*q* was found and in three other cases not known to be linked to either the HPT-JT or MEN1 loci gain of chromosome 19*p* was noted.

Familial hyperparathyroidism occurring as an isolated endocrinopathy in some cases appears to represent a variant of MEN1. Such families may be considered as MEN1 phenocopies analogous to the subset of MEN2 cases represented as familial medullary thyroid carcinoma (FMTC) which, like other MEN2 cases, have RET mutations (Eng *et al.,* 1996). In those FIHP families linkage to, or tumor allelic loss of, 11*q*13 markers was found (Kassem *et al.,* 1994, 2000; Teh *et al.,* 1998a). The disease was multiglandular with no sex difference in penetrance. In these families novel missense mutations in the *MEN1* gene were later identified. Several families with isolated hyperparathyroidism have now been tested for *MEN1* germline mutations and in some mutations were found and not in others (Agarwal *et al.,* 1997; and reviewed in Marx *et al.,* 1999). The existence of familial hyperparathyroidism distinct from other described inherited disorders remains the subject of debate. However, one kindred has been described with apparent autosomal recessive inheritance of the hyperparathyroidism and large recurrent adenomas (Law *et al.,* 1983). The mode of inheritance in this family would set it apart from the other autosomal dominant disorders, although it may prove with further study, to be autosomal dominant with decreased penetrance.

Molecular Pathogenesis of Parathyroid Carcinoma

The cyclin D1 oncoprotein, overexpressed in 20–40% of adenomas, was found to be overexpressed in 10 of 11 (91%) parathyroid carcinomas in one study (Vasef *et al.,* 1999) and in 2 of 3 in another (Hsi *et al.,* 1996). This observation, which should be confirmed and extended, raises the possibility that cyclin D1 may play a critical and consistent role in parathyroid cancers, and that patients with this disease might be considered for eventual inclusion in clinical trials once novel anti-cyclin D1 therapies have been developed. The tumor suppressor genes *p53* and *RB* have been examined for abnormalities in malignant, as well as benign, parathyroid tumors. Allelic loss of the *p53* gene has been found occasionally in parathyroid carcinomas but direct mutations have not been described, and *p53*′s overall contribution to parathyroid cancer is minimal at best (Cryns *et al.,* 1994a; Hakim and Levine, 1994). In contrast, loss of chromosome region 13*q* which contains *RB, BRCA2,* and other potential parathyroid tumor suppressors, is likely to be a key factor in the pathogenesis of many parathyroid carcinomas (Cryns *et al.,* 1994b; Dotzenrath *et al.,* 1996; Pearce *et al.,* 1996; Imanishi *et al.,* 1999b; Kytola *et al.,* 2000). Similarly, loss of putative tumor suppressor genes in genomic regions including 1*p,* 3*q,* 4*q,* and 21*q* may be involved as significant factors in malignant parathyroid tumors (Cryns *et al.,* 1995; Imanishi *et al.,* 1999b; Kytola *et al.,* 2000; Agarwal *et al.,* 1998). Specific chromosome regional gains, suggesting the involvement of oncogenes, have been found in parathyroid carcinomas by comparative genomic hybridization (Imanishi *et al.,* 1999b; Kytola *et al.,* 2000; Agarwal *et al.,* 1998), but the involved genomic locations show little consistency among the existing reports and the results require confirmation by complementary methods. Interestingly, the patterns of molecular allelic losses and chromosomal losses in carcinomas as compared with adenomas suggests that parathyroid carcinomas tend to arise *de novo* rather than evolve from preexisting adenomas. Furthermore, genetic lesions that are specific for the malignant phenotype may be useful in making a molecular diagnosis, which would aid in overcoming the well known difficulties in distinguishing malignant versus benign parathyroid tumors histopathologically.

Ectopic Secretion of PTH

The ectopic secretion of PTH by nonparathyroid tumors is an extremely rare cause of hyperparathyroidism. The use of modern immunometric assays for PTH which show no cross-reactivity with PTHrP, the major cause of the hypercalcemia of malignancy, combined, in some cases, with molecular analysis using specific human *PTH* gene probes (Hendy *et al.,* 1981; Vasicek *et al.,* 1983), have suggested or confirmed the occurrence of this syndrome in several cases (Schmelzer *et al.,* 1985; Yoshimoto *et al.,* 1989; Nussbaum *et al.,* 1990; Strewler *et al.,* 1993; Rizzoli *et al.,* 1994; Nielsen *et al.,* 1996; Iguchi *et al.,* 1998).

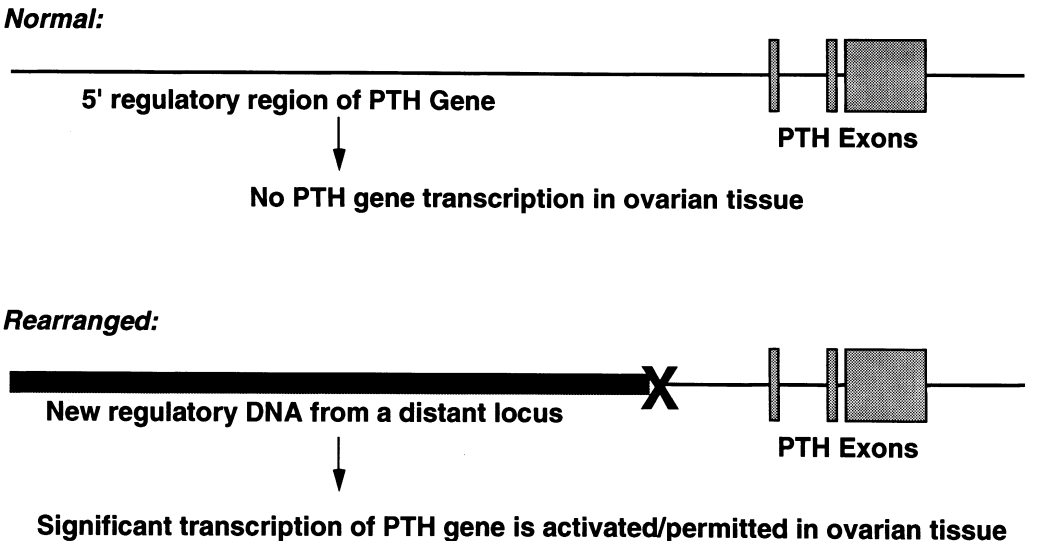

Figure 6 Molecular pathology of the ectopic production of PTH by an ovarian cancer. Schematic diagram of the normal PTH gene region (top) and the rearranged, amplified PTH gene region (bottom) in a PTH-secreting ovarian tumor. The bold "**X**" represents the breakpoint of the DNA rearrangement. Reproduced with permission from Arnold (1993).

In one case of an ovarian carcinoma (Nussbaum *et al.,* 1990), the molecular basis for the aberrant expression of PTH was determined. This involved a rearrangement (Fig. 6) and amplification of the *PTH* gene, such that it was no longer under the control of upstream regulatory elements which may normally act to silence *PTH* gene expression in nonparathyroid tissue. This case is also exceptional in its strong level of documentation that the tumor was in fact the source of the high circulating PTH levels and the patient's hypercalcemia.

Summary

Several advances have been achieved toward the goal of understanding the molecular basis of sporadic parathyroid tumorigenesis. The cyclin D1/*PRAD1* oncogene has been identified as a parathyroid oncogene, is overexpressed in 20–40% of parathyroid adenomas, and is also involved in the development of many additional tumor types. The gene responsible for MEN1 has been identified, and mutations in menin contribute to 12–17% of sporadic parathyroid adenomas. Mutations in the *RET* gene, the causal agent in MEN2, plus CASR and VDR, appear to contribute rarely if ever to the development of sporadic parathyroid tumors. The expected identification of additional major parathyroid tumor suppressor genes will no doubt provide further insights into parathyroid disease including parathyroid carcinoma. Ultimately, a description of parathyroid tumorigenesis will need to account for such features as the rarity of parathyroid carcinoma, the increased incidence of tumors after neck irradiation, and the increased frequency of hyperparathyroidism in postmenopausal women. In addition, the relationship between excessive cellular proliferation and an altered set-point in the mechanism linking extracellular calcium concentration to PTH secretion requires explanation and recently developed animal modeling of primary hyperparathyroidism may assist in this effort.

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CHAPTER 57

Familial Benign Hypocalciuric Hypercalcemia and Neonatal Primary Hyperparathyroidism

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Discovery and Initial Description

Before the ready availability of inexpensive and accurate measurements of serum calcium concentration, the diagnosis of hypercalcemia was generally considered only when there were overt signs or symptoms to suggest it. Thus, most cases of hypercalcemia that were identified were severe and presented with urolithiasis, nausea and vomiting, weight loss, or renal insufficiency (Albright and Reifenstein, 1948; Trigonis *et al.,* 1983). In the late 1960s, automated multichannel serum chemical analyzers became widely used in the United States and western Europe, leading to "routine" measurement of serum calcium in large numbers of adult medical patients, many having no specific symptoms. This large-scale screening of the population revealed a hitherto-unsuspected group of mildly to moderately hypercalcemic individuals, most of whom turned out to have primary hyperparathyroidism (1° HPT) (Heath *et al.,* 1980). During the past two decades, the literature on mild 1° HPT has expanded enormously, and the syndrome of virtually asymptomatic 1° HPT is now recognized to be the most common form of the disease (Bilezikian *et al.,* 1991; Heath, 1991). Some forms of 1° HPT are inherited, such as in multiple endocrine neoplasia types 1 or 2A (Gagel, 1994; Metz *et al.,* 1994), the hyperparathyroidism–jaw tumor syndrome (Szabo *et al.,* 1995), or isolated familial 1° HPT (Law *et al.,* 1983; Wassif *et al.,* 1993, 1999). However, investigators studying families in which hypercalcemia seemed to be transmitted as an autosomal dominant trait recognized nearly 30 years ago that some kindreds differed in important ways from those having inherited neoplastic parathyroid disease. Jackson and colleagues reported a hypercalcemic family (the WAL kindred) in which the clinical and biochemical characteristics shared certain features with 1° HPT, but in whom surgical exploration of affected individuals neither revealed parathyroid adenomas nor cured the hypercalcemia (Jackson and Boonstra, 1967). These investigators recognized that this family might have a novel hypercalcemic syndrome (Jackson and Boonstra, 1966), but did they not give the disorder a specific name. Only in 1972 did Foley *et al.* offer the first detailed studies, clear description, and distinct name for this syndrome of benign, life-long hypercalcemia that is not associated with clear-cut parathyroid hyperplasia or adenomatosis: familial benign hypercalcemia (Foley *et al.,* 1972). Subsequent studies (D. A. Heath, 1989; H. Heath, 1989;

Table I Comparison of Characteristic Features of Familial Benign Hypocalciuric Hypercalcemia (FBHH), Familial Isolated Primary Hyperparathyroidism (Familial HPT), and Sporadic Primary Hyperparathyroidism (Sporadic HPT)

Variable	FBHH	Familial HPT	Sporadic HPT
Age of onset	At birth	Variable	Usually >40 years
Symptoms	Usually none	Variable	—Asymptomatic in 80% —Cortical bone loss —Urolithiasis in 20%
Serum-plasma level			
Calcium	Elevated	Elevated	Elevated
Magnesium	Normal to elevated	Variable	Variable
Phosphorous	Normal to mild decrease	Normal to very low	Normal to very low
$1,25(OH)_2D$	Normal	Normal to increased	Normal to increased
Intact PTH	Normal (80–85%) Elevated (15–20%)	Elevated in >80%	Elevated in >80%
Urinary excretion			
Cyclic AMP	Normal to mildly increased	High normal to increased	High normal to increased
Calcium	Normal to low	Low to elevated	Low to elevated
Ca:Cr clearance ratio	Generally < 0.01	Generally >0.03	Generally >0.02
Magnesium	Low	Normal to high	Normal to high
Other findings	Possibly increased risk of chondrocalcinosis, gallstones	Typical findings of HPT	Typical findings of HPT

Heath, 1994; Law and Heath, 1985; Marx *et al.,* 1981a; Menko *et al.,* 1983) reinforced and extended Foley's original description, establishing the disorder as having the characteristics shown in Table I.

A related familial disorder occurring in neonates, characterized by severe hypercalcemia, bone demineralization, respiratory distress, and failure to thrive, had been described several decades earlier (Hillman *et al.,* 1964; Landon, 1932; Philips, 1948). Spiegel *et al.* were the first to recognize the relationship of this syndrome, now known as neonatal severe hyperparathyroidism (NSHPT), to familial benign hypercalcemia (Cooper *et al.,* 1986; Lillquist *et al.,* 1983; Marx *et al.,* 1982, 1985; Matsuo *et al.,* 1982; Page and Haddow, 1987; Spiegel *et al.,* 1977; Steinmann *et al.,* 1984). Almost a decade before the molecular basis for these two related syndromes was identified, Marx *et al.* suggested that they represented the expression of homozygous and heterozygous abnormalities in calcium-sensing, respectively (Fujimoto *et al.,* 1990; Marx *et al.,* 1982, 1985).

Familial Benign Hypocalciuric Hypercalcemia

As just noted, Foley and colleagues provided the first full clinical description of this syndrome (Foley *et al.,* 1972) as well as the name familial benign hypercalcemia (FBH)—a simple but very descriptive and reassuring name. Later, investigators at the National Institutes of Health reported the same condition as familial hypocalciuric hypercalcemia (FHH) (Marx *et al.,* 1977), in recognition of the unexpectedly low urinary excretion of calcium in affected persons. The literature is about evenly divided between the use of these two names, i.e., FBH or FHH, to describe this clinical entity (D. A. Heath, 1989; H. Heath, 1989; Heath, 1994; Law and Heath, 1985; Marx *et al.,* 1981a, 1977; Menko *et al.,* 1983). A unifying term describing the key features of the syndrome, familial benign hypocalciuric hypercalcemia (FBHH), was proposed by Heath (1989) and later adopted by Strewler (1994). This unifying term will be used in this chapter.

Clinical and Basic Laboratory Characteristics of FBHH

The earliest kindreds reported to have FBHH usually had one family member who had been misdiagnosed as having 1° HPT and, therefore, had undergone surgical exploration, only to have either no histologic abnormality (the commonest finding) or subtle "hyperplasia" of the parathyroids identified (Davies *et al.,* 1981; D. A. Heath, 1989; Heath, 1994; Law and Heath, 1985; Marx *et al.,* 1981a, 1977; Menko *et al.,* 1983; Paterson and Gunn, 1981; Sereni *et al.,* 1982). In any event, even subtotal parathyroidectomy failed to normalize serum calcium levels. Subsequent family investigations often revealed someone else with failed neck exploration and/or a large number of hypercalcemic, but asymptomatic, individuals of widely varying ages within a given kindred. Approximately 9% of patients referred to the National Institutes of Health in the 1970s with failed parathyroid surgery turned out to have FBHH (Marx *et al.,* 1980). As the syndrome of FBHH became more widely recognized in the 1980s, the

syndrome began to be diagnosed before any family members were subject to unnecessary parathyroid surgery.

The FBHH Syndrome Is Clinically Benign

This rare hypercalcemic syndrome is inherited in an autosomal dominant fashion and is usually characterized by lifelong asymptomatic hypercalcemia, relative hypocalciuria and the lack of the classical complications of hypercalcemia, including bone disease and renal stone disease. General morbidity and mortality appeared to be essentially normal in series from the Mayo Clinic, and National Institutes of Health, as well as in studies by other investigators (D. A. Heath, 1989; H. Heath, 1989; Heath, 1994; Law and Heath, 1985; Marx *et al.,* 1977, 1981a; Menko *et al.,* 1983). Furthermore, almost all affected persons probably lack symptoms of hypercalcemia (Foley *et al.,* 1972; D. A. Heath, 1989; Law and Heath, 1985; Marx *et al.,* 1977; Menko *et al.,* 1983), although symptoms consistent with hypercalcemia have been described in some probands (Marx *et al.,* 1981a). The studies of Law and Heath (1985) suggest that selection, referral, and "coaching" biases account for the presence of symptoms among probands; that is, the probands see physicians for various reasons that lead to biochemical screening and the incidental discovery of hypercalcemia. Probands may undergo repeated questioning about possible symptoms of hypercalcemia and be prompted inadvertently to answer in the affirmative. Thus, probands in FBHH kindreds often have a variety of symptoms, but affected family members detected by screening generally have no more symptoms than unaffected persons (see Associated Clinical Findings, below).

Associated Clinical Findings

Some observers have suggested that FBHH increases the risk of acute pancreatitis (Damoiseaux *et al.,* 1985; Davies *et al.,* 1981; Falko *et al.,* 1984; Robinson and Corall, 1990; Toss *et al.,* 1989), but others have questioned the validity of this claim (Stuckey *et al.,* 1990). Indeed, Stuckey *et al.* evaluated 10 cases with FBHH and pancreatitis: in 8/10 cases there were potential confounders for pancreatitis such as alcohol abuse ($n = 5$) and biliary pathology ($n = 3$) (Stuckey *et al.,* 1990). Clearly, there is a potential for ascertainment bias; that is, patients presenting with acute pancreatitis commonly are examined for hypercalcemia. Thus, hypercalcemia would be found incidentally in any patient with FBHH who developed pancreatitis for any reason. *Post hoc ergo propter hoc* reasoning would then lead the physician to conclude that FBHH caused the pancreatitis. This remains an unresolved issue. It is possible, nonetheless, that under certain conditions the hypercalcemia of FBHH predisposes to or aggravates acute pancreatitis. There is one report of increased prevalence of chondrocalcinosis in FBHH families (Marx *et al.,* 1981a); and cholithiasis appeared to occur at increased frequency in FBHH kindreds (Law and Heath, 1985). The association of symptoms associated with FBHH was systematically studied by Law and Heath who administered an interview to 15 well documented families with FBHH: 82 individuals were hypercalcemic and 52 were normocalcemic first-degree relatives. Whereas nocturia, arthritis, gallstones, and arterial hypertension were significantly more common in the index cases than in normocalcemic individuals, only gallstones were found to occur with increased frequency in cases found solely by family screening (Law and Heath, 1985). These associations require systematic reevaluation in a greater number of FBHH kindreds.

The skeleton in patients with FBHH generally appears to be normal histologically, radiographically, and by mineral densitometry (Abugassa *et al.,* 1992; Gilbert *et al.,* 1985; D. A. Heath, 1989; H. Heath, 1989; Heath, 1994; Law and Heath, 1985; Law *et al.,* 1984c; Marx *et al.,* 1977, 1981a; Menko *et al.,* 1983; Monfort-Gourand, 1993). Some case reports have described increased bone turnover on biopsy of FBHH patients (Alexandre *et al.,* 1982; Kristiansen *et al.,* 1987; Sereni *et al.,* 1982), but the data are very limited. As previously stated, serum alkaline phosphatase levels are normal in most patients with FBHH (D. A. Heath, 1989; Marx *et al.,* 1977, 1981a; Menko *et al.,* 1983). It is not clear if the bone in patients with FBHH is altered in a manner similar to that described by others in patients with 1° HPT, who exhibit increased or preserved iliac crest trabecular bone connectivity accompanied by cortical thinning (Christiansen *et al.,* 1992; Parisien *et al.,* 1990). While FBHH does not appear to threaten skeletal integrity, it does not confer immunity to osteoporosis; certainly, we have observed elderly members of some FBHH kindreds to have had osteoporosis that was clinically indistinguishable from that expected for their ages (Heath, H., III, unpublished observations).

Probably because of the relative hypocalciuria in patients with FBHH, nephrolithiasis is extremely uncommon, but has, however, been described in a few cases (see Renal Function and Ion Excretion, below).

Biochemical Findings in FBHH

The biochemical picture in FBHH is also benign, but can be indistinguishable from that of mild-to-moderate 1° HPT (H. Heath, 1989). The only absolute finding is hypercalcemia, which represents a true elevation of serum and total and ionized calcium; serum protein concentrations are normal, and calcium binding is normal (D. A. Heath, 1989; H. Heath, 1989; Heath, 1994; Law and Heath, 1985; Marx *et al.,* 1977, 1981a; Menko *et al.,* 1983). There is a variable degree of hypophosphatemia, and while this finding can occasionally be marked, absolutely low values for serum phosphorous are uncommon. Serum total magnesium levels are increased on average to about the upper limit of normal, being absolutely elevated in no more than half of affected individuals (Marx *et al.,* 1981a). Serum alkaline phosphatase activity has been reported as elevated in some cases (Marx *et al.,* 1981a), but is usually normal (D. A Heath, 1989; H. Heath,

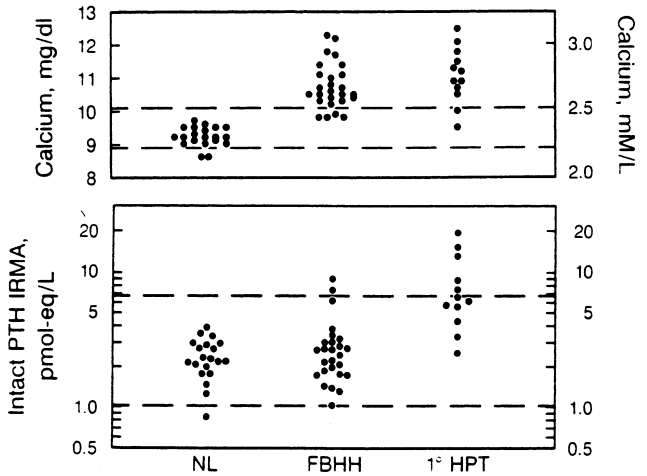

Figure 1 Serum calcium (top) and parathyroid hormone (PTH, bottom panel) concentrations in healthy adults (NL), patients with familial benign hypocalciuric hypercalcemia (FBHH), and patients with primary hyperparathyroidism (1 HPT) that was surgically verified. PTH was measured by a two-site immunoradiometric assay (Intact PTH, IRMA). Note the logarithmic scale for PTH. Normal ranges are indicated by horizontal dashed lines. Reprinted with permission from Rajala *et al.* (1991) *J. Bone Miner. Res.* **6,** 117–124.

1989; Heath, 1994; Law and Heath, 1985; Menko *et al.,* 1983). Other markers of bone turnover (e.g., osteocalcin or pyridinium cross-links) have not been reported in FBHH.

PTH levels have been measured in the serum of FBHH patients (Fig. 1) by first-generation radioimmunoassay (Kent *et al.,* 1987; Marx *et al.,* 1978b), cytochemical bioassay (Allgrove *et al.,* 1984), extraction-cell-based bioassay (Rajala *et al.,* 1991), and two-site immunoradiometric (Rajala *et al.,* 1991) and immunochemiluminometric assays (Firek *et al.,* 1991). The findings from all these methods are in general agreement: most persons affected with FBHH have paradoxically normal serum PTH values, and many are in the lower half of the normal range. However, 10–20% of FBHH patients have absolutely elevated serum PTH values (Fig. 1) (Firek *et al.,* 1991; Rajala *et al.,* 1991). In addition, one kindred coded as $FBHH_{Ok}$ had elevated serum PTH levels in affected individuals, specially the elderly (McMurtry *et al.,* 1992). In contrast, serum PTH values are supranormal in >85% of patients having 1° HPT (Firek *et al.,* 1991; Rajala *et al.,* 1991). One can imagine the ease with which mild to moderate hypercalcemia (say, 11.0 mg/dl), hypophosphatemia (perhaps 2.0–2.5 mg/dl), and slight elevation of serum intact PTH (70 pg/ml, as an example) could be mistakenly ascribed to 1° HPT in a patient who actually had FBHH.

Renal Function and Ion Excretion

The characteristic urinary mineral profile in FBHH led to the moniker "familial hypocalciuric hypocalcemia" (Marx *et al.,* 1978a); however, most affected persons are not truly "hypocalciuric" (below the normal range), but rather excrete less calcium than expected for their degree of hypercalcemia (a finding that has been termed "relative hypocalciuria"). About 75% of affected persons excrete less than 100 mg of calcium daily (Law and Heath, 1985), and some excrete strikingly low amounts of calcium in the face of hypercalcemia; values as low as 5 mg/day have been noted. However, the physician must be aware that a few individuals with FBHH may excrete as much as 250 mg of calcium daily (Law and Heath, 1985; Marx *et al.,* 1981a). The relatively low urinary excretion of calcium in FBHH results from avid renal tubular reabsorption of calcium, as detailed in the following section. Similarly, urinary excretion of magnesium is unexpectedly low given that the serum level is in the upper part of the normal range or, in some cases, mildly elevated (Kristiansen *et al.,* 1985, 1986; Marx *et al.,* 1978a).

The excretion of calcium in suspected FBHH cases may be expressed either as an absolute figure (mg/day) or as a unitless calcium:creatinine clearance ratio (fractional excretion of calcium), as advocated by Marx and colleagues (1980): [UCa.SCr]/[SCa.UCr], where UCa is the urinary calcium concentration, SCr the serum creatinine concentration, SCa the serum calcium concentration, and UCr the urinary creatinine concentration, all in milligrams per deciliter. Most patients having FBHH have urinary Ca:Cr clearance ratios below 0.01 (Law and Heath, 1985; Marx *et al.,* 1980, 1981a). There is considerable overlap between the absolute values for total urinary calcium excretion and Ca:Cr clearance ratios among persons having FBHH, their unaffected family members and unrelated patients having 1° HPT, as shown in Fig. 2. The ratio is most helpful in supporting a diagnosis of FBHH in a kindred, rather than in an individual patient, because the mean Ca:Cr clearance ratio generally will be less than 0.01 when averaged across affected members of the kindred.

Calcium infusions have been used to compare renal calcium handling in FBHH and hyperparathyroidism (Attie *et al.,* 1983; Davies *et al.,* 1984; Kristiansen *et al.,* 1986; Stuckey *et al.,* 1987). In response to a rising filtered calcium load, subjects with hyperparathyroidism increase their urinary calcium excretion more than FBHH patients do. This avid tubular calcium reabsorption persists even in FBHH patients who have been rendered surgically aparathyroid, pointing to a primary renal tubular abnormality in calcium reabsorption (Attie *et al.,* 1983; Davies *et al.,* 1984; Kristiansen *et al.,* 1986; Watanabe and Sutton, 1983). Renal function is well preserved in FBHH (D. A Heath, 1989, H. Heath, 1989; Heath, 1994; Law and Heath, 1985; Marx *et al.,* 1977, 1981a; Menko *et al.,* 1983), and there appears to be no more than the usual age-related loss of glomerular filtration rate. Moreover, the hypercalcemia of FBHH does not appear to alter renal sensitivity to vasopressin as it does in 1° HPT. Marx and colleagues demonstrated convincingly that reduced maximal urinary concentrating ability occurs in 1° HPT but not in FBHH (Marx *et al.,* 1981b). As stated above, urolithiasis is rare in patients having FBHH, but has been reported (Marx *et al.,* 1981a; Menko *et al.,* 1983; Toss

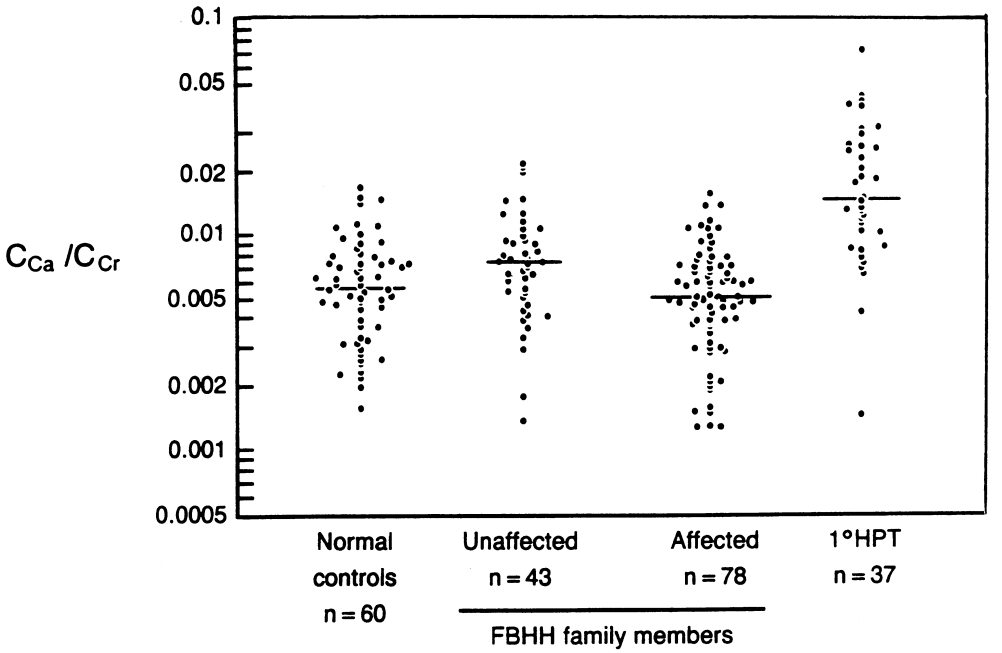

Figure 2 Ratios of calcium clearance to creatinine clearance in patients with familial benign hypocalciuric hypercalcemia (FBHH), unaffected family members, unrelated normal controls, and patients with surgically confirmed primary hyperparathyroidism (1° HPT). Note the logarithmic scale for C_{Ca}:C_{Cr} ratios. There is substantial overlap between FBHH and 1° HPT; however, a ratio of >0.02 is helpful in excluding FBHH. Reprinted with permission from Heath, H., III.

et al., 1989). A Swedish family with a syndrome that presented as a hybrid between 1° HPT and FBHH, with hypercalciuria that subsided after parathyroidectomy (Carling *et al.,* 2000).

In summary, the clinical and laboratory picture of FBHH is generally that of incidentally discovered hypercalcemia that bears no clear relationship to any of the patient's symptoms. The biochemical picture can be very similar to that of patients with asymptomatic 1° HPT, although elevation of serum PTH levels is much less common in FBHH than in 1° HPT (Allgrove *et al.,* 1984; Firek *et al.,* 1991; Kent *et al.,* 1987; Marx *et al.,* 1978b; Rajala *et al.,* 1991). While chondrocalcinosis, gallstones, and acute pancreatitis have been reported in FBHH, the relationship of these events to the FBHH is uncertain. Hyperparathyroid bone disease, hyposthenuria, and urolithiasis are conspicuously absent. The greatest importance of the FBHH syndrome to clinicians is that it is so easily misdiagnosed as mild to moderate 1° HPT, leading to excessive testing and to inappropriate, unnecessary, and potentially harmful cervical exploratory surgery.

Pathophysiologic Studies in FBHH

Extensive clinical research studies have clarified the organ-level pathogenesis of FBHH. Because the molecular basis of the predominant form of FBHH is now known (see the following), these studies now are mainly of value for providing a clear definition of the syndrome, but they also suggested basic research leads that ultimately resulted in determination of the molecular pathogenesis of one form of the syndrome.

Parameters of Systemic Plasma Calcium Regulation

PTH DYNAMICS

As previously stated, the hypercalcemia of FBHH is a "true" hypercalcemia, not an artifact of calcium-binding. It is stable across time and usually is reduced little or not at all by subtotal parathyroidectomy (Law and Heath, 1985; Marx *et al.,* 1981a). However, total parathyroidectomy results in permanent hypocalcemia that responds to the usual measures for treating hypoparathyroidism. The hypercalcemia of FBHH is therefore clearly PTH-dependent in a qualitative sense, but recent data show that this is also true in a quantitative sense (Firek *et al.,* 1991). Since the first application of PTH RIAs to the study of 1° HPT, a clear, direct relationship between the degree of hypercalcemia and the elevation of serum immunoreactive PTH has been evident (Arnaud *et al.,* 1971). Studies at the Mayo Clinic (Firek *et al.,* 1991) demonstrated a similar positive correlation between serum calcium and intact PTH levels in FBHH—but with a much shallower slope than the one described for hyperparathyroidism—and an inverse relationship was present between PTH and serum inorganic phosphorous levels. Clearly, hypercalcemia and hypophosphatemia are maintained in FBHH at

lower levels of ambient PTH than in 1° HPT, suggesting that inappropriately high PTH levels result from abnormal calcium-sensing at the level of the parathyroid gland. The reduced responsiveness of the parathyroid glands to changes in serum calcium has been documented carefully in FBHH. Induction of relative hypocalcemia by infusion of ethylenediaminetetraacetic acid (EDTA) or of relative hypercalcemia by calcium infusion causes appropriate increases and decreases of serum PTH concentrations (Auwerx *et al.,* 1984; Heath and Purnell, 1980; Khosla *et al.,* 1993). The decreases of PTH are greater in FBHH than in 1° HPT—in fact, indistinguishable from normal, although they occur at a higher level of serum calcium than do the corresponding reductions in PTH in normal persons—and increases are less than in 1° HPT. Systematic evaluation of PTH dynamics in FBHH patients compared to normal control is under way using consecutive citrate and calcium infusions. Preliminary results indicate a shift in the calcium–PTH relationship to the right, again consistent results with altered calcium-sensing (Fig. 3). PTH secretion responds in the usual inverse manner to changes in serum calcium concentration, but is reset so as to maintain a higher-than-normal level of plasma calcium. Interestingly, Foley *et al.* presciently interpreted their 1972 findings on FBHH as being consistent with a genetic abnormality of parathyroid calcium sensing (Foley *et al.,* 1972).

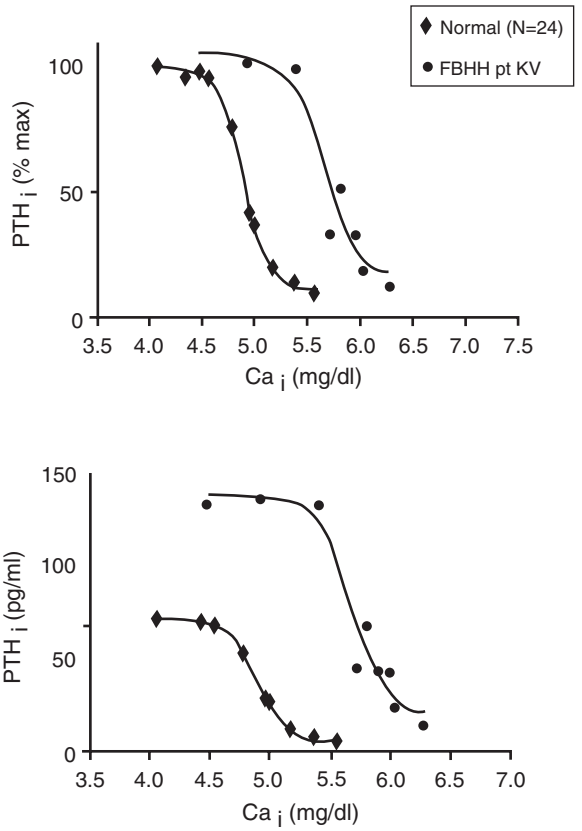

Figure 3 Inverse sigmoid curve between serum intact PTH and ionized calcium (Ca_i/PTH_i) in response to consecutive citrate and calcium infusions in patient KV with FBHH S. T. Haden, E. M. Brown, and G. El-Hajj Fuleihan, unpublished observation) in comparison to the Ca/PTH curve derived from 24 healthy subjects (adapted from Haden *et al.* (2000) *Clin. Endocrinol.* **52,** 329–338). There is a clear abnormality of PTH dynamics in the patient with FBHH compared to normal controls: a shift in the Ca/PTH curve to the right, increased PTH levels in response to hypocalcemia, and decreased suppression in response to hypercalcemia. The set-point, the calcium concentration at which there was 50% suppression in PTH levels, was 4.89 mg/dl in the curve derived from 24 healthy controls and 5.65 mg/dl in the patient with FBHH.

PARATHYROID GLANDS

The histologic findings in parathyroid glands of patients having FBHH are controversial. Most case and single-family reports have described unremarkable parathyroid tissue (Davies *et al.,* 1981; Paterson and Gunn, 1981; Sereni *et al.,* 1982, and reviewed in Law *et al.,* 1984b). These findings were corroborated by studies of large kindreds (Law and Heath, 1985; Menko *et al.,* 1983; Toss *et al.,* 1989). However, the two large histologic studies published to date focusing on parathyroid pathology yielded conflicting results. Thorgeirsson *et al.* reported that a highly variable parathyroid hyperplasia was typical in their examination of 55 parathyroid glands from 18 patients thought to have FBHH (Thorgeisson *et al.,* 1981). Conversely, Law *et al.* examined 28 parathyroid glands from 23 patients in 16 FBHH kindreds, quantifying fat to parathyroid parenchymal ratios in comparison with 82 normal glands from 47 control patients, and found no evidence to support the presence of parathyroid hyperplasia in FBHH (Law *et al.,* 1981). The discrepancy in the findings between these two large studies would be explained by referral bias or differences in the study populations (see Phenotype–Genotype Associations below).

OTHER CALCIOTROPIC HORMONES

Hypercalcemia in FBHH might be explained by increased formation of 25-hydroxyvitamin D or 1,25-dihydroxyvitamin D; however, plasma concentrations of these vitamin D metabolites are normal in FBHH patients (Davies *et al.,* 1983; Gilbert *et al.,* 1985; Kristiansen *et al.,* 1985; Law *et al.,* 1984a; Law and Heath., 1985; Lyons *et al.,* 1986), as is intestinal calcium absorption (Kristiansen *et al.,* 1985; Law and Heath 1985; Menko *et al.,* 1983). Thyroid C-cells increase calcitonin excretion in response to calcium; while calcitonin levels are inappropriately normal in FBHH for the degree of calcium elevation (Kristiansen *et al.,* 1985; Law and Heath, 1985; Menko *et al.,* 1983), they exhibit a normal response to other secretagogues (Rajala *et al.,* 1991).

THE KIDNEY

Two careful studies of very small numbers of patients have provided evidence for an intrinsic defect of renal tubular reabsorption of calcium in FBHH (Attie *et al.,* 1983; Davies *et al.,* 1984). These patients, who were later recognized to have FBHH, had inadvertently been rendered totally aparathyroid during surgical explorations for parathyroid tumors. Comparisons with individuals who became

hypoparathyroid after removal of typical sporadic parathyroid adenomas demonstrated greater PTH-independent, renal tubular reabsorption of calcium and magnesium in the FBHH patients than the sporadic hypoparathyroid controls (Attie *et al.,* 1983).

In summary, studies of the FBHH syndrome have almost uniformly suggested a genetic disorder of calcium sensing by the parathyroid gland paired with enhanced renal tubular reabsorption of calcium. PTH secretion persists at normal or slightly elevated values in the face of hypercalcemia, but without obvious disturbances of other calcium-regulating hormone concentrations. These pathophysiologic abnormalities would be most parsimoniously explained by dominant mutations of a single calcium-sensing receptor expressed both in parathyroid glands and in kidneys.

Genetic Studies in FBHH

No formal segregation analyses have been reported for FBHH kindreds, but inspection of numerous published and unpublished pedigrees shows beyond any doubt that the FBHH trait is inherited in an autosomal dominant pattern (Foley *et al.,* 1972; D. A. Heath, 1989; H. Heath, 1989; Heath 1994; Law and Heath, 1985; Marx *et al.,* 1977; 1981a; Menko *et al.,* 1983; Jackson and Boonstra, 1966; Toss *et al.,* 1989). Across numerous families, the sex distribution is essentially 1:1, and about half of all persons at risk manifest hypercalcemia. There is a considerable variation in clinical manifestation, that is, in the extent of hypercalcemia (there being almost no other clinical marker). Several groups have observed that the "hypercalcemia breeds true"; that is, there are FBHH families with relatively high and those with relatively low serum calcium concentrations (Marx *et al.,* 1981a; Rajala and Heath, 1987). There is, however, considerable variability of serum calcium values even within kindreds (Fig. 4). We have had the opportunity to follow affected women whose husbands were normocalcemic through three pregnancies and deliveries. Hypercalcemia was present in all three infants at birth, with serum calcium levels of up to 18 mg/dl observed in cord blood and during the first few days of life; subsequently, serum calcium declined by age 3–4 years to values similar to the affected mother's (unpublished results).

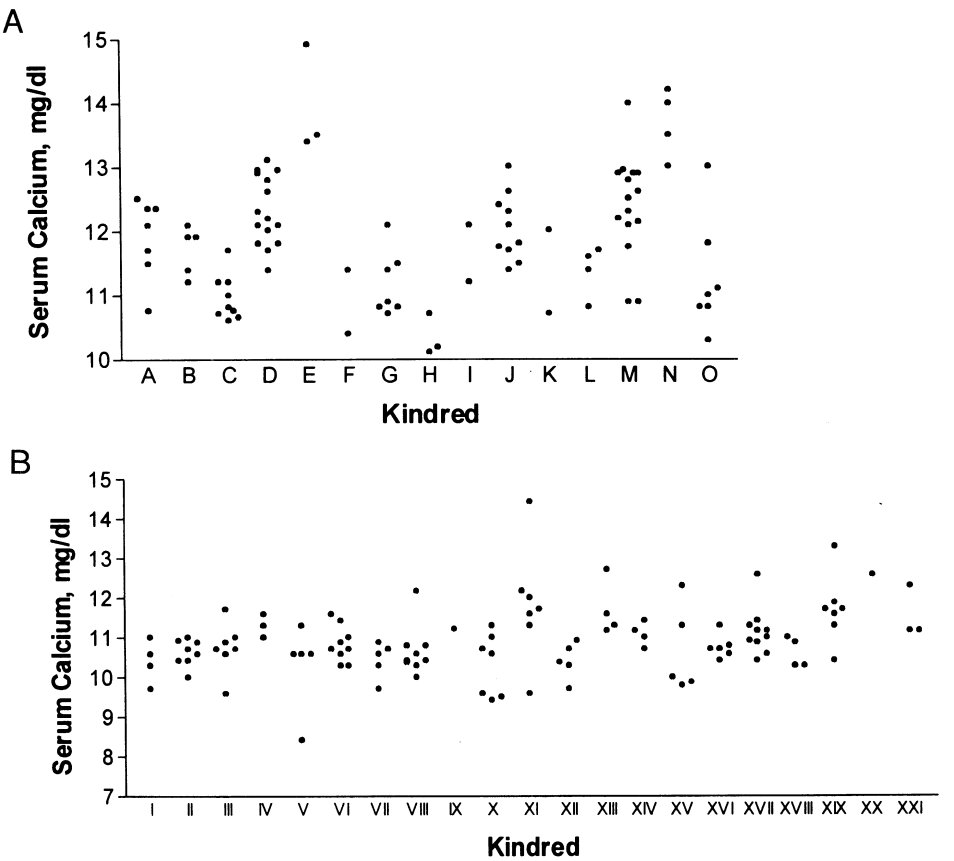

Figure 4 Scatter of serum calcium levels in FBHH kindreds. (A) Data from 15 kindreds reported by the National Institutes of Health group (adapted from Marx *et al.* (1981) *Medicine* **60,** 397, with permission). (B) Data from 21 kindreds reported by the Mayo Clinic group (adapted from Rajala and Heath (1987) *J. Clin. Endocrinol. Metab.* **65,** 1039, with permission). The mean ± SD calcium level in A is 12.1 ± 1.6 mg/dl and in B 10.9 ± 0.8, the mean serum calcium level from both studies is 11.4 ± 1.4 mg/dl.

Early genetic linkage studies excluded a number of candidate genes and loci as causes of FBHH, including the MEN 1 and MEN 2A loci, the PTH gene, and others (Heath and Leppert, 1992; Menko *et al.,* 1984; Paterson *et al.,* 1985). As it turns out, the FBHH syndrome is genetically heterogeneous: in the majority (over 85%) the FBHH phenotype links to a region of chromosome 3 that contains the calcium-sensing receptor sensor gene (CaSR), a disease subtype called $FBHH_{3q}$ (Heath *et al.,* 1993; Pearce *et al.,* 1995b). In two-thirds of $FBHH_{3q}$ families specific inactivating mutations of the CaSR were found to account for the hypercalcemia (Fig. 5). The remainder may have CaSR mutations outside of the coding region. In a smaller number of families the linkage of the trait was to markers on the short arm of chromosome 19 (Heath *et al.,* 1993), thus termed $FBHH_{19p}$ (Heath, 1994; Heath *et al.,* 1996; Strewler, 1994). Trump and colleagues carried out genetic linkage studies in the FBHH kindred described by Whyte *et al.* (Trump *et al.,* 1995), and found linkage neither to chromosome 3*q* nor chromosome 19*p* markers, establishing that there are three genetically distinct forms of the FBHH syndrome: $FBHH_{3q}$, $FBHH_{19p}$, and $FBHH_{OK}$ or $FBHH_{19q}$ ["OK" for Oklahoma, the family's state of residence (Trump *et al.,* 1995, Lloyd *et al.,* 1999)].

The Molecular Basis of $FBHH_{3q}$: Mutations in the CaSR

Chou *et al.* achieved the first linkage of the FBHH trait to markers on chromosome 3 (Chou *et al.,* 1992), specifically, $3q21-q24$. This linkage coincided with the cloning of the bovine parathyroid calcium-sensing receptor by Brown *et al.,* (1993), and the same group quickly demonstrated three distinct missense mutations in the calcium receptor genes in three separate families with $FBHH_{3q}$ (Pollack *et al.,* 1993). Several groups have since found point mutations of this gene that cosegregate with the $FBHH_{3q}$ trait (Aida *et al.,* 1995; Chou *et al.,* 1995; Heath *et al.,* 1996; Janicic *et al.,* 1995; Pearce *et al.,* 1995b).

The CaSR exhibits a modest degree of homology with the metabotropic glutamate receptors and has three domains: a large extracellular domain (612 aa), a seven-transmembrane-spanning segment that characterizes the G-protein-coupled receptor superfamily (250 aa), and an intracytoplasmic tail (222

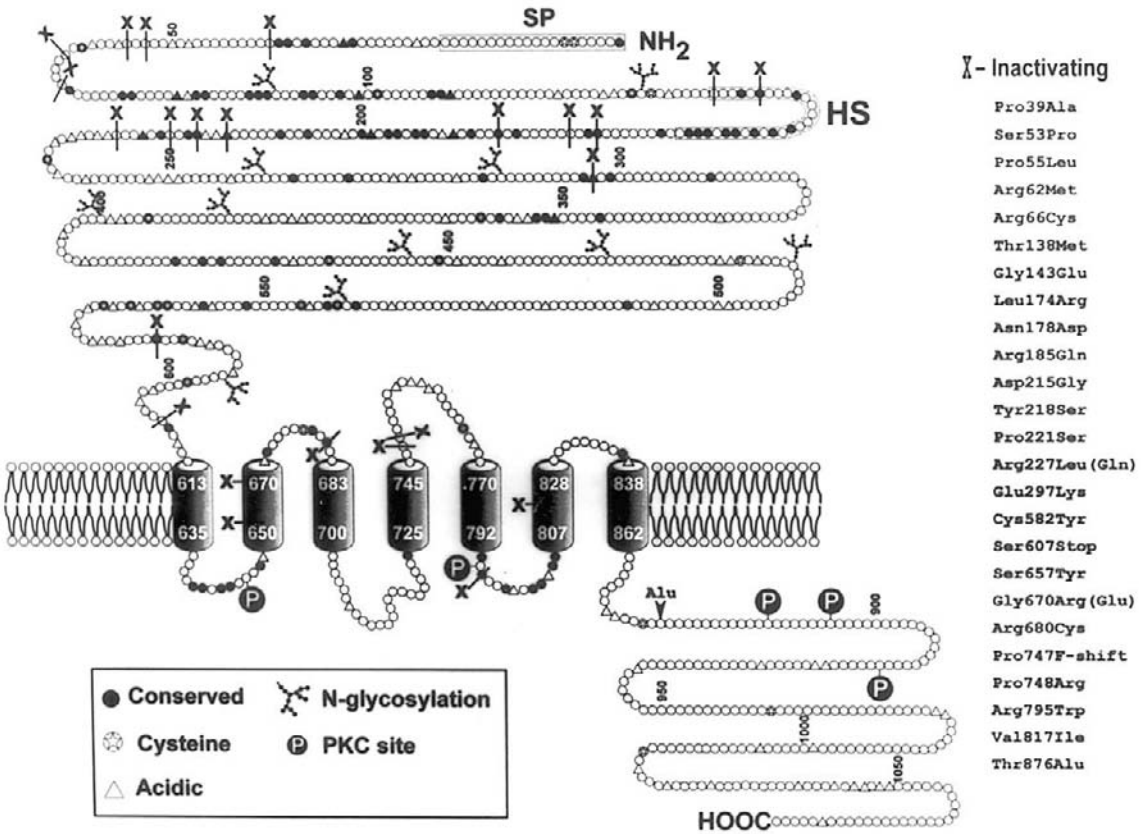

Figure 5 Schematic representation of the proposed structure of the extracellular Ca^{2+} calcium receptor cloned from human parathyroid gland. SP, signal peptide; HS hydrophobic substance. Also shown are 25 inactivating (missense and nonsense) mutations causing familial benign hypocalciuric hypercalcemia (FBHH). Mutations are indicated using the three-letter amino acid code, with the normal amino-acid indicated before and the FBHH mutation shown after the number of the relevant codon. Adapted from Bai *et al.* (1996), with permission.

aa). This receptor is expressed in many tissues but is found at the highest levels in the parathyroid gland, the thyroid C-cells and various regions of the kidney, especially the cortical thick ascending limb, a segment that plays a critical role in calcium handling (Brown *et al.,* 1995b). Activation of the normal calcium receptor suppresses parathyroid hormone secretion and enhances renal calcium excretion. Therefore, reduction or loss of the normal function of one allele of the calcium receptor, in FBHH, impairs calcium-sensing, with a resultant shift in the calcium–PTH curve to the right and reduced excretion of renal calcium at any given level of serum calcium concentration. Most of the inactivating mutations of the human CaSR gene found so far represent single amino-acid substitutions, but truncation (Pearce *et al.,* 1995b) and Alu insertion mutations (Janicic *et al.,* 1995) have also been described. In addition, "benign" polymorphisms have been described in up to one-third of unaffected subjects (Heath *et al.,* 1996). However, Cole *et al.* subsequently reported subtle abnormalities in serum calcium albeit within the normal range for the serum calcium concentration, in subjects with these "benign" polymorphisms (Cole *et al.,* 1999). To date, more than 25 different FBHH mutations of the CaSR gene are known. These $FBHH_{3q}$ mutations have been mostly in the extracellular or transmembrane part of the receptor (Fig. 5). Recently, however, a Swedish group described a kindred with a mutation in the cytoplasmic tail of the receptor, who presented with symptoms that combine those of FBHH and hyperparathyroidism (Carling *et al.,* 2000).

Several mechanisms have been suggested to account for the reduced activity of the CaSR and decreased calcium-sensing (Bai *et al.,* 1996):

1. Decreased affinity of the receptor for its physiological agonists, specifically calcium (the receptor also likely senses magnesium in a physiologically relevant manner).
2. Inability to form a fully active calcium receptor.
3. Failure of the receptor to couple normally to its respective signal-transduction pathway(s), e.g., activation of appropriate G-proteins.
4. A dominant-negative effect where in the mutated receptor interferes with the function of the normal receptor.

The role of the CaSR gene in controlling parathyroid function and the role of CaSR inactivation in causing FBHH have been elegantly supported by the mouse gene inactivation studies of Ho *et al.* (1995). Mice heterozygous for a disrupted CaSR gene had mild hypercalcemia [10.4 ± 0.55 mg/dl (mean ± SD)], non suppressed PTH levels, and reduced urinary calcium excretion compared to normal mice. The affected mice had normal radiographic skeletal morphology and parathyroid gland histology.

The Molecular Bases of $FBHH_{19p}$ and $FBHH_{OK}$

Two hypercalcemic syndromes that are similar to $FBHH_{3q}$ have been linked to loci on chromosomes 19*p* (Heath *et al.,* 1993) and 19*q*, the latter originally known as $FBHH_{OK}$ (Trump *et al.,* 1995; Lloyd *et al.,* 1999). At this writing, the molecular bases of the non-3*q* forms of FBHH are still unknown. However, the region mapped for $FBHH_{19p}$ (chromosome 19*p*ter) contains an interesting candidate gene, GNA11, which encodes a Gsa protein that is expressed in human parathyroid tissue (Varrault *et al.,* 1995). It is possible that GNA11 is the $FBHH_{19p}$ gene. The GNA11 gene product may be the Gsa protein acting in concert with the CaSR protein to control PTH secretion (Brown *et al.,* 1995b; Nemeth and Heath, 1995). Thus, inactivation either of one copy of the CaSR gene or of the GNA11 gene might confer the FBHH syndrome.

Autoimmune FBHH

Recently, two cases were described with autoimmune FBHH with biochemical features undistinguishable from usual FBHH (Kifor *et al.,* 2000). The two subjects had autoimmune disorders, thyroiditis and sprue, respectively. In those two subjects, FBHH was due to the presence of anti-CaSR antibodies that interfered with calcium-mediated suppression of PTH and calcium-sensing at the kidney.

POSSIBLE PHENOTYPE-GENOTYPE ASSOCIATIONS

When various missense or nonsense mutations identified in patients with FBHH are introduced into the normal CaSR by site-directed mutagenesis, a spectrum of CaSR activity emerges (Pearce *et al.,* 1995b), reflecting the spectrum in the syndrome's phenotypic expression. Hypercalcemia was noted to be the mildest in the $FBHH_{19p}$ kindred (Heath *et al.,* 1993); this observation is based on the evaluation of a single small family. In contrast, a putative "dominant negative" mutation is associated with more severe elevation of serum calcium levels through interference by the mutated receptor with the function of the wild-type allele (Bai *et al.,* 1996). This interference most likely occurs because the normal CaSR functions as a dimer, and functional interactions are known to occur between the two CaSR monomers within the dimer. However, as mentioned previously, within any given family there can a considerable variation in the degree of the hypercalcemia independent of the specific mutation (Fig. 4).

Some FBHH kindreds may be at increased risk for pancreatitis, and in three such kindreds Pearce found three heterozygous missense mutations in the extracellular domain of the calcium receptor (1996). This observation, in conjunction with that of an expression of the calcium receptor in the pancreatic duct (Bruce *et al.,* 1999), suggests the intriguing possibility that specific mutations may indeed carry a higher risk of pancreatitis.

The recent description by Carling *et al.,* of a novel mutation in the cytoplasmic carboxy-terminal tail of the calcium receptor, with a clinical and biochemical phenotype that is similar to primary hyperparathyroidism, with hypercalciuria and parathyroid hyperplasia, also raises the possibility that specific mutations may be associated with the unusual complications of stone disease and hyperplasia of the parathyroid glands that have been occasionally reported in FBHH

kindreds (Menko *et al.,* 1983; Toss *et al.,* 1989; Marx *et al.,* 1981a). Perhaps this mutant receptor couples differentially to its downstream effectors in parathyroid and kidney, conferring more resistance to extracellular calcium on parathyroid than on kidney cells. Similarly the presence of a receptor truncated in its carboxy-terminal tail owing to the insertion of an Alu element that produces a premature stop codon has also been associated with parathyroid hyperplasia (Cole *et al.,* 1997; Janicic *et al.,* 1995).

Finally, the $FBHH_{OK}$ syndrome has been associated with osteomalacia in one family for reasons that are yet to be unraveled since the genetic basis of that syndrome is still unknown (Trump *et al.,* 1995).

A Clinical Strategy for FBHH

The differential diagnosis of hypercalcemia is not the dilemma it once was; in patients with established hypercalcemia, a single measurement of serum PTH by an immunometric assay will allow a correct diagnosis of 1° HPT at least 80% of the time. However, many patients with 1° HPT are asymptomatic or oligosymptomatic, many have serum intact PTH concentrations within population normal ranges, and some have surprisingly low urinary calcium excretion (Marx *et al.,* 1977). A given patient, then, may present with asymptomatic, mild to moderate hypercalcemia, minimal or no hypophosphatemia, urinary calcium excretion below 100 mg/day (Law and Heath, 1985), and mild elevation of serum intact PTH, with no family history of hypercalcemia. The physician will have no simple or reliable way of determining if this patient has a parathyroid tumor or one of the variants of FBHH. What is the clinician to do? Genetic linkage studies are not practical, because of family sampling problems, cost, the large number of possible mutations, and the genetic heterogeneity of the syndrome, including the existence of FBHH variants whose molecular etiologies are not yet understood. Direct DNA screening studies of the CaSR gene could be informative, if abnormal, but even $FBHH_{3q}$ families do not have known mutations of the CaSR gene coding region in a substantial minority (about a third) of cases. The strategy to be followed must be based, for now, on clinical judgement.

For hypercalcemic patients over the age of 40, with no familial history of hypercalcemia, a clinical and biochemical picture consistent with 1° HPT is overwhelmingly likely to represent just that. The data of Marx *et al.* suggest odds of >1000:1 in favor of 1° HPT in this situation (Marx *et al.,* 1980). Obtaining a measurement of 24-hr urinary calcium could be helpful, hypercalciuria virtually excludes the diagnosis of FBHH, but normocalciuria or even low urinary calcium levels do not exclude 1° HPT, particularly in elderly women. Furthermore, families with FBHH and hypercalciuria have been described. A conservative approach, if the urinary calcium were very low, the serum PTH normal, and the patient clinically well, would be to sample available first-degree relatives for occult hypercalcemia. If surgical exploration were indicated for symptoms or complications [e.g., osteopenia (Nemeth and Heath, 1995), it could be done without great concern. In either case, long-term observation of untreated, uncomplicated 1° HPT and FBHH is a safe course of action. One's suspicion for FBHH would be heightened if the patient were young (under age 40, especially if under 30), if there were a family history of hypercalcemia, if anyone in the family had undergone unsuccessful neck exploration for hypercalcemia, if neonatal hyperparathyroidism had occurred in the family, and if the urinary excretion of calcium were particularly low (say, less than 50 mg/day) in the patient and other hypercalcemic relatives. In such case, one should defer surgical therapy and observe. The key point is that FBHH does not require treatment in the vast majority of cases. The goal in the clinic must be to identify FBHH before any family member has surgery, but this is regrettably not always going to be possible with our current state of knowledge.

Neonatal Primary Hyperparathyroidism

Clinical Characteristics

Neonatal primary hyperparathyroidism can be a mild syndrome representing the neonatal expression of FBHH, or it can present as a much more dramatic disease with high morbidity and even mortality if untreated, a syndrome known as neonatal severe primary hyperparathyroidism (NSHPT). Due to the severity of its symptoms, signs, and biochemical abnormalities, NSHPT was described several decades before FBHH. It manifests at birth, with severe hypercalcemia, hypotonia, osteitis fibrosa cystica, respiratory difficulty, failure to thrive, parathyroid hyperplasia, and, in general, markedly elevated serum levels of PTH (Fujita *et al.,* 1983; Marx *et al.,* 1982; Page and Haddow, 1987; Sereni *et al.,* 1982; Steinmann *et al.,* 1984). Additional clinical features include chest wall deformities with a flail chest syndrome secondary to multiple rib fractures, dysmorphic facies, and anovaginal and rectovaginal fistulas (Marx *et al.,* 1982; Spiegel *et al.,* 1977; Steinmann *et al.,* 1984). The hypercalcemia is usually severe, ranging from 14 to 20 mg/dl, one patient had a serum calcium of 30.8 mg/dl (Corbeel *et al.,* 1968), PTH levels are elevated by 5- to 10-fold (Fujimoto *et al.,* 1990) and examination of the parathyroid glands reveals four gland hyperplasia (Cooper *et al.,* 1986; Fujimoto *et al.,* 1990; Lutz *et al.,* 1986; Marx *et al.,* 1982; Matsuo *et al.,* 1982). Some affected infants have died from NSHPT, others have survived after total or subtotal parathyroidectomy, and, in more recent series, a substantial percentage of cases survived without parathyroid surgery. Indeed, some neonates with neonatal hyperparathyroidism who originally presented with respiratory compromise and demineralization have been reported to improve (Harris and D'Ercole, 1989; Orwoll *et al.,* 1982; Page and Haddow, 1987; Wilkinson and James, 1993). In

neonatal hyperparathyroidism, the serum calcium level is usually less elevated than in NSHPT and patients have no evidence of bone disease, the latter being a prominent feature of NSHPT. This probably reflects the fact that these neonates carry only one abnormal copy of the FBHH gene, whereas the severity of hypercalcemia and bone disease in NSHPT results from the inheritance of a double dose of the abnormal gene.

Genetic Basis of NSHPT

This syndrome may occur as a sporadic disorder, but has also been described in kindreds having FBHH (Fujita *et al.,* 1983; Marx *et al.,* 1982; Page and Haddow, 1987; Sereni *et al.,* 1982; Steinmann *et al.,* 1984). When NSHPT has occurred in FBHH families, in some cases, both parents have been hypercalcemic, suggesting the possibility that NSHPT is the clinical manifestation of homozygosity for an FBHH mutation (Chou *et al.,* 1995; Janicic *et al.,* 1995; Pollak *et al.,* 1993).

NSHPT can result from any of the following genetic alterations in the CaSR:

1. Homozygous form of FBHH: the neonate from a consanguineous marriage inherits a double dose of FBHH mutation, one from each heterozygous parent (Marx *et al.,* 1981, 1982, 1985; Pollak *et al.,* 1994).
2. Compound heterozygous form of FBHH: the neonate results from the marriage of two individuals with two different mutations in the CaSR (Kobayashi *et al.,* 1997).
3. Heterozygous form of NHPT: when the neonate occurs sporadically or comes from an FBHH family with only one parent affected (Harris and D'Ercole, 1989; Orwoll *et al.,* 1982; Page and Haddow, 1987; Powel *et al.,* 1993; Spiegel *et al.,* 1977; Wilkinson and James, 1993). Several potential explanations may be offered for that observation. The parents are both carriers of a CaSR mutation but only one is recognized, the offspring may experience a negative dominant effect of the abnormal gene on the normal one (Bai *et al.,* 1997) or the offspring carries *de novo* heterozygous mutations (Bai *et al.,* 1997; Pearce *et al.,* 1995b).

MICE MODELS OF NSHPT

Mice homozygous for a disrupted CaSR gene have more substantial hypercalcemia (14.8 $\pm$ 1.0 mg/dl) and increased parathyroid size compared to mice heterozygous for the mutated gene (Ho *et al.,* 1995) than did the heterozygous animals. The homozygotes grow poorly, have multiple bony abnormalities and usually die within 4 weeks (Ho *et al.,* 1995). Homozygous null mutation mice also have reduced skeletal radiodensity, kyphoscoliosis, and bowing of long bones, reminiscent of the skeletal anomalies in human NSHPT (Fujita *et al.,* 1983; Marx *et al.,* 1982; Page and Haddow, 1987; Sereni *et al.,* 1982; Steinmann *et al.,* 1984). These important data fulfill "Koch's postulates" for $FBHH_{3q}$ and make it almost certain that inactivating mutation of the human CaSR gene is the cause of FBHH and NSHPT in many if not most kindreds.

Management of Neonatal Hyperparathyroidism

In older reports, cases that were managed medically had a much worse prognosis than did those who underwent total parathyroidectomy (Cooper *et al.,* 1986; Fujimoto *et al.,* 1990; Hillman *et al.,* 1964; Marx *et al.,* 1982; Matsuo *et al.,* 1982; Spiegel *et al.,* 1977). Since 1980, milder forms of neonatal hyperparathyroidism have been noted that may improve with medical therapy alone. At this point it would be wise to aggressively support any neonate presenting with NSHPT or neonatal hyperparathyroidism with hydration, mechanical ventilation, and rely on surgery only in those who fail to improve with supportive measures. The surgical intervention of choice would be total parathyroidectomy with immediate or delayed auto-transplantation. Bony remineralization generally occurs within a matter of months(s) after successful parathyroid surgery (Cooper *et al.,* 1986; Fujimoto *et al.,* 1990; Lutz *et al.,* 1986). Any person known to have FBHH should ask his/her spouse to have a measurement of serum calcium performed; if the other spouse is hypercalcemic and both appear to have FBHH, then a pediatrician knowledgeable about NSHPT should be in attendance at the birth of any child. Consanguinity of the couple obviously would heighten concern about NSHPT, because there would be a 25% chance of their child having the disorder.

CaSR-based therapeutics are well into clinical trials (Silverberg *et al.,* 1997; Wada *et al.,* 1999). They may be useful tools not only in our understanding of the role of the CaSR in several organs, but also in the medical management of disorders of abnormal calcium-sensing. In NSHPT, as long as there is some residual function of the CaSR, calcium receptor agonists would presumably be helpful. They could also be used in the more severe variants of FBHH in the neonatal period as well as in adulthood—for instance in the most recent FBHH syndrome described (Carling *et al.,* 2000), as well as in autoimmune FBHH.

Conclusions

As predicted some years ago (Law and Heath, 1985), study of the FBHH syndrome has led to fascinating, important advances in our understanding of the regulation of systemic calcium metabolism (Brown *et al.,* 1995a; Pearce *et al.,* 1995a). What appeared just a few years ago to be a single, homogeneous hypercalcemic syndrome is now known to represent at least three genetically distinct disorders, $FBHH_{3q}$ (probably always a disorder of the parathyroid CaSR), $FBHH_{19p}$ (which may result from mutation of a gene encoding another component of the calcium receptor activation pathway, a $G_{s\alpha}$ protein gene), and $FBHH_{OK}$. $FBHH_{3q}$ accounts for the overwhelming majority of cases to date. It is possible that FBHH may also

map to as-yet-unidentified genes that encode for additional CaSR or for proteins that alter the function of these sensors. $FBHH_{3q}$ appears to be the clinical manifestation of heterozygous reduction or loss of CaSR function in the parathyroid glands and renal tubules, whereas NSHPT is the phenotype of homozygous loss or reduction of CaSR function. On the other end of the spectrum, activating mutation in the CaSR causes under expression of PTH, otherwise known as autosomal dominant hypocalcemia (see Chapter 62). Further insights into the structure–function relationship of the CaSR will undoubtedly enhance our understanding of the wide spectrum of the phenotypic expressions of FBHH and NSHPT. Furthermore, as-yet-unexplained phenotypic features of NSHPT may be providing subtle hints in this "experiment of nature" of currently unknown roles for the CaSR in nonclassical calcium-targeted organs. When the physician suspects FBHH in an individual patient, current knowledge permits only presumptive nongenetic diagnosis of the syndrome, but continuing research may soon lead to DNA-based screening studies not only for diagnosis of the FBHH syndrome, but also for determining the specific molecular basis, in individual patients. The goal of avoiding needless surgery in FBHH would then be achievable.

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CHAPTER 58

Multiple Endocrine Neoplasia Type 1

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Introduction

Multiple endocrine neoplasia type 1 (MEN1) syndrome is a rare disorder presenting with varying combinations of parathyroid adenoma, gastrinoma, and prolactinoma. MEN1 can be operationally defined as a case with two of these three principal MEN1-related endocrine tumors. Other endocrine and nonendocrine neoplasms such as foregut carcinoid, lipoma, and skin tumor are also common. Furthermore, other endocrine and nonendocrine tissues can be affected but with a lower frequency (Table I). Many tumors in MEN1 are benign, even if some entero-pancreatic neuroendocrine tumors (mainly gastrinomas) and foregut carcinoids are often malignant. Though often regarded as a treatable endocrinopathy, MEN1 can also be regarded as a cancer syndrome lacking effective prevention or cure for associated malignancies. MEN1 exhibits an autosomal dominant pattern of inheritance. There is growing evidence of sporadic cases, some associated with mutation of the *MEN1* gene. Familial MEN1 is defined as a family with one MEN1 case plus a first-degree relative, showing one of the three principal tumors. The tumor develops after loss of function via inactivation of both gene copies at chromosome $11q12-q13$, a process that led to initial localization of the *MEN1* gene and also that assisted later in *MEN1* gene discovery (Larsson *et al.,* 1988; Chandrasekarappa *et al.,* 1997).

The syndrome was originally described as an autopsy finding in a patient with acromegaly and four enlarged parathyroid glands (Erdheim, 1903). More than 20 years later, two groups reported a patient with the triad of parathyroid, pancreatic, and pituitary adenomas (Cushing and Davidoff, 1927). In 1953, Underdahl *et al.,* published an early review of related syndromes with a total of 14 cases (Underdahl *et al.,* 1953). However, it remained for Wermer to suggest that the trait was caused by an autosomal dominant gene with high penetrance (Wermer, 1954). This insight is commemorated by referring to MEN1 also as Wermer's syndrome.

The clinical features of the disorder were then clarified greatly after 1960, when radioimmunoassays for the various products of the affected endocrine tissues were developed (Yalow, 1978; Malarkey, 1979; Nussbaum and Potts, 1991). Progress in the management of MEN1 paralleled knowledge of the pathogenesis of common-variety endocrine tumors and included improving surgical and pharmacological interventions (Albright and Ellsworth, 1990; Bevan *et al.,* 1992; Schmid *et al.,* 1961).

Clinical Aspects

MEN1 Variants

Due to the constantly growing clinical and genetic knowledge about this syndrome, the MEN1-associated endocrinopathies, and their sporadic counterparts, it has been possible to recognize peculiar MEN1 variants and

Table I MEN1-Associated Tumors

Typical tumors (or more frequent)	Less prominent tumors	Rare tumors
	Endocrine	
Parathyroid adenoma (≈90%)	Foregut-derived carcinoid* (5%)	Pheochromocytoma
Anterior Pitutitary (≈25%)	Adrenal cortical (≈25%)	Ependymoma
(prevalently PRLoma) (20%)	(mainly nonfunctioning)	Leiomyoma
Neuroendocrine cells of g-I tract (gastrinoma, insulinoma, VIP-oma, glucagonoma, PP-oma) (≈50%)		Ductal adenocarcinoma of the pancreas
	Nonendocrine	
	Lipoma (visceral and cutaneous) (≈30%)	
	Skin tumor [facial angiofibroma (85%), collagenoma (70%)]	

phenocopies (phenocopy, similar phenotype but not from the same gene) (Marx, 2001).

Since primary hyperparathyroidism (PHPT) is the earliest and most frequent expression of MEN1, familial isolated hyperparathyroidism (FIHP) (Kassem *et al.,* 1994) in one-fifth of families with isolated hyperparathyroidism can be a prelude to typical MEN1 or an atypical expression of MEN1 (Marx *et al.,* 1982). However, in a much larger and more "elderly" family, isolated hyperparathyroidism could be a distinctive variant from *MEN1* mutation (Kassem *et al.,* 2000).

A variant (or phenotype or subtype), caused by *MEN1* mutation, termed the prolactinoma or "Burin" MEN1 variant shows high penetrance of hyperparathyroidism and of prolactinoma and low penetrance of gastrinoma (Olufemi *et al.,* 1998). The prolactinoma MEN1 variant has been associated with *MEN1* mutation or 11*q*13 linkage in each of three tested families (Agarwal *et al.,* 1997). The *MEN1* mutations in FIHP and "Burin" MEN1 phenotypes have not shown any informative pattern; thus, the causes of these phenotypes are not known. A family with an MEN1-variant and proven or likely *MEN1* mutation should remain under surveillance for other expressions of MEN1.

MEN1 Phenocopies

An MEN1 phenocopy is a trait that strongly resembles MEN1 in a patient or a family. It could be a case of FIHP in which *MEN1* mutation analysis failed to detect mutation; thus, other genes such as the calcium-sensing receptor gene (*CaSR*), accounting for familial hypocalciuric hypercalcemia (FHH) syndrome (Pollak *et al.,* 1993), and the putative gene responsible for the hyperparathyroidism–jaw tumor syndrome (HPT-JT) (Szabo *et al.,* 1994; Teh *et al.,* 1998a) might be involved.

Familial isolated pituitary tumor, generally somatotrophinoma, has so far yielded no *MEN1* mutation with a collective experience of more than 15 tested families (Tanaka *et al.,* 1998; Teh *et al.,* 1998b). Linkage analysis has been possible in only one large family, pointing to 11*q*13, and thereby suggesting undiscovered mutation of *MEN1* or a nearby gene (Gadelha *et al.,* 2000).

Primary Hyperparathyroidism in MEN1

Primary hyperparathyroidism is the most common endocrinopathy in MEN1, reaching nearly 100% by age 50 (Skarulis, 1998), and it is usually the first clinical manifestation, recognized by age 8 in several cases (Trump *et al.,* 1996).

The clinical features of the parathyroid hyperfunction in MEN1 show similarities to those of sporadic or common-variety primary hyperparathyroidism (Bilezikian *et al.,* 1995; Kleerekoper, 1995) with a long period of asymptomatic hypercalcemia and a low morbidity. The complications of the disorder are prevented by early intervention from an experienced surgeon.

In contrast to sporadic hyperparathyroidism, hyperparathyroidism (often asymptomatic) in patients with MEN1 develops at a younger age (Betts *et al.,* 1980; Skogseid *et al.,* 1991). Its average onset age is 20–25 years (Trump *et al.,* 1996; Skarulis *et al.,* 1998), three decades earlier than typical for sporadic parathyroid adenoma. Moreover, different from sporadic primary hyperparathyroidism (Heath *et al.,* 1980), primary hyperparathyroidism in MEN1 has a similar prevalence in females and males. Finally, unlike single adenoma of sporadic primary hyperparathyroidism, primary hyperparathyroidism in MEN1 is associated with multiple and asymmetric parathyroid gland enlargement (Hellman *et al.,* 1992; Marx *et al.,* 1991). MEN1-associated

hyperparathyroidism increases gastrin secretion from gastrinoma(s) and consequently a successful parathyroidectomy could decrease the severity of the Zollinger–Ellison (ZES) in MEN1 (Jensen, 1997). Still, because of excellent pharmacotherapy for ZES, ZES does not represent a sufficient indication for parathyroidectomy in MEN1.

After successful subtotal parathyroidectomy, hyperparathyroidism in MENI recurs progressively, reaching 50% by 8–12 years after surgery (Rizzoli *et al.,* 1985; Burgess *et al.,* 1998). It can be due to the growth of a neoplastic remnant of parathyroid tissue or to a new somatic mutation at 11*q*13 in the parathyroid remnant.

Burgess *et al.,* (1999) reported a reduction of bone mass in MEN1-affected women with hyperparathyroidism by 35 years of age (44%), with an increased likelihood of skeletal fractures. Parathyroidectomy and maintenance of a normocalcemic state resulted in improvement of both lumbar and femoral BMD.

Enteropancreatic and Foregut Carcinoid Manifestations of MEN1

The prevalence of enteropancreatic involvement in MEN1-affected individuals varies in different clinical series at 30–75% (Marx, 2001; Skogseid *et al.,* 1991; Vasen *et al.,* 1989) and approaches 80% in surgery or autopsy series. An enteropancreatic tumor is often recognized due to clinically overt features during the fifth decade but may be biochemically detected much earlier (third decade) in asymptomatic *MEN1* carriers.

Unlike nonfamilial variety endocrine tumor of the upper gastrointestinal tract, tumors in patients with MEN1 are usually multiple and develop at a younger age; MEN1-associated gastrinoma and insulinoma exhibit an average onset age 1 decade earlier than their sporadic counterparts (Skarulis, 1998). Moreover, the gastrointestinal manifestations are influenced by other endocrinopathies (mainly primary hyperparathyroidism) (Bone, 1990; Norton *et al.,* 1993).

Initial presentation of MEN1 as occurs only in a small proportion of patients (Benya *et al.,* 1993). The early presentation of these tumors is frequently associated with the lack of detection of the tumor by routine abdominal imaging techniques (Galiber *et al.,* 1988; Norton *et al.,* 1993). The majority of tumors with MEN1 ZES are in the duodenum, and are usually small (Ø <0.5 cm). The most frequent entero-pancreatic functional endocrine tumors in MEN1 are gastrinomas (54%) and benign insulinomas (21%) (Eberle and Grun, 1981; Jensen and Gardner, 1993; Metz and Jensen, 1993; Norton *et al.,* 1993). About 40% of MEN1 cases exhibit gastrinoma, and about one-fourth of all gastrinoma cases have MEN1. Most MEN1 gastrinomas are malignant; half have metastasized before diagnosis (Townsend *et al.,* 1990). One-third of sporadic and MEN1-associated ZES cases eventually die from the malignant aspects of their tumor(s). Poor prognosis is associated with pancreatic (not duodenal) primary, metastases (lymph node, liver, or bone), ectopic Cushing syndrome, or very high gastrin level (Yu *et al.,* 1999).

The diagnosis of gastrinoma is made by elevated serum gastrin and high gastric acid output, with or without symptoms. Confirmatory tests can include pentagastrin-stimulated acid output and secretin-stimulated gastrin levels (Wiedenmann *et al.,* 1998). A 72-hr fast protocol may be helpful in the diagnosis of insulinoma.

Glucagonomas, VIP-omas, GRF-omas, and somatostatinomas have also been described in MEN1 (Eberle and Grun, 1981; Norton *et al.,* 1993). However, nonfunctional[1] endocrine tumors are the most prevalent enteropancreatic endocrine neoplasms in MEN1, more than half being PP-omas (Eberle and Grun, 1981; Metz and Jensen, 1993; Norton *et al.,* 1993). Type II tumors of gastric histamine-secreting enterochromaffin-like cells (ECL-omas or gastric carcinoids) frequently accompany with Zollinger–Ellison-associated hypergastrinemia (Jensen and Gardner, 1991; Maton and Dayal, 1991). It is generally believed that prolonged hypergastrinemia causes ECL-omas (Frucht *et al.,* 1991; Maton and Dayal, 1991; Bordi *et al.,* 1998). This is particularly true for patients with MEN1 (Frucht *et al.,* 1991; Jensen and Gardner, 1991; Maton and Dayal, 1991).

Though sporadic carcinoid tumors are mainly in the midgut or hindgut, MEN1 carcinoids are all in the foregut (Duh, 1987; Godwin, 1975). Foregut carcinoids in MEN1 rarely oversecrete amine or peptide hormones. MENl bronchial carcinoid is mainly in females. MEN1 thymic carcinoid exhibits a male predominance. Cigarette smoking appears to be associated with a higher risk for thymic carcinoid (Teh *et al.,* 1997). Carcinoid tumors can be asymptomatic until a late stage, and their course appears more aggressive with than without MEN1.

Tumors of the Anterior Pituitary or the Adrenal in MEN1

An anterior pituitary tumor is the first clinical manifestation of MEN1 in less than 20% of cases (Carty *et al.,* 1998). The prevalence of pituitary tumor in MEN1 patients ranges from 10 to 50% (Metz *et al.,* 1994) of affected members, depending on the patients and methods utilized in the various studies. Microadenomas (Ø <1 cm) represent about 65% of the MEN1-associated pituitary tumors. Every type of anterior pituitary adenoma, except "true" gonadotropinoma, has been reported in MEN1 (Teh *et al.,* 1998b; Corbetta *et al.,* 1997). The frequency of plurihormonal tumors is greater in MEN1 tumors, with fewer null-cell tumors when compared to sporadic isolated pituitary tumors (Scheithauer *et al.,* 1987).

The mean age at the time of diagnosis of MEN1 pituitary tumors is about 40 years (Oberg *et al.,* 1989; Vasen *et al.,*

[1]Nonfunctional tumors are those that do not make a known hormone, make a hormone but do not secrete enough to cause a syndrome, or make and secrete a hormone (such as pancreatic polypeptide or calcitonin) that does not cause a syndrome.

1989; Skarulis, 1998), similar to that for sporadic isolated pituitary tumors (Scheithauer *et al.,* 1987). Prolactinoma (with or without simultaneous GH oversecretion) is the most frequent pituitary tumor in MEN1, followed by GH-oma (Scheithauer *et al.,* 1987).

Primary adrenocortical neoplasms are common in MEN1 (20–40%) and they are usually bilateral, hyperplastic, and nonfunctional (Skarulis, 1998; Skogseid *et al.,* 1992, 1995; Burgess *et al.,* 1996). Glucocorticoid excess, albeit rare, more often results from pituitary oversecretion of adrenocorticotropic hormone (ACTH) (Maton *et al.,* 1986). Hyperaldosteronism has been reported (Beckers *et al.,* 1992). Most of the adrenal enlargements exhibit an indolent clinical course (Metz *et al.,* 1994; Burgess *et al.,* 1996).

Unilateral pheochromocytoma is rare in MEN1. However, since 11*q*13 LOH is associated, pheochromocytoma should be considered a direct result of *MEN1* gene inactivation (Cote *et al.,* 1998).

Nonendocrine Tumors

Lipomas are frequently associated with MEN1 (20–30%) (Marx *et al.,* 1982; Metz *et al.,* 1994; Darling *et al.,* 1997). They are often multiple and can also occur viscerally. Lesions, often multicentric, may be small or large and cosmetically disturbing. They typically do not recur after removal. Multiple facial angiofibromas were observed in 40–80% of MEN1 patients, with half of the cases having five or more; Hoang-Xuan and Steger, 1999; Sakurai *et al.,* 2000); trunkal collagenomas were almost as common (Darling *et al.,* 1997). Angiofibromas are clinically and histologically similar in MEN1 or in tuberous sclerosis. Leiomyoma of the esophagus, uterus, or rectum has occurred; the frequency of leiomyoma in MEN1 has not been analyzed (Vortmeyer *et al.,* 1999; Dackiw *et al.,* 1999).

Treatments

Primary Hyperparathyroidism

Surgery for hyperparathyroidism in MEN1, more so than for the sporadic form, is the preferred treatment. The decision about timing for parathyroid surgery usually take into account the following criteria: (1) severity of symptoms or signs of HPT; (2) concentration of circulating PTH and calcium; (3) presence of MEN1-associated endocrinopathies, especially ZES; (4) patient age. Successful treatment of HPT is often followed by a decrease of the elevated circulating levels of gastrin. Persistence, late recurrence, and hypoparathyroidism are all more frequent after surgery for MEN1 than sporadic adenoma. Several approaches address these problems. First, surgery is delayed for stronger indications in MEN1 than sporadic cases. Second, several approaches to subtotal parathyroidectomy are aimed at limiting the frequency of hypoparathyroidism.

The development of a rapid intraoperative PTH assay makes it possible to monitor the correction of hyperparathyroidism, to assure when the important tumors have been removed, and to perform, when necessary, an immediate parathyroid autograft (Tonelli *et al.,* 2000).

Recently, calcium-sensing receptor agonists, a new and novel class of drugs, have been demonstrated to act directly on the parathyroid gland, decreasing PTH release (Silverberg *et al.,* 1997), and perhaps even decreasing parathyroid tumor growth. They might acquire an important role in treatment of hyperparathyroidism, including that from MEN1.

Subtotal Parathyroidectomy

Subtotal parathyroidectomy is the surgical ablation of three parathyroid glands and part of the fourth gland, leaving no more than 50 mg of tissue of the least compromised gland. Some authors suggest that the whole fourth parathyroid gland be saved when it appears to be of normal size. This kind of procedure has been proposed in order to avoid permanent hypoparathyroidism and to reduce the period of temporary postsurgical hypocalcemia. Rates of chronic hypocalcemia with this surgical approach range from 0 to 30% (Goretzki *et al.,* 1991; Grant and Weaver, 1994; Kraimps *et al.,* 1992; O'Riordain *et al.,* 1993; Thompson and Sandelin, 1994). A considerable rate of recurrence of hyperparathyroidism has been described after successful subtotal parathyroidectomy in MEN1. The incidence of recurrence increases proportionately with time after surgery (Hellman *et al.,* 1992; Kraimps *et al.,* 1992; Prinz *et al.,* 1981; Rizzoli *et al.,* 1985; Burgess *et al.,* 1998a). It is uncertain if the recurrence of hyperparathyroidism is due to the increased function of the tumor remnant or rather to new development of a tumor in a normal remaining ectopic or supernumerary parathyroid gland.

Total parathyroidectomy with autologous parathyroid graft (Wells *et al.,* 1980) is a more radical approach for MEN1-associated hyperparathyroidism. The whole parathyroid and thymic tissues are removed for the purpose of both avoiding subsequent cervical exploration and thymic carcinoid onset. The parathyroid graft is performed according to two main procedures. The first is the use of fresh autologous tissue stored in refrigerated saline solution and grafted immediately at the end of surgery. The advantage of this approach is that the graft is more likely to succeed. The disadvantage is not being able to verify the absence of functioning tissue remnant, normal remaining ectopic gland, or supernumerary gland. In the second procedure, implantation is performed some days or months after surgery using cryopreserved tissue. With this approach the lack of circulating PTH can be evaluated after parathyroidectomy. However, the viability of parathyroid tissue may be lower after cryopreservation. In both techniques, the parathyroid gland to be transplanted is the one that macroscopically exhibits the features closest to a normal gland possibly lacking nodular histopathology. The tissue to be transplanted varies from 5 to 20 1-mm^3-sized fragments. The graft is generally placed within the brachioradialis muscle so that it is possible to verify its function by simple blood sampling from the ipsilatered antecubital vein.

Because normal parathyroid glands can also be found in MEN1 patients, some authors believe that four-glands surgery is not always necessary. In this way the risk of permanent hypocalcemia is avoided. If partial parathyroidectomy is performed, an acceptable approach includes removal of the pathological and the normal homolateral gland, including thymectomy, and biopsy of the contralateral apparently normal parathyroids (Dralle and Scheumann, 1994). Consequently, the risk of persistent hyperparathyroidism is reduced and the lack or presence of multiglandular parathyroid involvement is determined. Whatever the approach to parathyroidectomy, an initial neck operation in MEN1 should include transcervical thymectomy. Most of the thymus can thereby be removed along with possibly included parathyroid tissue and carcinoid tissue.

Neuroendocrine Tumors

GASTRINOMA

Gastric acid hypersecretion in virtually all patients with hypergastrinemia can be effectively controlled using histamine H_2-receptor antagonists or H^+, K^+-ATPase inhibitors (Brunner *et al.,* 1989a,b; Jensen, 1997).

The surgical approach, if any, to be taken for gastrinoma(s) in MEN1 is controversial (Norton *et al.,* 1999). Some authors recommend surgery only in case of precise localization of gastrinoma, or in presence of particularly aggressive familial gastrinomas (Melvin *et al.,* 1993; Sheppard *et al.,* 1989), while other authors would perform surgery in all cases (Thompson *et al.,* 1993). Surgery has to follow the necessary procedures of tumor localization, such SRS and selective infusion of secretin or calcium in selective pancreatic arteries followed by hepatic vein sampling for gastrin measurement (Imamura and Takahashi, 1993). Even though none of these procedures is completely reliable for identifying sites of hypersecretion, *trans*-arterial challenge can indicate if excessive hormonal release is in the head or in the corpo-caudal segment. Small duodenal gastrinomas are not easily appreciated by external palpation, by intraoperative ultrasound, or by duodenal transillumination. The best approach appears to be intraluminal digital examination of tumors after large longitudinal exposure of the duodenum. The possibility that removal of duodenal gastrinomas and peripancreatic periduodenal lymph nodes might cure hypergastrinemia has been proposed. However, the experience is still insufficient to be certain. A more aggressive surgical approach is represented by the duodenum–cephalous pancreatectomy. This procedure should be used in those cases where gastrin production is clearly localized in the duodenum or head of pancreas. The procedure results in removal of duodenum, a frequent site of one or more gastrinomas, and removal of metastatic peripancreatic lymph nodes and of tumoral nodules from the pancreatic head. The great difficulty of this approach is principally represented by the high operative mortality and by postsurgical complications. In other functioning neuroendocrine tumors (glucagonoma, VIP-oma, PP-oma, and somatostatinoma) and nonfunctioning tumors, the therapeutic choice is easier, because the latter are generally represented by large neoplasms which are easily localized. The long-acting somatostatin analog octreotide is now considered to be the drug of choice for controling hormone secretion under conditions like glucagonoma and VIP-oma (Lamberts *et al.,* 1998; Maton *et al.,* 1989). Cases with systemic metastasis are usually excluded from surgery. Total pancreatectomy is generally not justified because of associated complications.

INSULINOMA

Although one-third of MEN1 microadenomas exhibit insulin immunoreactivity, only selected lesions larger than 5 mm are symptomatic (Kloppel *et al.,* 1986). Only rarely does insulinoma in MEN1 exhibit malignant degeneration, characterized by extrapancreatic infiltration and/or metastasis. Insulinoma surgery is mandatory for the following reasons: (a) generally hypoglycemia is not easily controlled by drugs; (b) during pancreatic surgery one or more incidental macroscopic lesions are found; (c) hypoglycemic syndrome is cured by radical resection of pancreatic macroscopical lesions (O'Riordain *et al.,* 1994) and; (d) malignant degeneration is prevented. Surgery has to be performed often with preoperative localization of a pancreatic nodule. The treatment of choice is removal of the main insulinoma with removal of residual tumors in the head of the pancreas. Splenic preservation is recommended when possible. Removal of nodules in the pancreatic head is needed in about 50% of patients. This surgical procedure can cause both pancreatic fistulas and pancreatitis. Intraoperative monitoring of glucose and insulin is helpful to verify removal of the tumor.

CARCINOIDS

Surgery remains the first choice of treatment of bronchial and thymic carcinoid tumors. In contrast to the surgical approach suggested for the tumors previously mentioned, the approach to ECL-omas in the setting of MEN1 is controversial, because of the multiplicity of gastric primary lesions and because of the uncertainties regarding the prospects of long-term cure as a result of partial or total gastrectomy. The treatment protocol for MEN1 ECL-omas should, therefore, be lowering of gastrin levels, intensified endoscopic surveillance therapy, and gastrectomy on appearance of macrolesions.

Somatostatin analogs have been successfully employed in the treatment of type II gastric carcinoids in three patients with MEN1 syndrome (Tomassetti *et al.,* 2000). During the treatment serum gastrin levels dramatically decreased and tumors regressed in all patients; one of the patients exhibited more than 30 foci widespread throughout the corpus and the fundus of the stomach that disappeared after 1 year of treatment. The regression of tumors was attributed to the decreasing of serum gastrin levels known to be an important factor in the pathogenesis of gastric carcinoid tumors in patients with MEN1 and ZES (Rindi *et al.,* 1996; Bordi *et al.,* 1998)

Pituitary Tumors

The therapy (medical, radiation, surgical) of pituitary tumors is the same as for sporadic pituitary tumors. Medical treatments with dopamine-agonists is the therapy of choice in patients with microprolactinoma.

Pathophysiology

MEN1 is an autosomal dominant disorder with penetrance that approaches 100% with increasing age and with a variable expression (different clinical phenotypes in affected members within a MEN1 kindred, in terms of tumor localization, onset age, and clinical aggressiveness) (Marx, in press). Pathogenic and pathophysiological issues include recent identification of the *MEN1*-encoded protein, named menin, and its largely unknown metabolic pathways at its target tissues.

Pathology/General Considerations

Pathological changes in the target organs of the MEN1 syndrome are more extended than suggested by clinical and biochemical features of the patients. Several lesions, including most pancreatic or gastric endocrine proliferations, can be clinically silent, and others may present overt symptomatology only at advanced stages. A review of 32 autopsy case reports (Majewski and Wilson, 1979) revealed that, by the age of 30, pathological lesions are consistently found in each of the three main involved glands (parathyroids, endocrine pancreas, pituitary), initiating the concept that the MEN1 syndrome is an "all-or-none" phenomenon. Whether this concept may apply to other organs whose involvement has become apparent since that review has not been clarified. These include the carcinoid tissues of the embryonic foregut (duodenum, stomach, lung, thymus), whose involvement may have serious clinical implications, as well as tissues that only rarely exhibit clinical morbidity in MEN1, such as adrenal cortex, adipose tissue, and skin (Friedman *et al.,* 1994; Padberg *et al.,* 1995; Darling *et al.,* 1997).

The sequence of events leading to neoplasia appears to be a subtle, diffuse, follows a similar pattern in all MEN1 target organs. On histopathological basis the initial lesion appears to be a diffuse proliferation of the affected endocrine tissue with bilateral involvement of paired organs and multifocal growth. Whether this change reflects polyclonal hyperplasia or *de novo,* arising multiple clonal lesions, however, has not been clarified yet.

The next step is development of multiple micro- and, eventually, macronodular lesions (Fig. 1). The transition between these lesions and true neoplasms is virtually unrecognizable on histopathological grounds. In fact, evidence from clonality studies in MEN1 parathyroids and pancreatic lesions revealed monoclonality of tissues classifiable as hyperplastic according to the established histopathological

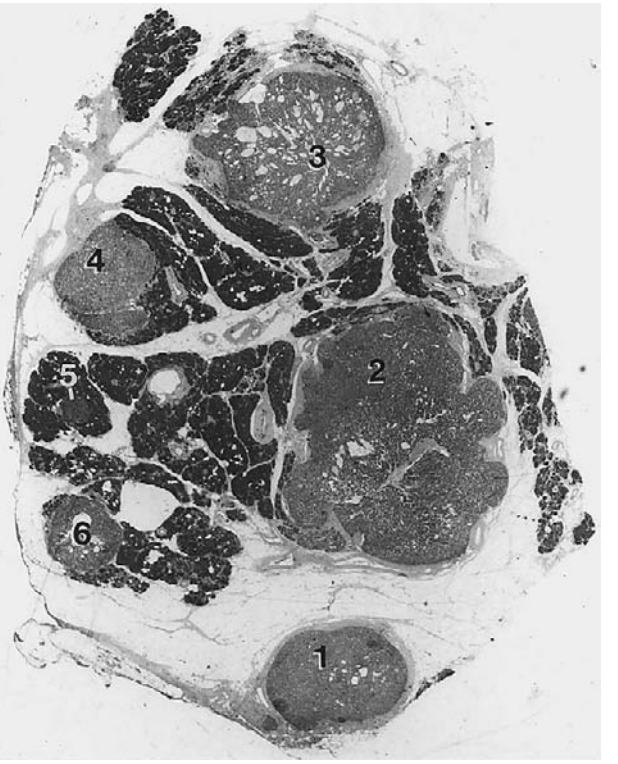

Figure 1 Numerous, scattered islet cell adenomas (numbered 1–6) of various size and architecture in a transverse section of the pancreas of a patient with MEN1 syndrome and ZES (H&E, 4). Reprinted with permission from Pilato *et al.* (1988).

criteria (Larsson *et al.,* 1988; Friedman *et al.,* 1989). LOH analysis of multiple tumors of the same organ, including parathyroids, pancreas, duodenum, and stomach (Morelli *et al.,* 1996; Lubensky *et al.,* 1996; Debelenko *et al.,* 1997a; Debelenko *et al.,* 1997b), consistently showed different patterns and sizes of chromosomal or subchromosomal deletions, indicating that each of multiple tumors is the result of an independent multinational event.

Malignancy in MEN1 syndrome is mostly confined to pancreatic, duodenal, thymic, and bronchial neoplasms. Notably, malignancy in the parathyroid is very rare in MEN1 (Sato *et al.,* 2000). Gastric carcinoid tumors may also pursue a very aggressive course (Bordi *et al.,* 1997; R. T. Jensen, pers. commun.). In a study of a large Tasmanian kindred enteropancreatic malignancies were found in 14 of 69 patients (20%) (Burgess *et al.,* 1998b). All but one of these patients also presented hypergastrinemia. Liver metastases are common in these cases but their origin may be difficult to ascertain. They may derive from either pancreatic (more commonly) or duodenal gastrinoma(s). However, ECL cell carcinoids of the stomach, which are dependent on the trophic stimulus of hypergastrinemia, may also contribute (Bordi *et al.,* 1998). Immunostaining for gastrin or for the isoform 2 of the vesicular monoamino transporter (VMAT-2), the latter of which is specific for ECL cells (Rindi *et al.,* 2000), may help to establish the primary tumor. As typical of endocrine tumors, histopathological

definition of malignancy based on traditional criteria is often flawed. The use of immunohistochemical markers of cell proliferation, however, is useful in this regard (Bordi and Viale, 1995).

Immunohistochemistry using markers of neuroendocrine cells, such as chromogranin A, synaptophysin, and neuron-specific enolase, as well as specific hormonal products of the individual endocrine glands is useful in tumor characterization and in the assessment of preneoplastic lesions. Due to the identification in serum of an MEN1 mitogenic factor (Brandi *et al.,* 1986), structurally similar to basic fibroblast growth factor (FGF) (Zimering *et al.,* 1993), immunohistochemistry, alone or in combination with molecular biology, has also been used to localize basic FGF in MEN1-related endocrine tissues. Ezzat *et al.* (1995) found high levels of basic FGF mRNA in adenomatous but not in normal pituitary glands, a finding consistent with the evidence of a pituitary origin of circulating basic FGF in MEN1 and sporadic pituitary tumor (Zimering *et al.,* 1993). Basic FGF mRNA levels correlated with *in vivo* and *in vitro* release of basic FGF but were consistently associated with lack of basic FGF immunoreactivity in tumor cells, suggesting a constitutive secretion of this peptide. However, basic FGF is elevated in many patients with MEN1 and may play a pathogenetic role (Hoang-Xuan and Steger, 1999). In contrast, immunohistochemical expression of basic FGF was well documented in the gastric proliferating ECL cells (Fig. 2) (Bordi *et al.,* 1994) and in pancreatic endocrine tumors (Chaudhry *et al.,* 1993) in both MEN1 and sporadic patients.

The expression of glycoprotein hormone α-subunit has been revealed by immunohistochemistry in all types of endocrine tumors involved in the MEN1 syndrome, including those of pituitary, pancreas, foregut, and parathyroids (Carlinfante *et al.,* 1998) (Fig. 3). Such expression has been regarded as a marker of malignancy in pancreatic (Heitz *et al.,* 1983) but not in gastric (Bordi *et al.,* 1995a) or pulmonary (Bonato *et al.,* 1992) endocrine tumors. Burgess *et al.* (1998b) reported that serum levels of this protein were elevated in 71% of MEN1 patients with metastatic tumors but only in 7% of those without metastatic neoplasms ($P < 0.05$).

Pathology of the Parathyroids

It is generally assumed that in MEN1 syndrome all parathyroid glands are pathologically involved. Size heterogeneity of parathyroid glands is, however, a consistent finding in MEN1 (Friedman *et al.,* 1994; Padberg *et al.,* 1995) with an average ratio of 9.5 between volumes of the largest and the smallest glands (Marx *et al.,* 1991). Small glands may show no detectable differences from normal glands, also when observed at the microscopic level (Harach and Jasani, 1992). When associated with asymmetric growth of one gland these findings rarely simulate adenoma (DeLellis, 1995). Parathyroid tumor within the thymus, in the thyroid gland, at the carotid bifurcation, or in the mediastinum may be responsible for surgical failures (Mallette, 1994).

By histology, the parathyroid changes are very similar to those of non-MEN1 hyperplasia or of secondary hyperparathyroidism although size variability is a more striking feature in MEN1 (Harach and Jasani, 1992). Both nodular and diffuse patterns of hyperplasia occur, the former being more frequently seen (DeLellis, 1995). In either pattern chief cells are the predominant cell type and are variously associated with oxyphil or transitional oxyphil cells. Neighboring nodules may strongly differ in their cytological and architectural features (Friedman *et al.,* 1994).

Morphological distinction between these hyperplastic nodules and true adenomas is difficult if not impossible (Friedman *et al.,* 1994; Padberg *et al.,* 1995). To date, the

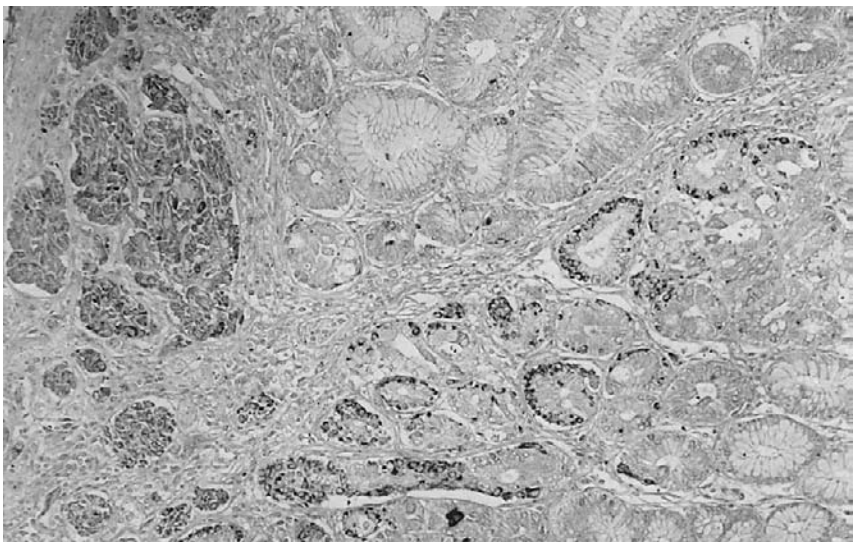

Figure 2 Immunostaining for basic FGF in the gastric endocrine cells of a patient with MEN1 syndrome and ZES. The peptide is expressed by both intraglandular cells showing hyperplastic pretumoral lesions (on the right) and carcinoid tumor (on the left) (immunoperoxidase, 100).

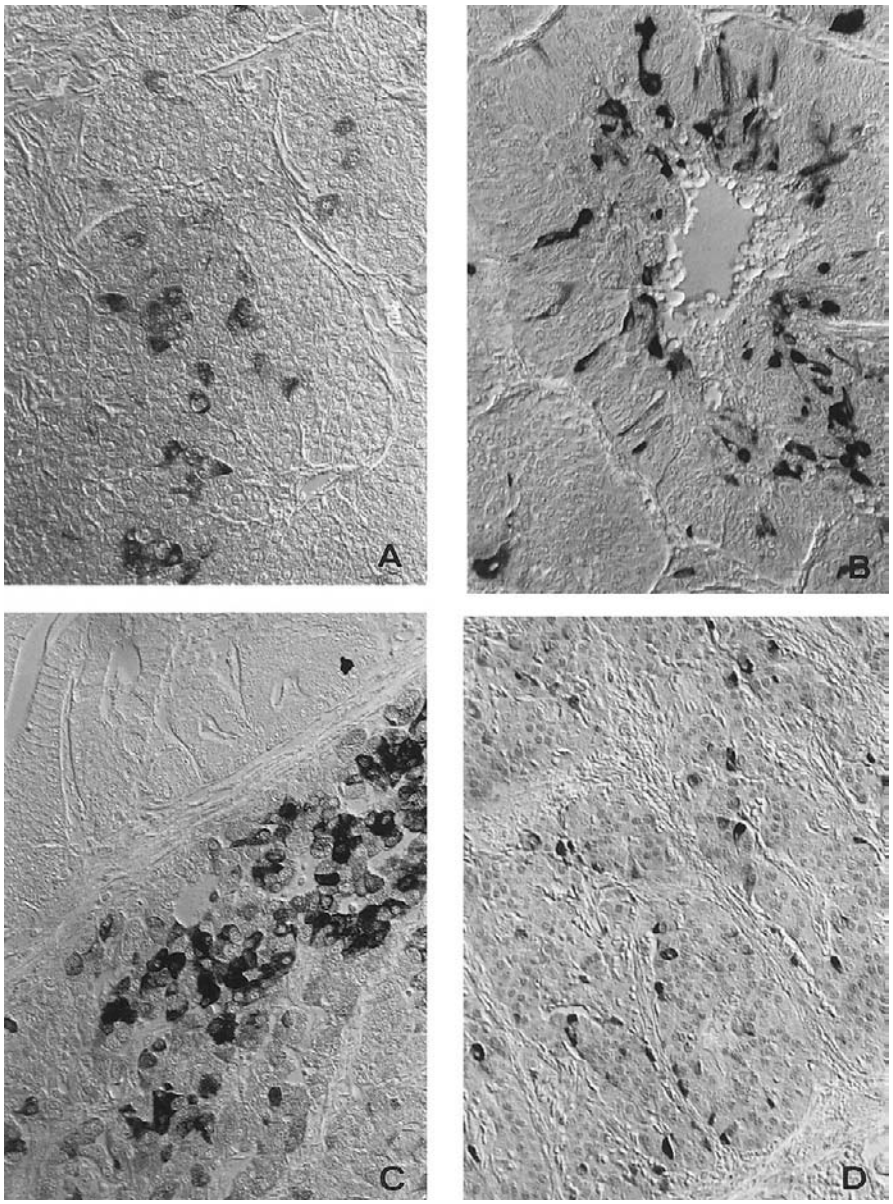

Figure 3 Immunohistochemical expression of γ-subunit of glycoprotein hormones in different types of tumors in MEN1 patients: parathyroid (A), lung carcinoid (B), gastric carcinoid (C), duodenal gastrinoma (D), (immunoperoxidase, 170).

issue has not been clarified even by clonality studies. As a rule the size of monoclonal lesions is significantly larger than that of polyclonal lesions (Friedman *et al.,* 1989; Falchetti *et al.,* 1993). Arnold *et al.,* (1995), using X-chromosome inactivation analysis in patients with either primary MEN1 and non-MEN1 parathyroid hyperplasia or uremic hyperparathyroidism demonstrated definite monoclonal status in 67% of glands with nodular hyperplasia and in 62% of glands with diffuse hyperplasia. These results cast doubts on the long-standing assumption that multiglandular parathyroid disease is a morphologic feature specific for polyclonal parathyroid hyperplasia.

The intensity and topographic distribution of parathyroid hormone immunoreactivity show pronounced variations in MEN1 parathyroid glands (Friedman *et al.,* 1994; Harach and Jasani, 1992). As a rule, hyperplastic areas reveal less immunoreactive hormone content than contiguous normal areas (Harach and Jasani, 1992), a finding consistent with defective mechanism(s) for hormone storage.

The endothelial cells of parathyroid glands, a target of the mitogenic activity of MEN1 growth factor (Brandi, 1991; Friedman *et al.,* 1994), have been investigated by ultrastructural morphometry in a series of MEN1 patients (D'Adda *et al.,* 1993). The parathyroid endothelial component was found to be significantly more expanded in MEN1 than in uremic hyperparathyroidism, indicating its potential role in the proliferation of parathyroid tissues in MEN1.

Pathology of Enteropancreatic Endocrine Tissues

Pathological changes of the pancreas in MEN1 patients have been often inconsistently defined, making evaluation of the actual incidence of specific lesions and of their relationships difficult and unreliable. Two basic changes showing specific functional and clinical characteristics, multiple islet cell microadenomas (microadenomatosis) and grossly single endocrine tumors, may be found (Friedman *et al.,* 1994; Klöppel *et al.,* 1986; Padberg *et al.,* 1995; Thompson *et al.,* 1984). Terms such as nesidioblastosis or islet cell hyperplasia should be avoided owing to their difficult histological identification and uncertain clinical significance.

Microadenomatosis is the most common pancreatic lesion in MEN1 cases. The multiple adenomas, scattered throughout the whole pancreatic gland (Fig. 1), may be very numerous (up to 100 in some cases) and range in size from microadenomas slightly larger than unaffected islets to occasional macroadenomas with a diameter larger that 0.5 cm. Like multiple nodules of parathyroid hyperplasia, even contiguous pancreatic lesions may exhibit striking variations in their histological arrangement, stromal content, and cytological characteristics. The adenomas are functionally silent and are consistently benign both histologically and clinically. They are composed exclusively of cell types normally present in the pancreas, often coexisting in single tumors but lacking the typical topographic distribution seen in normal islets. The immunoreactive cell composition of these adenomas is not random (Pilato *et al.,* 1988), a definite predominance of glucagon-, insulin-, and PP-producing cells being most often observed (Klöppel *et al.,* 1986; Pilato *et al.,* 1988; Padberg *et al.,* 1995; Le Bodic *et al.,* 1996). In small lesions such a predominance may be the only distinctive feature with respect to unaffected islets. In spite of their most frequent association with ZES, gastrin immunoreactivity is uncommonly found in these multiple adenomas, duodenal microgastrinomas, and/or their metastases being responsible for hypergastrinemia in these patients.

Large, macroscopically single endocrine tumors, indistinguishable from those found in those found without MEN1, are less frequently found in MEN1 syndrome. Usually they develop on the background of islet cell microadenomatosis. Like their sporadic counterparts, these neoplasms are frequently malignant and may metastatize to the liver. A large tumor size ($\geq$3 cm) is predictive of this metastatic evolution (Cadiot *et al.,* 1999). Macroscopically single pancreatic tumors are composed of cells producing either endogenous (insulin, glucagon, PP) or ectopic (gastrin, VIP, GHRF, calcitonin, PTH-related peptide) hormones and often show functional activity covering the wide spectrum of clinical syndromes associated with pancreatic endocrine tumors. It has been assumed that functioning insulinomas in MEN1 are more frequently single tumors curable with surgical resection (Klöppel *et al.,* 1986; Thompson *et al.,* 1984). However, others reported a frequent multicentricity of MEN1 insulinomas (DeLellis, 1995). When the one dominant insulinoma in MEN1 is removed with confirmation of its overproduction of insulin (preoperative insulin localization, intraoperative fine needle aspiration for insulin assay, intraoperative serum glucose and insulin), the nonfunctional feature of coexistent pancreatic nodules is evident (Libutti, S., unpublished).

Pathology of the Pituitary

At the pathological levels pituitary lesions are found in more than 60% of MEN1 patients (Scheithauer *et al.,* 1987; Capella *et al.,* 1995) and are often appreciated only when the gland is examined microscopically (Majewski and Wilson, 1979). They are usually represented by macroadenomas associated with diffuse or nodular hyperplasia of extratumoral pituitary cells (Capella *et al.,* 1995). The MEN1 tumors, like sporadic tumors, are grossly invasive in 10–15% of cases. By immunohistochemistry, they are most often found to produce GH or PRL, to be endocrinologically functioning, and to be plurihormonal more often than their sporadic counterparts.

Pathology of Diffuse Neuroendocrine System of Embryonic Foregut Origin

Duodenal gastrinomas are common MEN1 tumors, being the cause of the ZES in more than 90% of MEN1 cases (Padberg *et al.,* 1995). They are multiple tumors, often so small that they escape detection even at intraoperative inspection and so diffusely distributed that they might require removal of the whole duodenum for adequate correction of hypergastrinemia. Immunostaining for gastrin is usually strong. Metastases are frequent but mostly restricted to regional lymph nodes (Pipeleers-Marichal *et al.,* 1990).

Gastric, ECL cell carcinoids may develop in MEN1 patients affected by ZES through a sequence hyperplasia–dysplasia–neoplasia (Solcia *et al.,* 1988; Bordi *et al.,* 1998). In these cases hypergastrinemia acts as a potent promoter for ECL cell proliferation (Bordi *et al.,* 1995a). However, the lack of ECL cell carcinoids in cases of sporadic ZES indicates that biallelic inactivation of the *MEN1* gene (Cadiot *et al.,* 1993; Debelenko *et al.,* 1997a) is essential for the evolution of ECL cell hyperplasia into neoplasia (Solcia *et al.,* 1990; Bordi *et al.,* 1998). Malignant behavior is rare in these carcinoids (Rindi *et al.,* 1996). However, several recent cases of gastric endocrine tumors having aggressive behavior and very poor outcome have been observed (Bordi *et al.,* 1997; R. T. Jensen, pers. commun.). In one of these cases multiple benign gastric carcinoids coexisted with malignant poorly differentiated endocrine carcinomas causing the patient's death (Bordi *et al.,* 1997). 11*q*13 LOH was documented in these undifferentiated neoplasms.

Thymic and bronchial carcinoid tumors can also be regarded as an integral part of the MEN1 syndrome (Padberg *et al.,* 1995). The former are low-grade malignant neoplasms in more than 80% of cases, whereas 3/4 of the latter are benign (Duh *et al.,* 1987; Teh *et al.,* 1997).

Pathology of Other Organs

Various tumors of thyroid follicular cells, adrenal cortex, cutaneous, and visceral adipose tissue, and skin have been described in MEN1 patients (Brandi *et al.,* 1987; Friedman *et al.,* 1994; Padberg *et al.,* 1995). These tumors are mostly benign lesions. Multiple facial angiofibromas similar to those in tuberous sclerosis, and discrete papular collagenomas are commonly found in MEN1 patients (Darling *et al.,* 1997). Enlargement of adrenal glands *is* found in about 40% of MEN1 patients (Burgess *et al.,* 1996; Komminoth, 1997). The underlying pathologic lesions affect the adrenal cortex and are usually represented by diffuse or (macro)nodular hyperplasia or by adenoma although exceptional cases of carcinomas have been reported (Komminoth, 1997). Due to their common occurrence and lack of *MEN1* inactivation in the general population, thyroid lesions do not seem to be casually related to the MEN1 syndrome. In contrast, allelic loss at the *MEN1* locus has been reported in dermal angiofibroma, truncal collagenoma, esophageal leiomyoma and lipoma (Morelli *et al.,* 1995; Pack *et al.,* 1998; Vortmeyer *et al.,* 1999). Moreover, 11*q*13 LOH was found in rare adrenal medullary tumors (Skogseid *et al.,* 1992; Cote *et al.,* 1998b) but not found in the common adrenocortical hyperplasia of MEN1 patients (Skogseid *et al.,* 1992).

Miscellaneous Malignancies in Patients with MEN1 Syndrome

A number of malignant tumors of various, nonendocrine origin, including exocrine pancreas, kidney, bladder, endometrium, and skin have been reported in MEN1 patients (Bordi *et al.,* 1995b; Thompson *et al.,* 1984: Doherty *et al.,* 1998; Nord *et al.,* 2000). At least five cases of ductal pancreatic adenocarcinoma have been reported, suggesting an increased risk for this tumor in MEN1 (Bordi and Brandi, 1998). 11*q*13 LOH was not found in the single tumor investigated (Bordi *et al.,* 1995b). Malignant melanomas have been reported in seven typical patients from seven MEN-1 kindreds (Nord *et al.,* 2000). However, no role of the *MEN1* gene in the development of sporadic or familial melanomas is proven (Boni *et al.,* 1999; Nord *et al.,* 2000).

Biochemical and Radiological Tests for Tumors in Likely *MEN1* Mutation Carriers

Affected individuals may present with any of the MEN1-associated lesions in early teenage years or escape clinical symptoms for several decades (Skogseid *et al.,* 1991). A periodic investigation should be performed to look for the frequent endocrine and nonendocrine tumors associated with this syndrome in potentially affected individuals. MEN1-associated lesions may develop slowly in unaffected mutation carriers, despite earlier extensive biochemical testing. Early screening for MEN1 tumors in asymptomatic *MEN1* mutation carriers may help to reduce morbidity, but this is not proven. The overall age-related penetrance of tumors is near zero below age 5 years (Stratakis *et al.,* 2000), rising quickly to >50% by 20 years, and >95% by 40 years (Metz *et al.,* 1994; Trump *et al.,* 1996; Bassett *et al.,* 1998; Skarulis, 1998). Individuals at highest risk of developing MEN1 tumors (i.e., mutant *MEN1* carriers have near 100% lifetime risk) should be screened yearly for tumors. Screening should commence in early childhood, and it should continue for life (Trump *et al.,* 1996). However, screening for MEN1 tumors can be difficult and expensive because of large numbers of available and of recommended tests.

Primary hyperparathyroidism is often the first clinical manifestation and the most common pathology associated with the MEN1 syndrome (Lamers and Froeling, 1979; Marx *et al.,* 1986; Oberg *et al.,* 1982), being asymptomatic in 50% of hyperparathyroid cases. Accurate clinical and laboratory screening for parathyroid function (Table II) makes possible an early biochemical diagnosis. A bone mineral density evaluation by DEXA, QCT, or US, both at cortical and trabecular sites can define the fracture risk in cases with hyperparathyroidism. Preoperative parathyroid tumor imaging has little role in the unoperated case. Tc-99m Sesta-MIBI scan is the most useful among many methods prior to parathyroid reoperation. However, a well experienced neck surgeon remains the most important "clinical device" for successful surgery of hyperparathyroidism. Intraoperative measurement of PTH "on-line" is promising to test if deleterious amounts of parathyroid tumor remain during initial or repeat surgery (Tonelli *et al.,* 2000).

Gastrinoma constitutes the second commonest manifestation but the most morbid feature of MEN1 (Croisier *et al.,* 1971; Eberle and Grun, 1981). As for hyperparathyroidism, accurate clinical and biochemical screening could help the physician to detect enteropancreatic disease early, such as basal and maximal acid output, basal evaluation of gastrin, insulin, C-peptide, blood glucose, proinsulin, glucagon, PP, HCG, chromogranin A for an early detection of neoplasms (Skogseid *et al.,* 1987). Challenge tests (secretin or calcium stimulation for gastrin, standard meal for PP, supervised fasting for insulin) have been developed to increase sensitivity; they may be used selectively. Tests with abnormal levels should be repeated. False-positives include high proinsulin/-insulin levels in patients developing insulin resistance or

Table II Biochemical Screening of Primary Hyperparathyroidism in MEN1 Patients

Blood	Urine
Ca^{2+}	Calcium
Phosphate	Phosphate
Intact PTH	Pyridinium cross-links
Bone alkaline phosphatase	Cyclic AMP
Osteocalcin	Creatinine
25(OH)D	
$1,25(OH)_2D$	

hypergastrinemia in patients with hypochlorhydria. Somatostatin receptor scintigraphy ([^{111}In]DTPA-otreotide scan) and endoscopic ultrasound are proven pancreatic imaging methods; CAT or MRI are interchangeable here. Unfortunately, because of the small size and multiplicity of duodenal gastrinomas, these methods most sensitive for the pancreas have low sensitivity for MEN1 gastrinomas (Bansal *et al.,* 1999; Cadiot *et al.,* 1996).

Insulinoma negative to the preoperative imaging is usually readily identified by intraoperative ultrasonography (Bansal *et al.,* 1999). However, since radiology of the enteropancreatic lesion is neither sensitive nor specific, surgery should be done if the biochemical diagnosis is unequivocal, even without symptoms (Skogseid *et al.,* 1996; Granberg *et al.,* 1999).

Symptoms of pituitary neoplasms associated with the MEN1 syndrome depend on both the tumor volume and the hormonal secretion from the gland. Prolactin is the most frequent product of hypersecretion from the anterior pituitary (Croisier *et al.,* 1971; Eberle and Grun, 1981; Majewski and Wilson, 1979). Circulating prolactin measurement should be performed under basal conditions at 0 and 60 min in order to avoid an influence of stress on hormonal secretion. MRI is the preferred pituitary imaging method. Finally, even in cured patients, pituitary tumor screening should continue as *MEN1* somatic mutations ("second hits") in the remaining pituitary cells may predispose to recurrence or the development of new adenoma.

Computed tomography or magnetic resonance imaging of the chest are recommended for early diagnosis of thymic or bronchial carcinoids. Type Il gastric enterochromaffin-like (ECL) cell carcinoids are recognized mainly at gastric endoscopy incidental to evaluation for ZES.

Germline DNA-Based Tests

Human genetic diseases can be derived from different and complex molecular lesions. The simplest to explain are disorders from germline mutation of a single gene. Genetic diagnosis of monogenic disorders is made possible only if either cloned material from a specific gene is sequenced to allow mutation testing or the chromosomal localization of the specific gene is known, and tightly linked genetic markers have been characterized. Testing the *MEN1* mutation carrier state is possible by either approach.

The application of the techniques of molecular genetics has enabled the identification of the gene causing MEN1 syndrome and the detection of its mutations in patients. These recent advances led to experience with an *MEN1* mutation test for the identification of mutant *MEN1* gene carriers who are at a high risk of developing this disorder.

Most laboratories find an *MEN1* mutation in 70–100% of index cases in typical MEN1 families (Agarwal *et al.,* 1997; Teh *et al.,* 1998b; Marx, 2001). The positive rate is lower (20–80%) in index cases with sporadic MEN1. The positive rate is even lower (20%) in familial isolated hyperparathyroidism (Kassem *et al.,* 2000), and the rate is zero in familial somatotrophinoma (Tanaka *et al.,* 1998; Gadelha *et al.,* 2000). There has been no genotype–phenotype relationship, including unrevealing *MEN1* mutations in index cases for families with the prolactinoma variant of MEN1 (Agarwal *et al.,* 1997).

ADVANTAGES OF DNA-BASED ANALYSIS

The advantages of *MEN1* mutation analysis are first that it does not need to be repeated serially, unlike the biochemical screening test for ascertainment of MEN1 through tumor discovery. Second, most results of an *MEN1* mutation test influence long-range planning. Thus, a test identifying an *MEN1* mutant gene carrier may lead to earlier and more frequent biochemical screening for tumors. Screening for MEN1 tumors in asymptomatic mutation carriers may help to reduce morbidity. Moreover, finding family members at no risk for tumors because of no mutation may lead to a decision for no further screening.

LIMITATIONS OF DNA-BASED ANALYSIS

Unfortunately, the obtained information does not influence an immediate intervention or longevity. This reflects the lack of a therapy or prevention of proven value for MEN1-associated malignancy. Second, mutation analysis may provide false negatives since *MEN1* mutations are not detectable in 5–30% of MEN1 families. If large enough, virtually any MEN1 family is likely to be positive for an 11*q*13 haplotype (Larsson *et al.,* 1995; Courseaux *et al.,* 1996).

DNA-BASED ANALYSIS OF TUMORS

Tumor DNA has been evaluated extensively for 11*q*13 LOH and for *MEN1* mutation. These analyses have contributed importantly to research. In particular, analysis of 11*q*13 LOH contributed to narrowing the *MEN1* gene candidate interval for gene discovery (Emmert-Buck *et al.,* 1997; Chandrasekharappa *et al.,* 1997) and to understanding the clonality of certain lesions, such as parathyroid tumors and skin lesions in MEN1 (Friedman *et al.,* 1989; Pack *et al.,* 1998). *MEN1* mutation analysis confirmed a role of *MEN1* inactivation in oncogenesis of many sporadic tumors (Heppner *et al.,* 1997; Boni *et al.,* 1998). On the other hand, analysis of tumor DNA for *MEN1* involvement has little application in current clinical settings. It does not give a useful predictor of tumor aggressiveness, and it does not substitute for mutation analyses of germline DNA.

Early DNA-Based Studies of the *MEN1* Gene

CHROMOSOMAL LOCATION OF THE *MEN1* GENE

Combined studies of both linkage in MEN1 kindreds (Larsson *et al.,* 1988), and microdeletion analysis in tumors (Larsson *et al.,* 1988; Friedman *et al.,* 1989, Emmert-Buck *et al.,* 1997) demonstrated that the MEN1 trait was in close linkage to *PYGM*, the gene for muscle phosphorylase, at chromosome 11*q*13. The proposed model for tumorigenesis in familial MEN1 was according to Knudson's "two hit" hypothesis (Knudson, 1993). In fact, an affected *MEN1* copy is inherited in the germline from the affected parent

(first hit) while the wild copy, from the healthy parent, is eliminated at the somatic level (second hit). The second hit was indirectly suggested by loss of heterozygosity or allelic loss when tumoral DNA is compared to germline DNA. These findings strongly suggested gene inactivation as the mechanism of tumorigenesis from *MEN1*.

THE NORMAL *MEN1* GENE

In 1997 the *MEN1* gene was identified by positional cloning (Chandrasekharappa *et al.,* 1997; Lemmens *et al.,* 1997).

The gene spans 9 kb and consists of 10 exons with a 1830-bp coding region (Fig. 4) that encodes a novel 610-amino-acid protein, referred to as menin. The first exon and last part of exon 10 are not translated. A major transcript of 2.8 kb has been described in a large variety of human tissues (pancreas, thymus, adrenal glands, thyroid, testis, leukocytes, heart, brain, lung, muscle, small intestine, liver, and kidney, Lemmens *et al.* (1997)). Moreover, an additional transcript of approximately 4 kb has been detected in pancreas and thymus, suggesting a tissue-specific alternative splicing (Lemmens *et al.,* 1997).

MENIN: THE *MEN1* GENE-ENCODED PRODUCT

Initial analysis of the predicted amino acid sequence encoded by the *MEN1* transcript did not reveal homologies to any other proteins, sequence motifs, signal peptides, or consensus nuclear localization signals and thus the putative function of the protein menin could not be predicted (Guru *et al.,* 1998).

Intracellular Localization The first studies, based on immunofluorescence, revealed that menin was located primarily in the nucleus. Furthermore, at least two independent nuclear localisation signals (NLS) were identified and located in the C-terminal quarter of the protein (Guru *et al.,* 1998). All of the truncated *MEN1* proteins that would result from the nonsense and frameshift mutations, if expressed, would lack at least one of these NLS.

Menin Molecular Partner The nuclear localization of menin suggested that it may act either in the regulation of transcription, DNA replication, or in the cell cycle. Further studies performed in order to identify proteins that may interact with menin revealed that it directly interacts with the N-terminus of the AP-1 transcriptional factor (Karin *et al.,* 1997) JunD and that it can repress JunD-activated transcription (Agarwal *et al.,* 1999). Analysis of several *MEN1* missense and deletional mutations indicated that a central domain of menin and an amino-terminal domain of junD have a critical role in menin–JunD interaction (Agarwal *et al.,* 1999; Guru *et al.,* 1999). Menin might inhibit junD-activated transcription by recruiting histone deacetylase, a common mediator for many inhibitors of transcription, the so-called corepressors (Gobl *et al.,* 1999). Any role of the menin–JunD interaction remains to be proved. Separate studies have suggested that junD has growth suppressive activity (Agarwal *et al.,* 1999) and that homozygous knockout of the junD gene in the mouse does not cause tumors or tissue effects resembling those in MEN1 (Thepot *et al.,* 2000). Furthermore, the drosophila homolog of menin does not interact with the drosophila jun molecule or even with the human or mouse junD (Guru *et al.,* in press).

Lymphocytes from patient with heterozygous *MEN1* mutation exhibit a premature centromere division, suggesting a possible role of menin in controlling DNA integrity (Sakurai *et al.,* 1999). Hypersensitivity to alkylating agents occurs in lymphocytes from patients with MEN1 (Itakara *et al.,* 2000) with a possible role of menin as a negative regulator of cell proliferation after one type of DNA damage (Ikeo *et al.,* 2000).

MUTATION TYPES IN THE *MEN1* GENE

About 300 different mutations, both somatic and germline, of the *MEN1* gene have been identified. The mutation are varied and scattered throughout the coding region with no clear evidence for clustering (Fig. 4). Approximately half of mutations are unique. The other half are repeating mutations; the repeats reflect either a common founder or a mutational hotspot (Agarwal *et al.,* 1998). These potential mutational hot spots could be due to the presence in the vicinity of repeated long tracts of either single nucleotides or shorter elements. These repeated sequences may undergo misalignment during replication; a slipped strand mispairing model is the most likely mechanism to be associated with this sort of mutational hot spot (Agarwal *et al.,* 1999; Calender, 1999). All or most *MEN1* mutations are likely inactivating, as expected for a tumor suppressor gene: 60% of mutations are frameshift and 25% are nonsense. Truncating mutations predict an inactive or absent protein product. None of them could be associated to specific clinical manifestations of the disorder. Thus genotype/phenotype correlations appear to be absent in MEN1 (Teh *et al.,* 1998b; Agarwal *et al.,* 1997; Bassett *et al.,* 1998) (Fig. 4).

Mutational analysis of the *MEN1* gene in many centers failed to detect mutations in 5–30% of MEN1 patients. This failure could be explained if the mutation was a large deletion, causing the loss of the whole gene or a whole exon (Teh *et al.,* 1998b; Agarwal *et al.,* 1997; Bassett *et al.,* 1998). Consequently, the PCR amplification and mutational analysis shows only the normal allele. Alternatively, mutations may be within the untested parts of the gene, such as introns or the regulatory region. Thus, even though no *MEN1* gene mutation is found in some MEN1 index cases, this does not exclude its involvement. In fact, when MEN1 families have been large enough for linkage analysis, the trait has been linked to 11*q*13 in each (Larsson *et al.,* 1992; Corseaux *et al.,* 1996), suggesting cause by the same gene.

MEN1 GENE AND ENDOCRINE TUMORS

Endocrine tumors may occur either as part of MEN1 or, more commonly, as sporadic, nonfamilial, tumors.

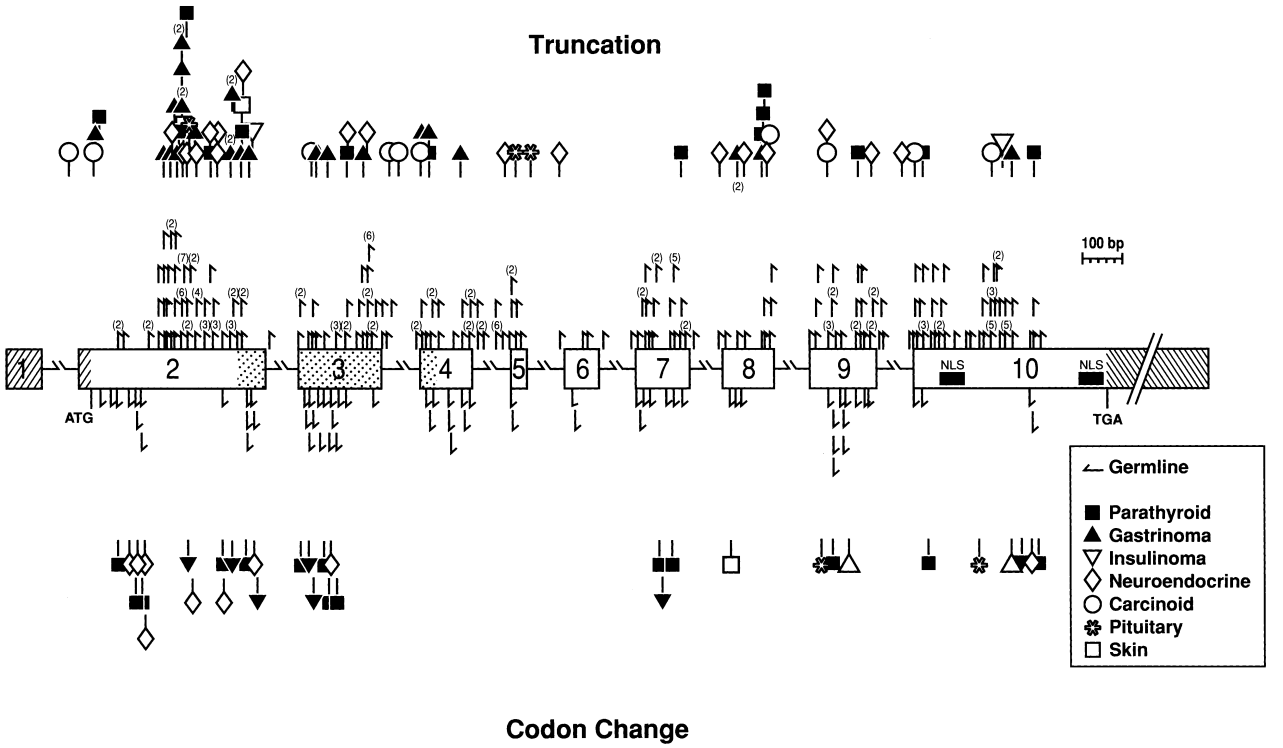

Figure 4 Genomic organization of the *MEN1* gene and of *MEN1* germline and somatic mutations, published as of June 2000. The gene contains 10 exons (with the first exon untranslated) and extends across 9 kb. Mutations shown above the exons cause menin truncation, and those shown below the exons cause a codon change. All unique mutations are represented; numbers in parenthesis designate multiple reports of the same mutation in presumed unrelated persons. The hatched areas indicate the untranslated regions. The location of the two nuclear localization signals (NLS), at codons 479–497 and 588–608, are indicated. Missense mutations in a region of menin (aa 139–242) (identified by stippling) prevented interaction with the AP1 transcription factor JunD.

Mutational analysis of MEN1-associated and sporadic endocrine tumors revealed that most tumors from MEN1 patients harbor the germline mutation together with a somatic LOH involving chromosome 11q13 (Marx *et al.,* 1998), as expected from Knudson's model and the proposed role of the *MEN1* gene as a tumor suppressor. However, LOH involving chromosome 11*q*13, which is the location of the *MEN1* gene, has also been observed in 5 to 50% of sporadic endocrine tumors, suggesting inactivation of the *MEN1* gene in the etiology of these tumors. Of those with 11*q*13 LOH, about half have shown somatic *MEN1* mutation; this includes sporadic parathyroid adenomas, sporadic gastrinomas, sporadic insulinomas, sporadic bronchial carcinoids, and sporadic anterior pituitary adenomas (Heppner *et al.,* 1997; Debelenko *et al.,* 1997; Zhuang *et al.,* 1997). Altogether, these observations establish that *MEN1* is the known gene most frequently mutated in nonfamilial variety endocrine tumors.

Nonendocrine Tumors

At least four different mesenchymal tumors can be caused by inactivation of the *MEN1* gene. These include skin/dermal lipoma, angiofibroma (Fig. 5), and collagenoma. In each case loss of the normal allele has been proven with material from MEN1 patients (Pack *et al.,* 1998). Furthermore sporadic lipoma and sporadic angiofibroma is occasionally associated with somatic *MEN1* mutation (Vortmeyer *et al.,* 1998; Boni *et al.,* 1998). Esophageal leiomyoma also was associated

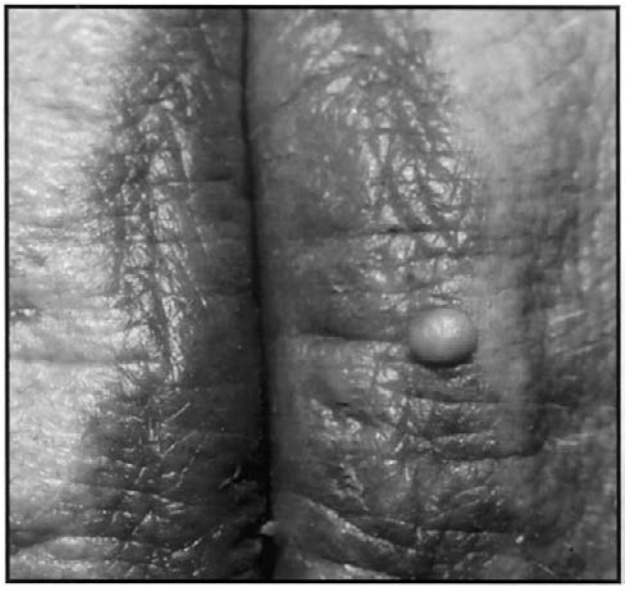

Figure 5 MEN1-associated angiofibroma.

with 11*q*13 LOH in one case (Vortmeyer *et al.,* 1999). This establishes that, like other multiple neoplasia syndromes, the tissue pattern of tumors in MEN1 does not follow a developmental distribution or any other recognizable distribution

Animal Models

Inactivation of certain other tumor suppressor genes alone or in combination can cause specific endocrine tumors in mice. In particular, mice with simultaneous homozygous knockout of two genes, $p18^{INK4c}$ and $p27^{KIP1}$, develop tumors of parathyroid, pituitary, pancreas islet, and duodenum (like MEN1). They also develop C-cell cancers and pheochromocytoma (like MEN2) (Franklin *et al.,* 2000). The knocked out genes encode members of the two cyclin-dependent kinase inhibitor families that participate in the G1 phase of the cell cycle, a phase that also includes retinoblastoma and cyclin D1. This syndromic resemblance raises the possibility that the pathways of MEN1 and/or MEN2 interact with the cell cycling pathway and perhaps with each other.

The mouse *Men1* gene is highly homologous to *MEN1*. Heterozygous knockout of *Men1* in the mouse results in a promising model of MEN1 (Crabtree *et al.,* 2000). Those mice develop tumors of the parathyroids, pancreatic islets, and pituitary. Unlike the pituitary intermediate lobe tumors with G1 phase tumor suppressor gene knockouts, these pituitary tumors are prolactinomas. The mouse tumors are associated with LOH at the mouse *Men1* locus.

Conclusion

The discovery of the *MEN1* gene has led to changes in clinical management and to new insights about normal and abnormal functions. New tools are in place to answer many questions shortly. Similarly there are prospects for novel treatments based upon DNA, RNA, or even other small molecules.

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CHAPTER 59

The Role of the *RET* Protooncogene in Multiple Endocrine Neoplasia Type 2

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Introduction

The *RET* proto-oncogene is a tyrosine kinase receptor that regulates neuronal differentiation and kidney development. It is expressed in the neural crest during early embryonic life. Migration of *RET*-positive cells from the neural crest leads to development of the gastrointestinal neuronal system and the dorsal sympathetic system. In an unexpected twist, mutations of the *RET* proto-oncogene were identified in multiple endocrine neoplasia type 2A (MEN 2A), a syndrome composed of medullary thyroid carcinoma (MTC), pheochromocytoma, and hyperparathyroidism (Sipple, 1961). The identification of these activating mutations and a parallel series of fundamental observations seeking to clarify the role of glial cell-derived neurotrophic factor (GDNF) has defined a new receptor system that is important for development and differentiation of neural crest-derived cells. This chapter will outline the substantial progress over the past 5 years toward an understanding of the *RET* receptor system.

Multiple Endocrine Neoplasia Type 2 (MEN 2)

Sipple was the first to describe an association between thyroid carcinoma and pheochromocytoma (Sipple, 1961), leading to a decade in which the clinical and heritable features of this syndrome were defined (Steiner *et al.,* 1968; Melvin *et al.,* 1972). Williams and his coworkers (Williams and Pollock, 1966) are generally credited with the reidentification of the mucosal neuroma syndrome (MEN 2B) first described earlier (Froboese, 1923) and the recognition that this clinical syndrome is associated with MTC and pheochromocytoma. Additional variants of MEN 2 include familial medullary thyroid carcinoma (FMTC) with no other manifestations of MEN 2 (Farndon *et al.,* 1986), MEN 2A in association with cutaneous lichen amyloidosis (Gagel *et al.,* 1989; Nunziata *et al.,* 1989), and MEN 2A in association with Hirschsprung disease (Table I) (Verdy *et al.,* 1982).

Multiple Endocrine Neoplasia Type 2A

The term Sipple syndrome is most correctly applied to the association of MTC, pheochromocytoma, and parathyroid neoplasia (Table I), now known as MEN 2A. Our thinking about this syndrome has evolved over the past several decades. During the first decade after Sipple's report, the three major components of the syndrome were characterized (Schimke *et al.,* 1965, 1968). The convergence of several observations led to the use of provocative tests for diagnosis of early MTC. These included the identification of the peptide hormone calcitonin (Copp *et al.,* 1961; Hirsch *et al.,* 1964), the identification of the C cell (thyroid parafollicular cell) as the transformed cell type in MTC (Williams, 1966), the recognition it produces calcitonin (CT) (Foster *et al.,* 1964), and the development of radioimmunoassays for calcitonin

Table I Multiple Endocrine Neoplasia Type 2 (MEN 2) Classification

MEN 2A
MTC
Pheochromocytoma
Parathyroid neoplasia
Variants of MEN 2A
MEN 2A in association with Hirschsprung's disease
MEN 2A in association with cutaneous lichen amyloidosis
Familial MTC only (FMTC)
MEN 2B
MTC
Pheochromocytoma
Mucosal and aliamentary tract ganglioneuromatosis
Marfanoid features
Absence of parathyroid neoplasia

(Deftos *et al.,* 1968; Melvin *et al.,* 1972). The observation that either intravenous calcium or pentagastrin could be used to stimulate calcitonin release provided early investigators with a tool for identifying early C cell abnormalities (Cooper *et al.,* 1971; Melvin *et al.,* 1971; Hennessey *et al.,* 1973, 1974). These provocative tests were subsequently used by others to screen MEN 2 kindreds for early C cell abnormalities (Wells *et al.,* 1975; Leape *et al.,* 1976; Graze *et al.,* 1978; Gagel *et al.,* 1988). In parallel studies, hyperplasia of the adrenal medulla was identified as a precursor lesion (Carney *et al.,* 1976; DeLellis *et al.,* 1976). Implementation of prospective screening studies over the next two decades resulted in the routine identification of MTC and pheochromocyoma at early developmental stages. It is now reasonable to believe that death related to either of these manifestations can be prevented in most patients by surgical intervention at an appropriate point during the natural history of this clinical syndrome (Cance and Wells, 1985; Gagel *et al.,* 1988).

The Three Components of MEN 2

Neoplastic Transformation of the C Cell

The parafollicular cells are neural crest-derived neuroendocrine cells located adjacent to the thyroid follicle (Fig. 1). These cells produce CT, a small peptide that binds to specific receptors on osteoclasts and inhibit bone resorption. The C cells are distributed within the thyroid gland in a characteristic pattern. The greatest concentration is found in the upper one-third of each lobe along a hypothetical cephalad–caudal axis. It is therefore not surprising that hereditary MTC, in which each cell has an activating mutation, occurs most frequently at this anatomic location. There is a progression of histologic events in the development of MTC: C cells progress through several stages including hyperplasia, nodular hyperplasia (a single focus of C cells that displace a thyroid follicle, giving the appearance of a nodule of cells within the follicle), and microscopic MTC (Wolfe *et al.,* 1973; Carney *et al.,* 1979; Wolfe *et al.,* 1981). At some point along this pathway of progression metastasis may occur (Graham *et al.,* 1987; Niccoli-Sire *et al.,* 1999). Separate foci at different stages of neoplastic development are found commonly within the thyroid gland of a gene carrier. Metastasis to central lymph nodes of the neck is found most commonly

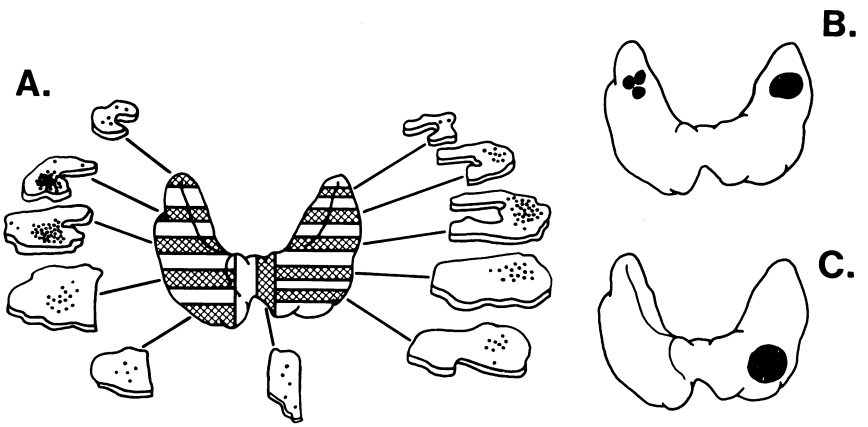

Figure 1 Distribution of C cells in the thyroid gland. (A) Reconstruction of the distribution of C cells in the thyroid gland. The greatest concentration of C cells occurs at the junction of the upper one-half and lower-two thirds of the gland. This distribution explains the characteristic location of hereditary medullary thyroid carcinoma. (Modified from Wolfe H. J., Voelkel, E. F., Tashjian, A. H., Jr: Distribution of calcitonin containing cells in the normal adult human thyroid gland: a correlation of morphology with peptide content. *J. Clin. Endocrinol. Metab.* **38,** 688–694, 1974.) (B) Hereditary medullary thyroid carcinoma is almost always bilateral, although the extent of involvement may not be equal. (C) Sporadic medullary thyroid carcinoma is most commonly a unilateral process that may develop at any location within the thyroid gland.

in patients with a visible focus of MTC, although it has been reported with microscopic MTC (Niccoli-Sire *et al.,* 1999). It is thought that local lymph node metastasis precedes distant metastasis in most cases, because surgical resection of lymph nodes metastasis in patients with early MTC will cure about 25% of patients (Miller *et al.,* 1972; Gagel *et al.,* 1988).

Hereditary MTC is characteristically multifocal. Each tumor appears as a whitish-yellow circumscribed lesion on a background of thyroid tissue. The characteristic histologic features of the tumor include a nested endocrine-type appearance or sheets of cells interspersed with amyloid which is derived at least in part from calcitonin gene products (Livolsi, 1990).

Tumor markers that are useful for management of hereditary or sporadic MTC include CT and carcinoembryonic antigen (Baylin *et al.,* 1972). A variety of other peptides are produced by MTC including somatostatin (Gagel *et al.,* 1986), chromogrannin A (Deftos *et al.,* 1988), histaminase (Baylin *et al.,* 1970), and DOPA decarboxylase (Baylin *et al.,* 1979).

Adrenal Medullary Abnormalities

There are several features that differentiate pheochromocytomas associated with the MEN 2 from sporadic pheochromocytomas. These include the development of pheochromocytoma on a background of medullary hyperplasia, production of a greater proportion of epinephrine than is seen in sporadic pheochromocytomas, and bilaterality. Analogous to the thyroid, pheochromocytomas in MEN 2 evolve out of medullary hyperplasia and it is not uncommon to identify multiple discrete pheochromocytomas set on a background of hyperplasia. MEN 2-related pheochromocytomas, unlike sporadic pheochromocytomas or those associated with von Hippel Lindau syndrome, express the enzyme phenylethanolamine-*N*-methyltransferase at higher levels and therefore produce disproportionate amounts of epinephrine. The first abnormality in a MEN 2A-related pheochromocytoma is an absolute increase of epinephrine production (Gagel *et al.,* 1975; Hamilton *et al.,* 1978; Miyauchi *et al.,* 1982; Telenius-Berg *et al.,* 1987) or an increase in plasma normetanephrine or metanephrine values (Eisenhofer *et al.,* 1999). These biochemical findings lead to symptoms of epinephrine excess including palpitations, nervousness, jitteriness, and the absence of hypertension. There may be subsequent development of hypertenision as norepinephrine production increases. Most MEN 2 pheochromocytomas are intraadrenal lesions. Extraadrenal pheochromocytomas in this syndrome are thought to have arisen from adrenal rest tissue or represent recurrence of a pheochromocytoma in the anatomic region of the adrenal gland (Lips *et al.,* 1981; Modigliani *et al.,* 1995). Pheochromocytomas occur in approximately 50% of gene carriers and are bilateral in approximately one-half (Steiner *et al.,* 1968; Lips *et al.,* 1981; Gagel *et al.,* 1988; Howe *et al.,* 1993; Modigliani *et al.,* 1995).

Parathyroid Abnormalities

There is a low incidence of hyperparathyroidism in MEN 2A (5–20%). There are several unique features. Hyperparathyroidism occurs in some kindreds with greater frequency than others (Wells *et al.,* 1975). In addition, early prophylactic thyroidectomy for MTC appears to have resulted in a lower incidence of hyperparathyroidism despite follow-up periods that now exceed 25 years in some kindreds (Gagel *et al.,* 1988; Bone *et al.,* 1992). Development of hyperparathyroidism following total thyroidectomy in a single member of a kindred in which there is a high incidence of this disorder suggests this is not always the case (author's experience). Is it unclear whether the observed lower incidence of hyperparathyroidism in some kindreds treated by prophylactic thyroidectomy is related to a growth stimulatory factor produced by the thyroid gland or has resulted from the inevitable loss of parathyroid tissue during total thyroidectomy. Pheochromocytoma may rarely cause hypercalcemia, possibly related to production of parathyroid hormone-related protein, and should be excluded prior to surgical exploration (Gagel *et al.,* 1988).

Hyperplasia of multiple parathyroid glands is the most common abnormality found in hyperparathyroidism associated with MEN 2, although many reports emphasize the occurrence of multiple parathyroid adenomas in individuals who present with the fully developed MEN 2 syndrome, generally after the age of 35 years. Most surgeons treat hyperparathyroidism by multiple gland resection, although in kindreds where hyperparathyroidism is a significant problem, total parathyroidectomy with transplantation of a normal amount of tissue to the nondominant forearm may be appropriate.

Familial Medullary Thyroid Carcinoma

Familial MTC is defined as the occurrence of hereditary MTC without other manifestations of MEN 2A and represents 10–15% of all hereditary cases. The MTC tends to develop later and is less likely to behave aggressively (Farndon *et al.,* 1986). Categorization as FMTC should be made only after observing several generations; pheochromocytoma and hyperparathyroidism are much less penetrant in classic MEN 2A (50 and 15%, respectively) and it is possible to incorrectly asssign the term FMTC to small families in which these manifestations have not developed. This distinction is important to ensure appropriate screening for pheochromocytoma.

The MEN 2A/Hirschsprung's Phenotype

Classic Hirschsprung disease has been observed in association with MEN2A in 20–40 kindreds (Wohllk *et al.,* 1996; Decker *et al.,* 1998). The Hirschsprung disease associated with MEN 2A does not differ from other forms of

childhood Hirschsprung disease and is usually correctable surgically. In contrast MEN 2B, described below, is characterized by neuromas in the oral cavity and throughout the gastrointestinal tract.

MEN 2A with Cutaneous Lichen Amyloidosis

In fewer than 20 kindreds with MEN 2A a unilateral or bilateral pruritic skin lesion located over the upper back (dermatomes C6-T5) has been identified (Gagel *et al.,* 1989; Nunziata *et al.,* 1989). Clinical features in these kindreds are identical to classic MEN 2A, although there may be a lower incidence of parathyroid disease (Ferrer *et al.,* 1991; Chabre *et al.,* 1992b; Robinson *et al.,* 1992). The characteristic feature of this variant is intense intermittent periods of prurritus that may develop in childhood and be a premonitory indication of gene carrier status (Chabre *et al.,* 1992a). Biopsy of advanced forms of this lesion has demonstrated typical findings of cutaneous lichen amyloidosis (Gagel *et al.,* 1989), whereas amyloid is infrequent in early skin lesions (Gagel *et al.,* 1989; Ferrer *et al.,* 1991; Chabre *et al.,* 1992b). Several explanations have been considered to explain the presence of amyloid. Friction amyloidosis, caused by the incessant scratching, appears to be the most logical mechanism for amyloid accumulation (Chabre *et al.,* 1992a). This is supported by the finding of positive immunohistochemical staining for keratin in the amyloid and the absence of CT gene products (Donovan *et al.,* 1989). The dermatome-like distribution of the skin lesion, which matches the normal expression pattern of *RET* (Durbec *et al.,* 1996), suggests an effect of *RET* on neuronal firing, leading to the prurritic symptomatology. Abnormal cutaneous innervation in *RET* knockout mice provides independent support for this hypothesis (Fundin *et al.,* 1999).

Multiple Endocrine Neoplasia Type 2B

The clinical findings of MTC, pheochromcytoma, mucosal neuromatosis, skeletal abnormalities suggestive of Marfan's syndrome, and the absence of hyperparathyroidism define the clinical features of multiple endocrine neoplasia type 2B (MEN 2B). Although there is wide variability in clinical presentation, most patients with MEN 2B have mucosal neuromas on the tip of the tongue, on the conjunctival surface of the eye lids and throughout the gastrointestinal tract. Children most commonly present with abdominal pain or pseudoobstruction (Carney *et al.,* 1976). The Marafanoid-like features of longer arms and legs, pectus abnormalities, long, thin fingers, hyperextensibility of joints, and epiphyseal abnormalities combined with mucosal neuromas are pathognomonic for this clinical syndrome (Carney *et al.,* 1978, 1981; Williams and Pollock, 1966). Lens ectopia, cataracts and dissecting aortic aneurysm, frequent findings in Marfan syndrome, have never been reported in MEN 2B, although the author has seen a patient who inherited the genes for MEN 2A and Marfan syndrome from separate parents.

Penetrance of MTC in MEN 2B is high; metastasis has been described during the first year of life. Medullary thyroid carcinoma in the context of MEN 2B commonly pursues an aggressive course, although this is not uniformly the case. A number of multigenerational kindreds with this disorder have been described, suggesting a more benign course in some (Sizemore *et al.,* 1992; Vasen *et al.,* 1992). Hyperparathyroidism is a rare finding in MEN 2B (Carney *et al.,* 1980).

Genetic Linkage Analysis and the RET Receptor System

The gene for MEN 2 was localized to centromeric chromosome 10 in 1987 (Mathew *et al.,* 1987; Simpson *et al.,* 1987) and the mutations of the *RET* proto-oncogene were identified in 1993 (Donis-Keller *et al.,* 1993; Mulligan *et al.,* 1993). In the period since 1993 the individual components of the *RET* receptor system have been identified (Fig. 2). The first is the RET tyrosine kinase receptor, identified in 1985 (Takahashi *et al.,* 1985, 1987). Two other components of this receptor system, a ligand (glial cell-derived neurotrophic factor or GDNF) and a second extracellular component of the receptor system (glial cell-derived neurotrophic factor receptor alpha-1 or GFRα-1) have been identified (Fig. 2) (Hoff *et al.,* 2000). Individual disruption of each of the three components of the receptor system in mice leads to nearly identical phenotypes that include failure of normal kidney development, a Hirschsprung-like neuronal phenotype in the gastrointestinal tract (disordered neuronal development), and abnormal dorsal sympathetic development (Schuchardt *et al.,* 1995; Durbec *et al.,* 1996b; Jing *et al.,* 1996; Moore *et al.,* 1996; Pichel *et al.,* 1996; Sanchez *et al.,* 1996; Treanor *et al.,* 1996). Subsequent studies showed that GDNF is a ligand for RET (Durbec *et al.,* 1996a; Treanor *et al.,* 1996) and identified GFRα-1, a protein that is tethered to the extracellular membrane, binds GDNF, but contains no transmembrane or intracellular domain. Together, RET and GFRα-1 form a receptor system for GDNF.

Mutations of *RET* Cause Multiple Endocrine Neoplasia Type 2

Two broad categories of mutations cause hereditary MTC or MEN 2. The most common are extracellular mutations of the cysteine-rich dimerization domain; less common are mutations that change single amino acids in the intracellular tyrosine kinase domain (Table I). The extracellular domain mutations associated with MEN 2 or familial MTC affect codons 609, 611, 618, 620, 630, and 634 and change a highly conserved cysteine to another amino acid (Table I). Mutations at codon 634 mutations are most common, accounting for approximately 80% of all mutations in MEN 2; a single

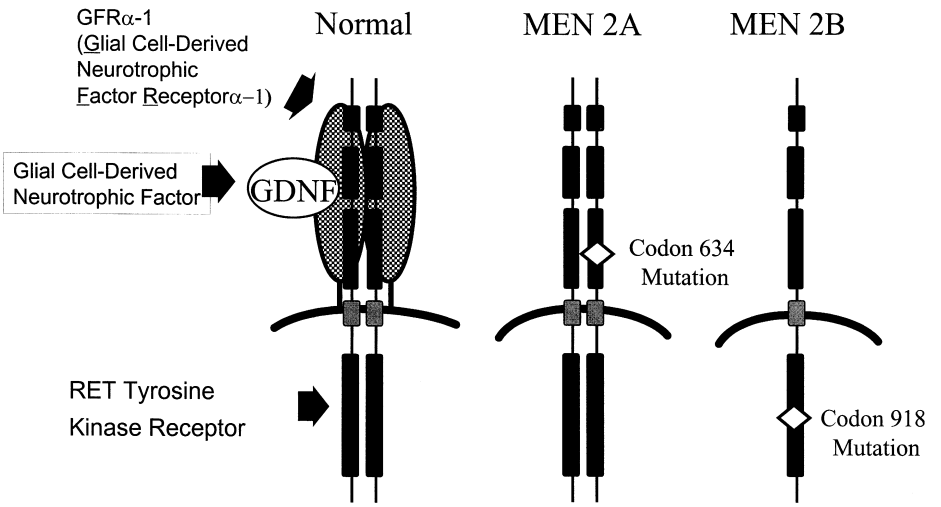

Figure 2 The RET receptor complex and mechanism of RET activation. Normal: Glial cell-derived neurotrophic factor (GDNF) is a small peptide that promotes neuronal survival. It interacts with a receptor complex formed by the RET tyrosine kinase receptor (RET) and the glial cell-derived neurotrophic factor receptor (GFRα-1). GDNF and RET/GFRα-1 interactions promote development of the collecting system of the kidney, normal migration of neurons from the neural crest into the gastrointestinal tract, and normal sympathetic nervous development in those neurons derived from the first six somites. MEN 2A: Mutations of highly conserved cysteines in a region immediately outside the extracellular membrane promote RET dimerization and activation of downstream signaling pathways. MEN 2B: Mutation of codon 918 (methonine to threonine) results in activation of the tyrosine kinase domain in the absence of either ligand or dimerization of the receptor.

change (TGC to CGC; cysteine to arginine) accounts for approximately 50% of all *RET* mutations found in hereditary MTC. The extracellular domain mutations cause dimerization of the RET receptor complex, autophosphorylation, and activation of downstream tyrosine kinase pathways in the absence of GDNF. Intracellular mutations change codons 768, 790, 791, 804, 891, 883, and 918. The intracellular changes activate the intracellular kinase domain directly without the necessity for receptor dimerization (Asai *et al.,* 1995; Santoro *et al.,* 1995).

There are mutation-specific correlations between codon substitutions and the clinical phenotype (Table I). A codon 634 mutation is most commonly associated with classic MEN 2A (Eng *et al.,* 1996). MEN 2A has also been found in patients with mutations of codons 609, 611, 618, 620 (Donis-Keller *et al.,* 1993; Mulligan *et al.,* 1994), 791 (Berndt *et al.,* 1998), and V804L (Nilsson *et al.,* 1999). Kindreds with the MEN 2A/cutaneous lichen amyloidosis variant invariably have a mutation at codon 634 (Eng *et al.,* 1996), whereas individuals with the MEN 2A/Hirschsprung variant have codon 609, 618, or 620 mutations (Lacroix *et al.,* 1994; Mulligan *et al.,* 1994; Decker *et al.,* 1998).

Kindreds in which there is multigeneration FMTC may also have codon 609, 611, 618, or 620 *RET* mutations 620 (Donis-Keller *et al.,* 1993; Mulligan *et al.,* 1994), although mutations at these same codons may also be associated with MEN 2A (Table I). FMTC is uniquely associated with mutations at codons 768 (Boccia *et al.,* 1997), 790 (Berndt *et al.,* 1998), V804M (Bolino *et al.,* 1995; Miyauchi *et al.,* 1999), and 891 (Hofstra *et al.,* 1997; Dang *et al.,* 1999).

MEN 2B is most commonly associated with a codon met918thr substitution (Carlson *et al.,* 1994; Hofstra *et al.,* 1994). There have been a handful of MEN 2B patients described with codon 883 substitutions (Gimm *et al.,* 1997; Smith *et al.,* 1997). The majority of MEN 2B mutations occur *de novo* (neither parent carries a germline mutation), although a small number of multigeneration MEN 2B kindreds exist. Most of the *de novo* mutations occur in the paternal allele (Carlson *et al.,* 1994).

There are still a handful of kindreds with MEN 2 or FMTC ($<$2% of the total) in which no *RET* mutation has been identified. This suggests that there are other as yet unidentified mutations of *RET* or that mutations of a second gene may be involved in this hereditary syndrome. It is important to keep this point in mind when evaluating a kindred with hereditary MTC; although there may be no identifiable mutation at present, consideration should be given to retesting at periodic intervals as new mutations are identified.

The Mechanism of Transformation

Missense mutations of *RET* cause transformation by one of two different mechanisms. Mutations of the extracellular domain (609, 611, 618, 620, 630, and 634) cause dimerization and activation of the receptor (Fig. 2), presumably in the absence of GDNF (Asai *et al.,* 1995; Santoro *et al.,* 1995). In contrast, dimerization of the receptor is not required for transformation caused by a codon 918 mutation

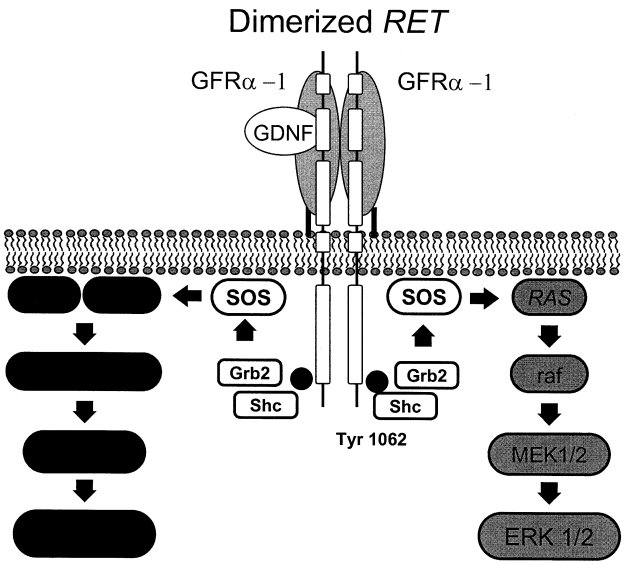

Figure 3 Intracellular signaling pathways activated by the GDNF–GFRa-1/Ret signaling system. Two different intracellular signaling pathways (JNK/SAPK and ERK 1/2) are activated by this the the GDNF-GFRa-1/Ret receptor complex.

(Fig. 2), the most common intracellular domain mutation (Asai *et al.,* 1995; Santoro *et al.,* 1995). Both intracellular and extracellular mutations cause receptor autophosphorylation and phosphorylation of different sets of downstream proteins in the signaling cascade. The tyrosine at codon 1062 (a *Shc* binding site) is autophosphorylated, interacts with c-*src,* and appears to mediate most of the transforming activity caused by either codon 634 or codon 918 mutations (Asai *et al.,* 1996; Ohiwa *et al.,* 1997; Santoro *et al.,* 1999; De Vita *et al.,* 2000). Activation of a JNK and ERK 1 pathways mediates the transforming effects of both types of mutations (Fig. 3) (Chiariello *et al.,* 1998).

Other Genetic Mechanisms in MEN 2

Recent studies suggest that either loss of the normal copy of *RET* (loss of the normal chromosome) or reduplication of the mutant chromosome 10 (Fig. 4; trisomy 10) are found in a high percentage of MEN 2-related tumors (Huang *et al.,* 2000). These findings suggest that the ratio of mutant to normal RET expression may be an independent factor (a second hit) in the transformation process. These findings imply that a homodimer containing two mutant copies of RET have a greater transforming effect than a receptor complex consisting of a heterodimerized receptor.

In addition, genetic abnormalities at other loci are found in MEN 2-related tumors. These include a loss of heterozygosity (LOH) on chromosome 1*p* in MTC (Mathew *et al.,* 1987; Moley *et al.,* 1992), 3*p* [Cooley *et al.,* 1995; (Khosla *et al.,* 1991), and chromosome 22 (Takai *et al.,* 1987; Cooley *et al.,* 1995). Specific genes at these loci have not been identified.

The Role of *RET* in Normal Development

The GDNF/RET/GFRα-1 receptor system is a major determinant of neuronal migration from neural crest tissue. RET-positive cells presage the development of the facial, glossopharyngeal, trigeminal, vagus cranial, and sympathetic ganglia, and spinal motor neurons (Tsuzuki *et al.,* 1995). RET-positive cells also migrate from the neural crest into the developing gastrointestinal tract and are found in the developing ureteric bud. Targeted deletion of the GDNF (Moore *et al.,* 1996; Pichel *et al.,* 1996; Sanchez *et al.,* 1996), *RET* (Schuchardt *et al.,* 1995; Durbec *et al.,* 1996a), or GFRα-1 (Cacalano *et al.,* 1998) genes in mice cause major abnormalities of the sympathetic nervous system and cause a Hirschsprung-like phenotype in the gastrointestinal tract. Indeed, this finding predicted the presence of inactivating RET mutations in one-half of patients with familial Hirschsprung disease, although more recent studies suggest a role for rare polymorphisms of *RET* in nonfamilial Hirschsprung disease (Borrego *et al.,* 1999). Other phenotypic abnormalities in knockout animals include diminished ganglionic development and a failure of normal kidney development, a defect caused by a failure of interaction between RET (expressed in the ureteric bud) and GDNF (expressed in the renal mesoderm).

Use of Genetic Information in the Clinical Management of MEN 2

Genetic testing in MEN 2 has quickly become one of the more successful examples of use of this technology for management of cancer. This differs from the experience with other malignancies such as breast or colon cancer where use of genetic testing is utilized to focus screening efforts or exclude individuals with negative tests from the risk pool.

Rapid acceptance of genetic testing for management of this disorder occurred for several reasons. The most important was a 25-year experience utilizing provocative CT testing for early diagnosis of MTC in MEN 2 (Cance and Wells, 1985; Gagel *et al.,* 1988). These studies demonstrated the long-term effectiveness of early thyroidectomy in the management of this malignancy. Approximately one-half of children identified by this approach had C cell hyperplasia, a premalignant lesion and the remainder were found to have microscopic MTC (Gagel *et al.,* 1988). A second component in the rapid acceptance of this approach is how well tolerated thyroidectomy is in children. Although there is concern that failure to take thyroid hormone as directed could lead to altered intellectual development, there is little evidence that this is a real concern. Hypoparathyroidism, although rare following thyroidectomy in this age group, remains a greater concern.

It is important to recognize, however, that not all children who received early thyroidectomy (mean age of 13 years) were cured of their disease. Recurrent disease has been reported in 15–25% of individuals treated by thyroidectomy before the age of 20 years. The finding of metastatic MTC

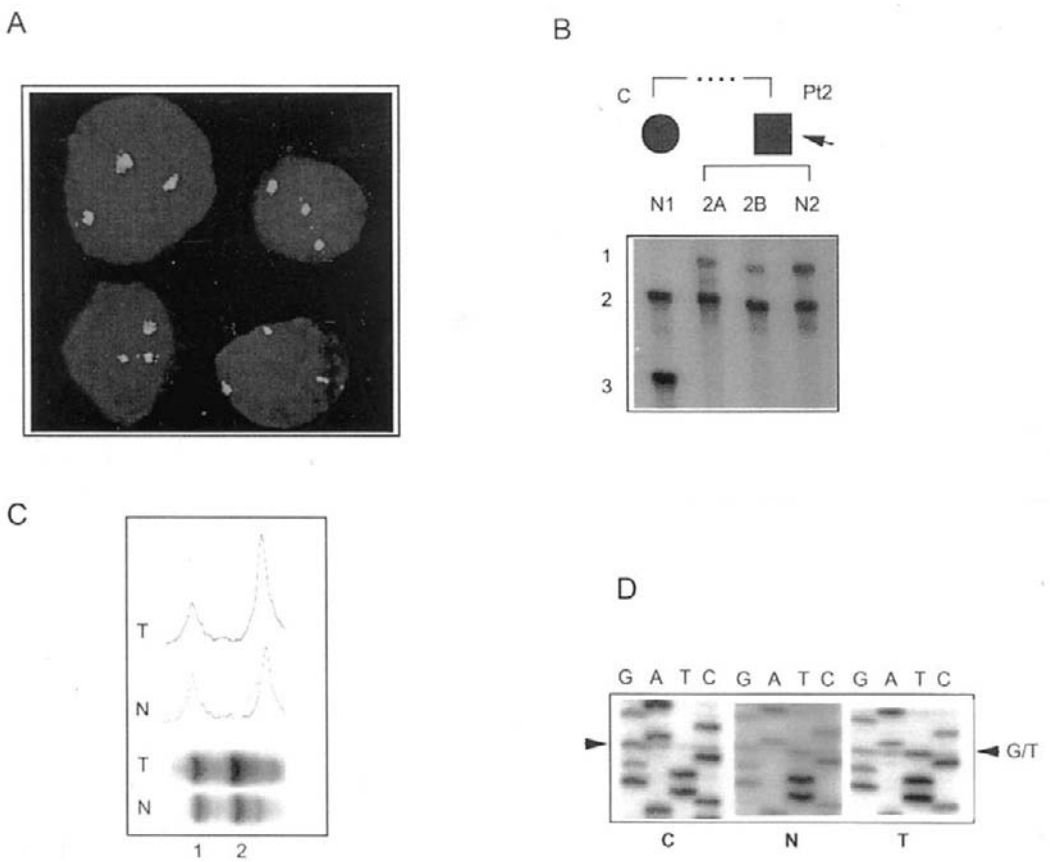

Figure 4 Trisomy 10 with nonrandom duplication of the mutant *RET* allele in MEN 2-associated pheochromocytoma. (A) Representative interphase FISH analysis on tumor touch. Three copies of chromosome 10 are shown using a centromeric α-satel-ite probe (FITC, green signal) specific for chromosome 10. (B) Combined pedigree and tumor allelic analysis. Arrow, affected patient. Filled symbols, individuals with microsatellite marker *D10S1239* linked to the *RET* locus. Allele 2 of *D10S1239* is coinherited with the disease in this patient's family. In the affected patient, allele 2 shows a greater intensity in lanes 2A and 2B (tumors) than allele 1, representing the wild-type allele, as compared with lane N2 (blood DNA). Lane N1 (blood DNA) shows equal intensities of mutant and wild-type allele in the patient's affected cousin (C). (C) Representative results of microsatellite and phosphorimage analyses. After PCR amplification using marker *D10S1239,* quantitative measurement of allelic intensity was performed using phosphorimage analysis. In tumor tissue (D), allele 2 is more intense than allele 1. Phosphorimage densitometry shows a 2:1 imbalance between the two alleles in tumor (*T*) as compared with the normal tissue (N). D, representative results of sequencing analysis of *RET* in tumor 3A. Blood DNA from an unaffected/healthy individual (C, left) shows the wild-type *RET* seqeunce (codon 631 GAC). Blood DNA from patient 3 (N, middle) shows the germ-line mutation (G/T). Tumor DNA (T, right) shows a higher intensity of the mutant nucleotide (T) compared with the wild-type nucleotide (G). Reprinted with permission from Huang *et al.* (2000).

in children as young as 3 years of age or younger has led clinicians who care for these children to advocate earlier thyroidectomy based on genetic testing (Niccoli-Sire *et al.,* 1999). Experience with families with specific mutations over the past 8 years suggests that there is a correlation between mutations at specific codons and MTC aggressiveness although the experience is incomplete and certainly imperfect. In addition, there is a growing *in vitro* body of information describing the relative ability of specific mutations to cause *in vitro* transformation. For example, there is evidence that a codon 634 mutation has greater transforming capacity than codon 609, 611, 618 or 620 mutations (Carlomagno *et al.,* 1997; Takahashi *et al.,* 1998). Based on the combined clinical and *in vitro* eperience there has been a stratification into three broad groups of aggressiveness.

MTC caused by a codon 918 mutation (MEN 2B) is generally considered to be the most aggressive form of this disease. Children with this mutation should have a total thyroidectomy and central node dissection performed during the first month of life or at the earliest time of detection. Cure is difficult because these children frequently come to attention between the ages of 5 and 10 years. In children with gross MTC an extensive lymph node dissection will improve the chances of a surgical cure (Moley *et al.,* 1997, 1998; Evans *et al.,* 1999).

Children with codon 611, 618, 620, and codon 634 mutations are considered to have a high risk of developing aggressive MTC. In these children there is a general recommendation for thyroidectomy at the age of 5–6 years of age, although recent reports of metastatic MTC in children

ages 2–4 suggests that earlier thyroidectomy may be required to cure most children. A total thyroidectomy with central lymph node dissection is generally considered to be appropriate therapy for these children (Lips *et al.,* 1994; Gagel *et al.,* 1995; Dralle *et al.,* 1998). A minority of surgeons perform a total thyroidectomy, central neck dissection, and concomitant transplantation of parathyroid tissue to the nondominant arm to lower the risk of hypoparathyroidism (Wells *et al.,* 1994; Dralle *et al.,* 1998).

Finally, children with 609, 768, 790, 791, 804, or 891 mutations are considered to have an intermediate risk of aggressive disease. Although deaths from MTC have been reported in kindreds with all of these mutations except for codon 790 or 791 mutations and there are kindreds where metastasis has occurred in family members before the age of 30 years (Feldman *et al.,* 2000), it is also clear there are some kindreds with one of these mutations in which death from MTC has never been reported. It is difficult to convince a parent with one of these mutations and a clearly defined family history of benignity to have younger child with a mutant *RET* gene undergo early thyroidectomy. As a result, clinicians caring for kindreds with these mutations have continued provocative testing for calcitonin abnormalities, delaying a thyroidectomy until the development of an abnormal test result. This type of approach may delay thyroidectomy until the mid-teenage years or later. A total thyroidectomy with central lymph node dissection is generally appropriate therapy for these cases.

There is at present no evidence that earlier intervention will further improve outcomes in children with germline mutations. Initial reports suggest that early intervention is associated with a high rate of success (Lips *et al.,* 1994; Wells *et al.,* 1994; Dralle *et al.,* 1998; Niccoli-Sire *et al.,* 1999) However, this enthusiasm must be tempered by the reality that the genetic abnormality in MEN 2 affects all cells. Any remaining C cell has the potential for subsequent transformation. This underscores the importance of completeness of the thyroidectomy and the need for long-term follow-up. Most of these children are under the age of 5 years at the time of thyroidectomy and will be under the age of 30 years at the time of 25-year follow-up.

The Future

Effective therapeutic options for treatment of the neoplastic components of MEN 2 have been limited to surgical removal of neoplastic components. The recent demonstration that an inhibitor of tyrosine phosphorylation was used successfully to treat two malignancies caused by activating mutations of a tyrosine kinase provides clear direction for investigators in this field (Druker *et al.,* 2001a,b; Joensuu *et al.,* 2001). The findings that the first and second genetic "hit" in MEN 2 activates a tyrosine kinase receptor, that tumor development proceeds over years, and that transformation can be prevented by inactivation of the tyrosine kinase receptor suggest that this disorder could be prevented or treated by a similar approach.

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CHAPTER 60

Systemic Factors in Skeletal Manifestations of Malignancy

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Malignancy-Associated Hypercalcemia

History

ALBRIGHT'S HYPOTHESIS

The association between elevated blood calcium and neoplastic disease was first noted in the 1920s by a group of German physicians (Zondek *et al.,* 1923), who believed that release of calcium from bone by the direct osteolytic action of malignant cells was responsible for hypercalcemia associated with malignancy. However, hypercalcemia was often accompanied by hypophosphatemia, the hallmark of hyperparathyroidism (HPT), and also occurred in the absence of skeletal metastases. This constellation of abnormalities was frequently seen in association with certain histological types of tumors, notably renal cell carcinomas and squamous cell cancers originating in a variety of primary sites. It was Fuller Albright in 1941 who hypothesized that a malignant tumor might release a systemically active factor resulting in hypercalcemia (Albright, 1941). The hypothesis was raised when a patient with a renal carcinoma, metastatic to bone, presented with hypercalcemia and hypophosphatemia in the absence of any obvious parathyroid dysfunction. The patient's biochemical abnormalities were temporarily normalized following tumor reduction but became apparent again as the tumor resumed its growth. Dr. Albright reasoned that since hyperphosphatemia did not accompany hypercalcemia, despite evidence of osteolysis, perhaps the tumor was secreting a phosphaturic substance like parathyroid hormone (PTH). Although no bioassayable hormone was detected in the tumor, the important and insightful observation had been made that hypercalcemia associated with malignancy could have a humoral basis and that the factor might be related to PTH. The term humoral hypercalcemia of malignancy (HHM) or malignancy-associated hypercalcemia (MAH) was later coined to distinguish this syndrome, commonly seen in patients with renal cell and squamous carcinoma, from hypercalcemia caused by the direct lytic action of tumor cells in bone, which was commonly associated with hematologic malignancies and with metastatic breast disease. Nevertheless, it is now clear that the "direct" lytic action of tumor cells is also mediated by humoral factors and that these locally acting factors may include those which also act on a systemic basis.

CHARACTERISITICS OF HYPERCALCEMIA OF MALIGNANCY

Despite the striking similarities between the biochemical manifestations of HPT and MAH that precipitated Albright's hypothesis, it subsequently became apparent that there were also a number of important differences between these syndromes. Early attempts at the differential diagnosis of these hypercalcemic disorders identified not only normal to subnormal concentrations of immunoreactive PTH but also a mild hypokalemic alkalosis in patients with MAH compared with the elevated PTH and mild metabolic acidosis associated with HPT (Lafferty, 1966). In addition, circulating $1,25(OH)_2D_3$ levels were often low in patients with malignancy and elevated in patients with HPT (Stewart *et al.,* 1980). Histomorphometric evaluation of bone biopsies also revealed discrepancies in bone formation, which appeared to be decreased in hypercalcemic cancer patients but increased in those

with parathyroid disease (Stewart *et al.,* 1982), whereas catabolic activity was significantly increased in both disorders. These apparent discrepancies were not resolved by early animal models of MAH in which circulating $1,25(OH)_2D_3$ levels were high and bone turnover appeared to be coupled (Sica *et al.,* 1983; Strewler *et al.,* 1986). Neither was the problem solved by *in vivo* and *in vitro* studies performed with synthetic amino terminal fragments of PTHrP, which suggested that the peptides had identical bioactivity (Chorev and Rosenblatt, 1994; Fraher *et al.,* 1992; Rabbani *et al.,* 1988). These issues were later revisited in a longitudinal study using nude rats implanted with a human PTHrP-producing tumor (Yamamoto *et al.,* 1995). Unlike the dramatic uncoupling of bone formation and resorption observed in the terminal stages of human disease, a substantial increase in both indices was observed at an earlier time point, prior to a detectable elevation in circulating PTHrP or hypercalcemia. Taken together with evidence that serum $1,25(OH)_2D_3$was also elevated in the tumor-bearing rats during the early stage of disease, these observations suggested that PTHrP and PTH do indeed elicit the same biological activity in bone. Furthermore, the discovery that PTH/PTHrP receptors were present on osteoblasts and their precursors (Rouleau *et al.,* 1988), but not on osteoclasts, suggested that cells of the osteoblast lineage ultimately regulated the catabolic activity of PTHrP in bone.

With the advent of sensitive and specific immunochemical and molecular biochemical assays for PTHrP it became apparent that a wide variety of tumors, including those of breast, hematologic and endocrine origin expressed the protein. (Asa *et al.,* 1990; Henderson *et al.,* 1990). Moreover, 60% of primary breast tumors and 90% of metastatic foci in bone were shown to be PTHrP positive (Powell *et al.,* 1991), which caused a reassessment of the relative contributions of "humoral" and "local" factors to MAH.

Purification and Cloning of PTHrP

Until the 1980s investigators continued with their attempts to identify PTH as the causative agent in MAH. However, immunoreactive PTH could not be detected in the circulation (Powell *et al.,* 1973) or PTH mRNA in the tumors (Simpson *et al.,* 1983) of hypercalcemic cancer patients. The search for a novel protein with PTH-like bioactivity intensified using several bioassays that had been developed for PTH (Goltzman *et al.,* 1981; Nissenson *et al.,* 1981; Rodan *et al.,* 1983). Using both *in vivo* and *in vitro* approaches, PTH-like bioactivity was identified in the blood and in tumor extracts from patients with MAH. These same bioassays were used in various combinations during the purification of a novel protein, named parathyroid-hormone-like peptide (PLP), from tumor tissue (Burtis *et al.,* 1987; Moseley *et al.,* 1987; Strewler *et al.,* 1987). When the gene was cloned and demonstrated to be structurally homologous to *PTH,* the protein was renamed parathyroid-hormone-related protein, or PTHrP (Mangin *et al.,* 1989; Suva *et al.,* 1987).

Molecular and Cellular Biology of PTHrP

Characterisitics of the PTHrP Gene and Its Protein Products

Localization of the genes encoding PTH and PTHrP to the short arms of human chromosomes 11 and 12, respectively, placed them among syntenic groups of functionally related genes and suggested a common ancestral origin (Goltzman *et al.,* 1989). Similarities in their structural organization and in the functional properties of their amino termini provide further support for the hypothesis that *PTH* and *PTHrP* are members of a single gene family. The human *PTHrP* gene is a complex unit that spans more than 15 kb of DNA. Its mRNA is transcribed from at least three promoters and undergoes differential splicing, giving rise to heterogenous mRNA species. The cDNA encodes a prototypical secretory protein with predicted mature isoforms of 139, 141, and 173 amino acids. The rat *PTHrP* gene is driven by a single promoter, and encodes a protein of 141 amino acids with marked sequence homology to human PTHrP up to residue 111, which suggests conserved functionality (Yasuda *et al.,* 1989). These proteins appear to undergo endoproteolytic cleavage in the secretory pathway, resulting in the release of fragments 1–36, 1–86, 38–94 and 38–101 (Rabbani *et al.,* 1993; Wu *et al.,* 1996).

PTHrP Overproduction in Malignancy

In view of its broad distribution pattern in embryonic and adult tissues, overproduction of PTHrP by malignant cells most likely results from deregulated expression of an endogenous protein during the process of malignant transformation, that is "eutopic" rather than "ectopic" production. Whereas tumor-bearing animal models have contributed enormously to our knowledge of the pathogenesis and treatment of MAH, *in vitro* cell models of carcinogenesis and solution biochemistry have provided valuable information regarding the molecular control of PTHrP expression and metabolism.

Transcriptional Regulation At the level of transcription a number of potential mechanisms have been invoked to explain overexpression of PTHrP in malignant cells, including gene amplification (Sidler *et al.,* 1996) and alterations in the methylation status of critical regulatory regions of the gene during neoplastic transformation (Broadus and Stewart, 1994; Ganderton and Briggs, 1997). Furthermore, the complex organization of the human *PTHrP* gene suggests that changes in tissue-specific promoter usage or splice variants might also contribute to overproduction of PTHrP during the process of malignant transformation. An extensive clinical study examined a variety of tumors, including 13 breast malignancies, with exon-specific probes to identify transcripts arising from all three promoters and from the different 3′ splice variants (Southby *et al.,* 1995). Although the authors failed to identify tissue-specific or tumor-specific transcripts, they did show that PTHrP was transcribed from multiple promoters in tumor samples, compared with a single promoter in normal tissue harvested from the same individual. The resulting overall increase in transcription could

then lead to cumulative overexpression of the protein in neoplastic tissue.

Adult T-cell leukemia-lymphoma (ATL) is an aggressive malignancy that is endemic to parts of Japan, Africa, the Carribean, and the United States. The disease develops after 20–30 years of latency in about 5% of individuals infected with human T-cell leukemia virus type I (HTLV-I) (Ejima *et al.,* 1995). PTHrP mRNA has been identified in samples harvested from asymptomatic HTLV-I carriers as well as from leukemic cells of ATL patients (Motokura *et al.,* 1989), and elevated circulating levels of PTHrP were detected in most hypercalcemic individuals in the acute phase of the disease (Ikeda *et al.,* 1994). In view of the documented role of HTLV-I infection in the pathogenesis of this disorder, and the high incidence of PTHrP overexpression in these patients, it was proposed that the viral protein TAX may stimulate PTHrP gene transcription. TAX is a 40-kDa nuclear phosphoprotein that transactivates its own promoter as well as those of a number of cellular genes. It interacts with a variety of transcription complexes that bind to DNA consensus elements in the *PTHrP* promoter including the cAMP response element, Ets-1, serum response element and the AP-1 binding site (Dittmer *et al.,* 1993; Ejima *et al.,* 1993). The MT-2 cell line, in which TAX is overexpressed, was used to determine that maximal stimulation of *PTHrP* involved activation of PKA and PKC (Ikeda *et al.,* 1995).

Regulation by Hormones and Growth Factors The apparent association between PTHrP overproduction and a malignant phenotype prompted investigators to use cell culture models of malignant progression to examine the regulation of PTHrP by hormones and growth factors. A variety of growth factors were found to be potent stimulators of *PTHrP* gene transcription (Kremer *et al.,* 1991; Sebag *et al.,* 1994), whereas $1,25(OH)_2(D)_3$ was found to be a potent inhibitor (Kremer *et al.,* 1991; Sebag *et al.,* 1992).

With the knowledge that keratinocytes were the first nontransformed cells in which PTHrP bioactivity was identified (Merendino *et al.,* 1986), a model of human epithelial cell carcinogenesis was chosen to examine the regulation of PTHrP production by hormones and growth factors (Henderson *et al.,* 1991; Kremer *et al.,* 1991; Sebag *et al.,* 1992). Basal levels of mRNA and unstimulated release of PTHrP into conditioned medium were both significantly higher in Ras-transformed keratinocytes compared with their nontransformed counterpart. In addition, the response to both positive (serum, EGF) and negative ($1,25(OH)_2D_3$) regulators of PTHrP production was significantly blunted in the malignant keratinocytes compared with either established or normal cells. The deregulated response to $1,25(OH)_2D_3$ was not a function of altered receptor status but was associated with a decrease in the ability of the steroid to inhibit mitogen-induced c-*myc* expression and progression through the cell cycle. Similar experiments performed on normal, human mammary epithelial cells indicated that the induction of PTHrP expression by serum and growth factors, and its repression by $1,25(OH)_2D_3$, were partially regulated at the level of gene transcription (Sebag *et al.,* 1994).

A $1,25(OH)_2D_3$ responsive repressor sequence (Fig. 1) was later identified between bases –1121 to –1075 upstream of the single promoter in the rat *PTHrP* gene (Kremer *et al.,* 1996). Using nuclear extracts prepared from normal human keratinocytes in mobility shift analyses, it was determined that the PTHrP repressor site bound a vitamin D receptor (VDR)–retinoid X receptor (RXR) heterodimer. The VDR–RXR complex was also identified in extracts of nuclei from immortalized keratinocytes but not from the Ras transformed cells that had previously shown resistance to $1,25(OH)_2D_3$ induced inhibition of PTHrP expression (Solomon *et al.,* 1998). Expression of wild-type RXRα in the transformed cells resulted in reconstitution of the VDR–RXR heterodimer. Subsequent work by the same group determined that disruption of the VDR–RXR complex in Ras transformed keratinocytes resulted from mitogen-activated protein kinase (MAPK)-stimulated phosphorylation of RXRα by the activated Ras/Raf/MAPK pathway (Solomon *et al.,* 1999). Taken together, these studies indicated that increased PTHrP production can result from alterations in the interaction between stimulatory and inhibitory signaling pathways in malignant cells.

In view of the evidence that signaling by growth factors through receptor tyrosine kinases (RTKs) was an important mechanism for regulating PTHrP gene expression, and that oncogenic transformation by the Ras component of the RTK signal transduction pathway was associated with overproduction of PTHrP by many malignant cells, studies were undertaken to examine the molecular mechanisms that linked PTHrP overexpression with RTK signaling. Transfection of a constitutively active derivative of the hepatocyte growth factor receptor known as Tpr-Met into PTHrP-producing cells resulted in a substantial increase in the expression and release of PTHrP (Aklilu *et al.,* 1996). Introduction of a point mutation into Tpr-Met, which prevented its association with the Ras signaling pathway, led to a significant reduction in PTHrP expression and release. Similar reductions were observed in cells treated with agents that inhibit Ras function by preventing it from anchoring to the cell membrane and transducing a signal (Aklilu *et al.,* 1997, 2000). These studies demonstrated the central role of Ras in enhancing PTHrP production in transformed cells and suggested a potential therapeutic role for Ras inhibitors in the treatment of MAH.

Processing and Degradation of PTHrP The Fischer rat implanted with the Rice-H500 Leydig cell tumor is a well characterized model of the human syndrome of MAH. The central role played by amino-terminal PTHrP in the pathogenesis of MAH in this model was demonstrated by passive immunization of tumor-bearing rats with antiserum raised against synthetic PTHrP-(1–34) (Henderson *et al.,* 1990). Hypercalcemic rats treated with PTHrP antiserum

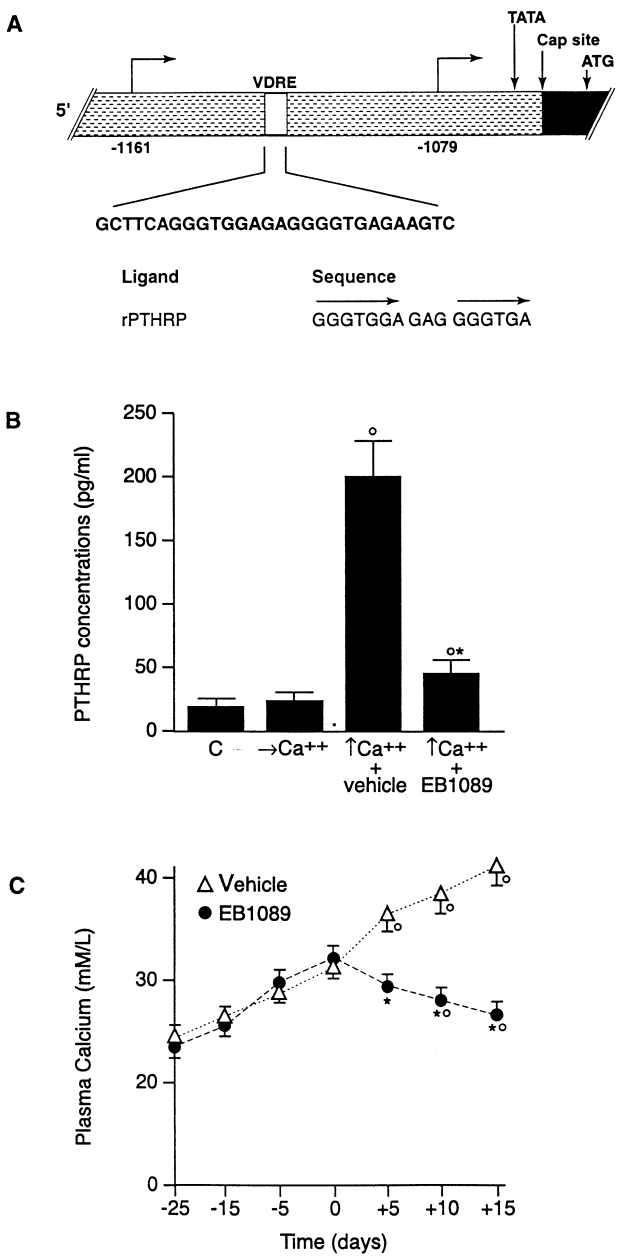

Figure 1 Repression of PTHrP and hypercalcemia by the vitamin D analog EB1089. $1,25(OH)_2D_3$ is a potent inhibitor of PTHrP production *in vitro* and *in vivo* and EB1089 is a low-calcemic analog of this active metabolite of vitamin D. (A) A consensus vitamin D response element (VDRE) in the *PTHrP* promoter was shown to bind a VDR–RXR heterodimer, which mediated repression of gene transcription *in vitro.* Infusion of EB1089 into hypercalcemic mice carrying a PTHrP-producing human squamous carcinoma resulted in a decline in systemic levels of immunoreactive PTHrP and in plasma calcium. (B) Plasma PTHrP was significantly reduced in EB1089-treated mice compared with vehicle treated control mice (*), although it was still elevated compared with control mice that did not carry tumors (O). (C) Plasma calcium levels continued to rise in the mice treated with vehicle whereas they approached near normal levels 15 days after commencing treatment with EB1089. Adapted in part from Kremer *et al.* (1996) and Abdaimi *et al.* (1999).

demonstrated a rapid and prolonged reversal of biochemical abnormalities, while those treated with preimmune serum or anti-PTH-(1–34) antiserum soon died of complications resulting from hypercalcemia. The Rice H-500 Leydig tumor cells were subsequently used to investigate the regulation of PTHrP production (Liu *et al.,* 1993) and metabolism (Rabbani *et al.,* 1993). Biosynthetic labeling of nascent PTHrP revealed rapid processing into three distinct amino-terminal species of 1–36, 1–86, and 1–141 amino acids, which were constitutively released into the extracellular environment. These observations were in agreement with similar studies that had been performed in malignant human cells (Soifer *et al.,* 1992).

Like PTH, PTHrP is synthesized as a prohormone with an amino-terminal extension. The biological potency of pro-PTHrP is considerably less than that of PTHrP-(1–34) (Liu *et al.,* 1995). The presence of a furin recognition sequence between the propeptide and the mature protein, and the knowledge that mature PTHrP was released from the cell through a constitutive pathway, predicted that pro-PTHrP was a substrate for the prohormone convertase furin. Transfection of COS-7 cells with a pro-PTHrP expression vector resulted in high levels of immunoreactive and bioactive PTHrP being released into the culture medium (Liu *et al.,* 1995). Coexpression of pro-PTHrP and anti-sense furin resulted in marked inhibition of furin expression and secretion of a PTHrP species that comigrated with synthetic pro-PTHrP and exhibited a marked reduction in its capacity to stimulate adenylate cyclase. This work supported the hypothesis that PTHrP is a furin substrate and raised the possibility that reducing furin activity inhibited production of bioactive PTHrP by malignant tissues.

From the preceding passages it is apparent that PTHrP shares certain properties with oncofetal proteins, transcription factors and cell cycle regulatory proteins, all of which are induced in a rapid and transient manner in response to mitogenic stimuli and are overexpressed in transformed cells. Taken together with evidence that PTHrP localizes to the nuclear compartment in a cell-cycle dependent manner, these observations suggest that the intracellular degradation of PTHrP might represent an important means by which cell proliferation and cell death are kept in check. Credence to this hypothesis was provided by studies that identified prepro-PTHrP as a target for multiubiquitination and ubiquitin-dependent proteolysis by the 26S protease complex (Meerovitch *et al.,* 1997). The ubiquitin pathway is most commonly associated with the rapid degradation of defective proteins and of short-lived cytosolic and nuclear proteins. However, ubiquitin and its activating enzymes have also been localized to a post ER compartment of the secretory pathway, and to the 26S proteasome at the cytosolic face of ER membranes. Recent studies have suggested that PTHrP might exit the secretory pathway between the ER and the Golgi to undergo ubiquitin-mediated degradation in the cytosol (Meerovitch *et al.,* 1998). This degradative pathway might also target PTHrP that reenters the cell following release into the extracellular environment (Aarts *et al.,* 1999) and suggests a potential mechanism that could regulate the balance between intracellular and secreted forms of PTHrP.

Mechanisms of Action of PTHrP

INTERACTION OF NH_2 TERMINAL PTHRP WITH CELL SURFACE RECEPTORS.

PTHrP was originally identified by virtue of its capacity to activate the classic G-protein-coupled PTH receptor (PTH1R) in kidney and bone. Thus, an elevation in the circulating level of PTHrP in patients with MAH initially results in calcium retention and phosphate wasting by the kidney, in association with an increase in the nephrogenous component of excreted cAMP. The increase in circulating PTHrP also stimulates bone resorption, which results in release of calcium and phosphate into the circulation. The excess phosphate is cleared through the action of PTHrP in the kidney, while calcium is retained, and the patient develops hypercalcemia and hypophosphatemia. Hypercalciuria ensues when the filtered load of calcium exceeds the reabsorptive capacity of the kidney. While PTH and PTHrP appear to influence the renal handling of calcium and phosphate in a similar manner, PTHrP may have a distinct mechanism of action in regulating the renal 1α hydroxylase enzyme. It has been pointed out that circulating levels of $1,25(OH)_2D_3$ are often low in advanced stages of MAH. Hypotheses that have been forwarded to explain this anomalous decrease include cosecretion of a specific 1α hydroxylase inhibitory factor from the tumor along with PTHrP, direct inhibition of the 1α hydroxylase enzyme by severe hypercalcemia or direct inhibition of the enzyme by non-amino terminal species of PTHrP (Kremer *et al.,* 1996)

Sequence homology between PTH and PTHrP is restricted to 8 of the first 13 residues, which include those required for activation of signal transduction cascades through adenylate cyclase and phospholipase C (Chorev and Rosenblatt, 1994). Additional conformational similarities in the 14–34 region permit amino-terminal fragments of the proteins to act as equivalent agonists for their common receptor, PTH1R. Whereas PTH1R is expressed in a wide variety of embryonic and adult tissues, including cartilage and bone, the recently identified PTH2R (see Chapter 24) appears to be preferentially expressed in the brain, where its principal ligand appears to be a hypothalamic peptide called tuberoinfundibular protein (TIP) (Usdin, 1997). In addition to these receptors for amino terminal species of PTHrP, several reports suggest the presence of cell surface binding proteins for carboxyl terminal fragments of PTHrP on skeletal cells (Orloff and Stewart, 1995) where they are believed to mediate anti-resorptive functions.

MECHANISM OF ACTION OF COOH-TERMINAL PTHRP

In the past, several investigators have reported a variety of functions for fragments of PTHrP that share no homology with PTH. These include the pentapeptide PTHrP-(107–111), which was named osteostatin for its potential to inhibit osteoclastic bone resorption in culture (Fenton *et al.,* 1991). Other studies using carboxy-terminal fragments of PTHrP have shown that they inhibit production of the early osteoblast marker osteopontin (Seitz *et al.,* 1995) in isolated osteoblasts and to be almost as effective as PTHrP-(1–34) in stimulating functional osteoclast formation from progenitor cells, both in the presence and absence of osteoblastic cells (Kaji *et al.,* 1995). These studies support *in vivo* observations demonstrating decreased osteoblastic and increased osteoclastic activity in association with elevated circulating levels of carboxy-terminal fragments of PTHrP in patients with MAH (Burtis *et al.,* 1994). In light of the considerable advances that have recently been made in the mechanisms that regulate bone turnover (see Chapter 58), further examination of the effects of carboxy-terminal fragments of PTHrP on bone cells may well be warranted at this time.

INTRACELLULAR MECHANISM OF PTHRP ACTION

Most of the cellular actions of PTHrP have been attributed to the interaction of its amino-terminus with the common PTH1R receptor and activation of signal transduction cascades through cAMP, inositol phosphates and DAG. However, similar to other growth regulatory proteins (Henderson, 1997), increasing evidence now points to a direct, intracellular mechanism of PTHrP action mediated through residues 87–107 in the mid-region of the protein. This region shares sequence homology with a lysine-rich bipartite nuclear targeting sequence (NTS) in nucleolin (Schmidt-Zachmann and Nigg, 1993), and with an arginine-rich NTS in the retroviral regulatory protein TAT (Dang and Lee, 1989). The PTHrP NTS is both necessary and sufficient to direct the passage of transfected PTHrP, as well as the cytoplasmic protein β-galctosidase, to the nuclear compartment of transfected cells and to localize endogenously expressed PTHrP to nucleoli in chondrocytes and osteoblasts *in vitro* and *in vivo* (Henderson *et al.,* 1995). Endogenous PTHrP has been identified in the coarse fibrillar component of nucleoli by immunoelectron microscopy. This area is occupied by complexes of newly transcribed 45s ribosomal RNA and protein that are destined for assembly into ribosomes. The biological relevance of nucleolar PTHrP was demonstrated when chondrocytes expressing wild-type PTHrP were protected from apoptosis induced by serum deprivation, whereas cells expressing the protein without the NTS were not.

These initial studies were corroborated by subsequent work that has implicated nuclear PTHrP in events that regulate cell cycle progression in vascular smooth muscle cells (Massfelder *et al.,* 1997) and keratinocytes and in differentiation in chondrocytes (Henderson *et al.,* 1996). Taken together, the observations have changed the way in which PTHrP is viewed and have paved the way for studies that have examined the molecular mechanisms that mediate its intracellular localization and action. The work is of physiological importance to cartilage and bone development as well as of pathophysiological importance in defining the role of PTHrP in MAH. One of the questions currently being addressed relates to the translocation of a prototypical secretory protein into the cytoplasic compartment of target cells. Three potential routes of entry are being evaluated. One involves retrograde transport of nascent PTHrP from the endoplasmic reticulum (Meerovitch *et al.,* 1998), a second involves internalization of secreted PTHrP (Aarts *et al.,* 1999), and a third involves

the alternative initiation of translation to exclude the "pre" sequence, which is necessary for entry into the endoplasmic reticulum.

In the original work using transfected COS-1 cells, the protein was detected in the secretory pathway of 100% of PTHrP-expressing cells, but only 15% of cells had nucleolar PTHrP. This suggested that PTHrP might be secreted and then reenter a subpopulation of randomly cycling cells. Using immunofluorescence microscopy and immunoblot analysis, full-length wild-type PTHrP was found on the cell surface, in the cytoplasm, and in the nuclear compartment of target cells *in vitro.* Furthermore, nuclear localization occurred in the presence and in the absence of PTH1R, which supports the previous studies suggesting the presence of an alternative cell surface binding protein for PTHrP (Aarts *et al.,* 1999).

Some insight into the regulated entry of cytoplasmic PTHrP into the nucleus was recently provided by studies performed on immortalized keratinocytes, which showed that PTHrP was a substrate for cell cycle associated kinases (Lam *et al.,* 1999). Phosphorylation of PTHrP at Thr^{85} by CDC2–CDK2 was proposed to anchor the protein in the cytoplasm during G2/M, whereas dephosphorylated PTHrP localized to nucleoli during other stages of the cell cycle. A similar mechanism of cytoplasmic anchoring has been proposed for SV40T antigen, which contains a bipartite NTS similar to that seen in PTHrP.

A third series of experiments has addressed the functional role of PTHrP in the nuclear compartment. The work was based on the original observation that PTHrP localized to a region of the nucleus that is rich in RNA–protein complexes. This led to the hypothesis that nucleolar PTHrP could interact with RNA and that it might play a role in ribosome biogenesis. Wild-type PTHrP was subsequently shown to bind to GC-rich homopolymeric RNA and to total cellular RNA with specificity and with high affinity (Aarts *et al.,* 1999). The PTHrP NTS peptide was effective in competing for this binding, whereas the equally basic nucleolin NTS was not. Furthermore, exogenous PTHrP NTS peptide translocated to the nucleolus of chondrocytes in culture, whereas a similar basic peptide corresponding to the nucleolin NTS was not internalized itself and could not prevent internalization of the PTHrP NTS peptide by competitive inhibition. These data suggested that the PTHrP NTS was recognized specifically by a cell surface binding protein. Site-directed mutagenesis identified a conserved GxKKxxK motif within the PTHrP NTS that was critical for both cell surface attachment and for RNA binding. These properties of the PTHrP NTS, namely to mediate internalization, nuclear translocation and RNA binding, are reminiscent of those bestowed on the RNA binding protein TAT by its NTS. Ultimately, however, *in vivo* studies will be required to determine the role of nuclear PTHrP in the physiology and pathophysiology of the skeleton.

PTHrP Action in Bone

Indirect Action of PTHrP on Osteoclasts Under physiological circumstances, PTH and PTHrP bind to receptors on preosteoblastic stromal cells and to mature osteoblasts (Rouleau *et al.,* 1990, 1988). Activation of PTH1R then promotes proliferation of committed osteoprogenitor cells and mediates coupled bone turnover. One of the most striking features of patients with MAH is the apparent dissociation of this coupled activity in favor of catabolic activity, which ultimately results in massive bone destruction and osteopenia.

Although it was recognized early on that stimulation of PTH1R on osteoblastic cells provided a signal to osteoclasts to resorb bone, it was not apparent that the two cells needed to be in physical contact for the signal to be generated. In retrospect, it was not surprising that early attempts to examine catabolic activity by isolated osteoclasts consistently failed, whereas coculture of cells derived from fetal mouse calvaria (source of osteoblasts) and mouse spleen (source of osteoclasts) resulted in the formation of multinucleated, bone resorbing cells (Takahashi *et al.,* 1988). These cells expressed tartrate-resistant acid phosphatase (TRAP), which is a recognized marker for osteoclasts *in vivo,* and were seen only in cultures treated with $1,25(OH)_2D_3$ in which osteoblastic and spleen cells were not physically separated by a porous membrane. These studies, therefore, indicated that osteolytic factors, after binding to preosteoblastic cells, could mediate the differentiation of hematopoietic mononuclear precursor cells into mature, multinucleated, functional osteoclasts and that the process required contact between the two cell lineages. The KS-4 cell line was cloned from fetal mouse calvaria and characterized as preosteoblastic on the basis of type I collagen expression and alkaline phosphatase activity. It was subsequently used to examine osteoclast formation and bone resorption *in vitro* in response to osteolytic factors like PTH, vitamin D and calcitonin (Yamashita *et al.,* 1990).

The RANKL/RANK/OPG System Some of the most significant advances in bone cell biology over the past few years have emerged as a consequence of studies that examined the basic signaling molecules that couple anabolic to catabolic activity in bone. In 1997 a novel, secreted member of the tumor necrosis factor receptor superfamily was identified in rat intestine (Simonet *et al.,* 1997). This soluble binding protein was named osteoprotegerin (OPG) when it was subsequently discovered that the most pronounced phenotypic trait of transgenic mice with high circulating levels of OPG was osteopetrosis caused by a dramatic decrease in bone resorbing osteoclasts. Administration of the recombinant protein to normal mice elicited a similar phenotype and inhibited the formation of osteoclasts *in vitro.* The same protein was independently purified from human embryonic lung fibroblasts and called osteoclastogenesis inhibitory factor (OIF), which was shown to inhibit PTH and $1,25(OH_2)D_3$-induced osteoclast formation (Tsuda *et al.,* 1997). The endogenous ligand for OPG/OIF was subsequently cloned by the same two groups who named it OPG ligand (OPGL) (Lacey *et al.,* 1998) and osteoclast differentiation factor (ODF) (Yasuda *et al.,* 1998), respectively. This novel TNF family member, now named RANKL is attached to the surface of cells of

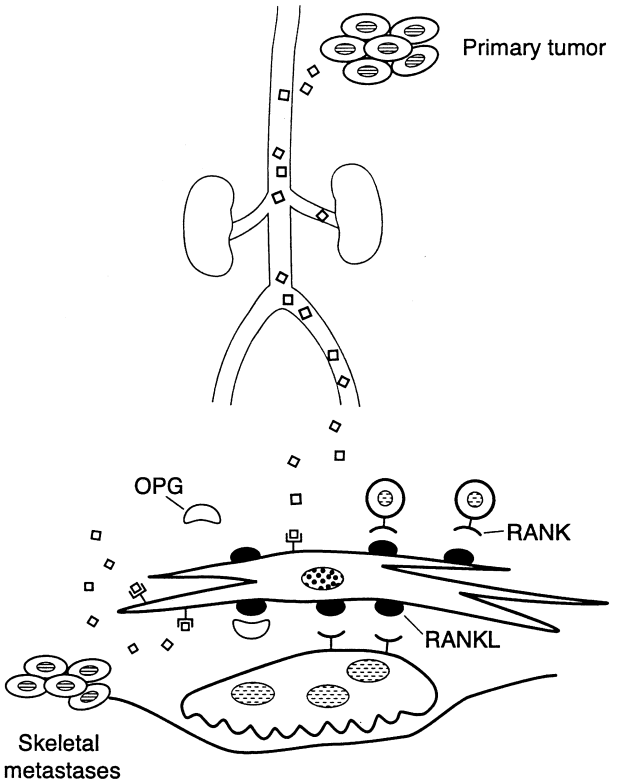

Figure 2 Systemic and local actions of tumor-derived PTHrP. Many primary tumors of diverse histological origin have been shown to release PTHrP into the systemic circulation, which carries the hormone to its primary target tissues of kidney and bone. Hypercalcemia ensues when PTHrP stimulates the renal reabsorption of calcium and promotes osteoclastic bone resorption, in part via its influence on the RANKL/RANK/OPG system. PTHrP is also released within the bone microenvironment from metastatic tumor cells. Binding of PTHrP to PTH1R on preosteoblastic stromal cells upregulates RANKL and downregulates the soluble RANKL receptor OPG. The interaction between RANKL and its receptor RANK on preosteoclasts and osteoclasts leads to an increase in the number of mature osteoclasts as well as an increase in their activity.

the osteoblast lineage and induces osteoclast differentiation and activation by binding to a receptor on the surface of osteoclast precursors and mature osteoclasts. Systemic administration of RANKL to normal mice resulted in bone destruction and hypercalcemia as a consequence of increased bone resorption with no associated increase in bone formation. The cell surface receptor for RANKL was later identified as receptor activator of NF-κB or RANK (Hsu *et al.,* 1999). Binding of RANKL to RANK results in the differentiation of pre-osteoclasts into mature osteoclasts and stimulates the activity of mature, bone-resorbing cells (Lacey *et al.,* 1998). The necessity of a functional RANKL/RANK/OPG system for osteoblast-mediated activation of bone was clearly demonstrated in mice with a targeted disruption of the gene encoding RANKL (Kong *et al.,* 1999). Mice lacking a functional *RANKL* gene exhibited severe osteopetrosis and defective tooth eruption, which were attributed to the complete absence of osteoclasts.

The physical association of osteoblast and osteoclast precusors in the bone microenvironment was, therefore, necessary for osteoclast differentiation and induction of bone resorption. Thus RANKL, while bound to the surface of osteoblastic cells, interacts with its cognate receptor RANK on the surface of preosteoclastic and osteoclastic cells to intiate bone resorption. On the other hand, the soluble receptor OPG can nonproductively bind to RANKL and prevent its interaction with the cell surface receptor RANK.

Regulation of the RANKL/RANK/OPG System by Calciotropic Hormones A local increase in calciotropic factors such as PTH, PTHrP, or $1,25(OH)_2D_3$ results in suppression of OPG and an increase in RANKL, which then binds its cognate receptor RANK to promote bone resorption (Lee and Lorenzo, 1999). Fragments of PTH with an intact amino-terminus were recently shown to inhibit OPG expression in osteoblastic cells in culture by a cAMP-dependent pathway (Kanzawa *et al.,* 2000). Experiments using bone marrow stromal cells have demonstrated an inverse relationship between the stage of differentiation and stimulation of RANKL expression by $1,25(OH)_2D_3$ (Menaa *et al.,* 2000).

MAH and the RANKL/RANK/OPG System It is readily apparent that the implications of the RANKL/RANK/OPG system for the pathogenesis of MAH are far-reaching (Fig 2). The vast majority of cases of MAH are thought to be caused by release of PTHrP from tumor cells, whether they are in the form of a solid tumor, a metastatic lesion in bone, or a hematologic malignancy. Tumor-bearing animal models, which were initially used to characterize the biochemical and histologic abnormalities associated with PTHrP-producing tumors in the 1980s and 1990s, are now in use to examine the relationships between PTHrP, the RANKL/RANK/OPG system and bone resorption. One such study used tumor-bearing mice to investigate the capacity of recombinant OPG to prevent, or inhibit, PTHrP-induced hypercalcemia (Capparelli *et al.,* 2000). Daily subcutaneous administration of 2.5 mg of recombinant protein prevented the onset of hypercalcemia in tumor-bearing mice and rapidly reduced blood calcium in mice with established hypercalcemia. These changes were associated with a reduction in osteoclast numbers, as assessed by histomorphometry, to well below the level seen in control mice. However, this treatment protocol did not affect the tumor burden, plasma PTHrP or cachexia in tumor-bearing mice. Although administration of 0.5 mg/day OPG was sufficient to reduce osteoclast numbers to below normal it did not prevent hypercalcemia in the tumor-bearing mice, which suggests the presence of a significant renal component to hypercalcemia associated with PTHrP overproduction by tumors. This later observation is in keeping with previous work using rats carrying the Rice-500 Leydig cell tumor as a model of MAH. Hypercalcemic, tumor-bearing rats treated with a bolus dose of anti-PTHrP-(1–34) antiserum sustained a rapid decrease in plasma calcium, associated with an increase in urinary calcium, followed by a sustained period of normocalcemia (Henderson *et al.,* 1990). The biphasic

response was attributed to an initial neutralizing effect of PTHrP bioactivity on calcium reabsorption in the kidney followed by sustained suppression of its catabolic activity in bone. In the mouse model, treatment with OPG would adequately suppress osteoclastic bone resorption but could not antagonize the renal component, which would result in residual hypercalcemia. These studies therefore reemphasize the importance of developing therapeutic strategies that take into account the dual role of PTHrP in the kidney and in bone, and which are aimed primarily at removing PTHrP as the principal pathogenetic agent in MAH.

Hypercalcemia associated with hematologic malignancies has traditionally been viewed as a consequence of the local lytic activity of tumor cells in bone. However, elevated circulating levels of PTHrP have been reported in hypercalcemic patients with a variety of hematologic malignancies (Burtis *et al.,* 1990; Henderson *et al.,* 1990). Further analysis of patients with lymphoma revealed similar results to those found in patients with breast cancer, in that high plasma PTHrP was associated with severe bone destruction and was most often associated with late-stage disease and high-grade pathology (Kremer *et al.,* 1996). In retrospect, these observations are not surprising. In both cases, bone destruction is mediated by the direct action of systemic and locally derived PTHrP on osteoblastic cells, to inhibit OPG and to stimulate RANKL, with a subsequent increase in bone resorption. Malignant cells also adhere to the marrow stroma, causing release of cytokines such as interleukins and tumor necrosis factor (TNF), which also stimulate RANKL production. In the case of multiple myeloma, residual OPG is inactivated by binding to syndecan-1, which is overproduced by the malignant B cells (Tricot, 2000).

Recently a soluble form of RANKL was discovered (sODF) in SCC-4 cells, which also secrete high levels of PTHrP and were derived from a tumor removed from a patient with MAH (Nagai *et al.,* 2000). The reading frame of the novel protein lacks a transmembrane domain and is homologous with the 74–318 region of the membrane-bound form of RANKL. Hence, solid tumors expressing both PTHrP and soluble RANKL pose a double "long-distance" threat to skeletal metabolism by releasing both of these catabolic agents into the systemic circulation. Indeed, the "uncoupling" of bone formation and resorption in some patients with MAH could conceivably be due to the predominant production of such a soluble RANKL, which would selectively activate the resorptive pathway in bone.

Experimental Approaches to Controlling Overproduction of PTHrP in MAH

Inhibition of Signaling through Receptor Tyrosine Kinases

Growth factors acting via receptor tyrosine kinases (RTKs) have been shown to be potent stimuli of *PTHrP* gene transcription. One of the best characterized signal transduction pathways downstream of RTKs is the Ras/Raf/Mek pathway. Activation of this pathway by growth factors or activated Ras was shown to increase PTHrP expression and release from a variety of cells in culture. Conversely, inhibition of the pathway through pharmacologic intervention successfully reduced PTHrP production *in vitro.* This suggested that specific inhibitors of components of the Ras signaling pathway could be used for therapeutic intervention to prevent the hypercalcemic syndrome associated with PTHrP overproduction *in vivo.* The direct role of Ras signaling in overproduction of PTHrP was investigated in rat 3T3 fibroblasts stably transfected with oncogenic Ras (Aklilu *et al.,* 1997). Release of PTHrP was increased up to 10-fold in the Ras-transformed cells, which rapidly formed tumors and induced MAH when implanted into nude mice. B-1086 is a small molecule that acts as a competitive inhibitor of Ras farnesylation, which is one of the metabolic conversions that is required for Ras to be anchored to the inner aspect of the cell membrane and transmit a signal downstream. Treatment of the Ras-3T3 fibroblasts *in vitro,* or of the Ras-3T3 tumor-bearing rats *in vivo,* with B-1086 led to a significant reduction in PTHrP secretion. In hypercalcemic mice this was reflected in a decrease in tumor growth and reversal of the biochemical abnormalities associated with MAH. Constitutive expression and release of PTHrP from the rat H-500 Leydig cell tumor could also be diminished using dominant negative forms of Ras and Raf (Aklilu *et al.,* 2000).

Vitamin D-Mediated Repression of PTHrP

$1,25(OH)_2D_3$ is a potent inhibitor of PTHrP in a variety of culture systems including keratinocytes, Leydig cells, prostate cells, and mammary and cervical epithelial cells. This inhibitory activity is mediated through the classic VDR, which also mediates the effects of $1,25(OH)_2D_3$ on calcium homeostasis. For this reason, administation of $1,25(OH)_2D_3$ in cases of MAH is counterproductive due to its intrinsic hypercalcemic effects. A concentrated effort has, therefore, been focused on the development of low-calcemic analogs of $1,25(OH)_2D_3$ that will effectively suppress PTHrP production but will not stimulate calcium absorption by the gut, calcium reabsorption by the kidney and, most importantly, bone resorption.

EB1089 and MC903 are two $1,25(OH)_2D_3$ analogs with conservative side-chain modifications that have been used to inhibit PTHrP production *in vitro,* and with variable degrees of success to prevent the hypercalcemic syndrome in animal models of MAH. Both EB1089 and MC903 were equipotent with $1,25(OH)_2D_3$ in their capacity to inhibit the release of PTHrP from immortalized and malignant keratinocytes in a previously characterized model of tumor progression (Yu *et al.,* 1995). Using the H-500 Leydig tumor model, constant infusion of either $1,25(OH)_2D_3$ or EB1089 inhibited the early increase in PTHrP expression and release from the tumors following implantation into syngeneic rats (Haq *et al.,* 1993). EB1089 was also effective in reversing hypercalcemia in nude mice carrying human squamous (Abdaimi *et al.,* 1999) and breast (El Abdaimi *et al.,* 2000) tumors (Fig. 1). Ras-transformed keratinocytes expressing high

levels of PTHrP rapidly developed into palpable tumors that continued to grow throughout the 100-day duration of the experiment. However, plasma calcium did not rise above the upper limit of normal until the final 20 days before sacrifice. Initiation of treatment by constant infusion of EB1089 at this stage resulted in a decline in serum calcium and PTHrP to near normal levels over the following weeks. However, the primary tumor continued to grow, suggesting that factors other than PTHrP were involved in tumor growth or that EB1089 had a greater effect on PTHrP release than it did on PTHrP expression in this case. Nude mice injected with MD-MB-231 human breast cancer cells and treated from the outset with EB 1089 exhibited a significant reduction in skeletal tumor burden over the 5-week experimental time course. These studies suggested that low calcemic vitamin D analogs may prove useful in the management of MAH and in inhibiting skeletal metastases associated with breast cancer.

ANTISENSE INHIBITION OF PTHRP PRODUCTION

An alternative approach for diminishing PTHrP overproduction in malignancy employed antisense technology to inhibit endogenous PTHrP expression. Transduction of H-500 rat Leydig tumor cells with antisense PTHrP followed by implantation of the transduced tumor cells into Fischer rats led to diminished hypercalcemia, decreased tumor cell proliferation, and prolonged survival of the host animal (Rabbani *et al.,* 1995). In view of evidence demonstrating that antisense furin could also reduce the production of bioactive PTHrP *in vitro,* presumably by inhibiting processing of mature PTHrP from its relatively inert precursor, it was decided to examine the effect of this maneuver *in vivo.* When Leydig tumor cells transduced with antisense furin were implanted *in vivo,* the host animals remained normocalcemic and tumor growth was reduced. Furthermore, survival of the animals was markedly prolonged (Liu *et al.,* 1995).

Overall these studies suggest that knowledge of the regulation of PTHrP production and processing can identify targets that may be useful for the development of agents to reduce circulating concentrations of bioactive PTHrP *in vivo* and to diminish hypercalcemia. Given the reduced size of tumors expressing antisense PTHrP, such agents may also reduce PTHrP levels indirectly by inhibiting tumor growth.

Role of 1,25-Dihydroxyvitamin D in MAH

Until quite recently the site of conversion of 25(OH)D to its active metabolite, $1,25(OH)_2D_3$, by the 25(OH)D 1 α-hydroxylase enzyme was thought to be restricted to the kidney. Although the kidney is still recognized as the primary site of $1,25(OH)_2D_3$ production *in vivo,* several extrarenal sites of 1 α-hydroxylase activity have been identified. These include cells of the hematopoietic and immune systems, the skin, the placenta and the developing skeleton . In contrast to the systemic role played by kidney-derived $1,25(OH)_2D_3$ in calcium homeostasis, locally produced hormone is thought to regulate cell proliferation and differentiation (Holick, 1999).

In contrast to the low circulating levels seen in the majority of hypercalcemic cancer patients, elevated serum concentrations of $1,25(OH)_2D_3$ have been reported in some cases of non-Hodgkin's (Breslau *et al.,* 1984) and Hodgkin's lymphoma (Jacobson *et al.,* 1988). This increase in circulating $1,25(OH)_2D_3$ was often seen in the presence of renal impairment, suggesting an extrarenal source of the hormone. More recently a human small cell lung cancer cell line, NCI H82, was shown to synthesize a vitamin D metabolite with similar biochemical properties and bioactivity to authentic $1,25(OH)_2D_3$ (Mawer *et al.,* 1994). Whether or not the systemic increase in $1,25(OH)_2D_3$ makes a significant contribution to hypercalcemia in these isolated cases remains a question of debate as the same malignancies also release cytokines and PTHrP, which are known mediators of MAH. On the contrary, in view of the recognized inhibitory activity of $1,25(OH)_2D_3$ on tumor growth and PTHrP production, coproduction of these factors by a tumor might be considered a good prognostic indicator, although this hypothesis has not yet been tested.

Cytokines as Systemic Mediators of MAH

Circulating proinflammatory cytokines most probably contribute to a number of the systemic manifestations of malignancy such as anorexia, dehydration, and cachexia (Ogata, 2000). Mounting evidence also suggests that cytokines released into the systemic circulation by a variety of solid tumors, which often coexpress PTHrP, contribute to the bone destruction associated with MAH. In this respect it is interesting to note that circulating concentrations of IL-6 correlate in a positive manner with tumor burden in patients with squamous and renal cell carcinoma, which represent the 2 prototypical malignancies associated with hypercalcemia and elevated circulating levels of PTHrP (Costes *et al.,* 1997; Nagai *et al.,* 1998).

Cytokines such as interleukin (IL)-1, IL-6, and TNF have been identified as physiological regulators of skeletal metabolism. They are produced by cells in the bone microenvironment, where they regulate their own and each other's expression and activity, as well as that of their cell surface receptors and soluble binding proteins (see Chapter 53 for details). The overall effect of this complex, interdependent activity is to stimulate bone resorption and to inhibit bone formation. Thus, the apparent uncoupling of resorption and formation in malignancies like myeloma that localize to bone has been attributed in large part to increased production of these cytokines in the bone microenvironment by the malignant cells (see Chapter 61 for details).

The relative contributions of systemically active PTHrP and cytokines to MAH has been examined in immune-compromised rodents carrying human tumors that overexpress both factors (Nagai *et al.,* 1998). Nude rats transplanted with a human squamous carcinoma of the oral cavity (OCC), which was shown to overproduce both PTHrP and

IL-6, rapidly developed severe hypercalcemia in association with high circulating levels of both PTHrP and IL-6. Hypercalcemic animals immunized with anti-IL-6 monoclonal antibody demonstrated a complete reversal in the biochemical abnormalities associated with elevated circulating IL-6, but only a small reduction in serum calcium. As measured by quantitative histomorphometry, there was a significant decrease in indices of bone resorption, as well as an increase in the mineral apposition rate in rats that received the neutralizing antibody. These results suggest that although IL-6 contributed to the skeletal abnormalities seen in OCC tumor-bearing rats, its contribution to the hypercalcemic syndrome was minor compared with that of PTHrP.

Using an alternate model, others demonstrated that mice carrying a human esophageal tumor (EC-GI), which coexpresses PTHrP and IL-1, develop a modest elevation in blood calcium (Sato *et al.,* 1989). A comparable level of hypercalcemia was observed in normal mice receiving a continuous infusion of IL-1. However, a significant increase in blood calcium was noted in mice that received a minimal daily dose of PTHrP in addition to the IL-1 infusion, suggesting a synergistic effect of PTHrP and IL-1 on bone resorption in this model. This hypothesis was supported by experiments in which addition of a small amount of synthetic PTHrP to the culture medium greatly enhanced ^{45}Ca release from prelabeled mouse bones, in response to recombinant IL-1.

This work using animal models of human disease predicts that PTHrP is the principal mediator of MAH. The hypothesis is further supported by clinical studies in which elevated circulating levels of PTHrP correlate strongly with hypercalcemia in patients with tumors of widely diverse histological origin (Ogata, 2000). No such correlation has been demonstrated for hypercalcemia and elevated circulating levels of cytokines such as IL-6 or IL-1. However, it has been proposed that a systemic increase in PTHrP not only promotes hypercalcemia but also stimulates normal cells to produce factors such as TNF-α, IL-1, IL-5, IL-6, and IL-8 in patients presenting with end-stage malignancy. The observation that infusion of anti-PTHrP antibody into mice with high circulating levels of PTHrP and IL-6 resulted in a prolonged decrease in the concentration of both factors in the bloodstream lends some support to this hypothesis.

Focal Osteolysis

Although PTHrP is more commonly recognized as a mediator of MAH while acting as an endocrine factor, increasing evidence suggests that it may also be a mediator of focal osteolysis induced by skeletal metastases while acting in a paracrine mode. Evidence for this has been presented in models of MDA-MB-231 breast cancer (El Abdaimi *et al.,* 2000; Guise *et al.,* 1996) and prostate cancer (Rabbani *et al.,* 1999). Indeed, it has been postulated that growth factors released from bone, notably transforming growth factor (TGF)-β, may increase PTHrP production and release from breast tumor metastases, further enhancing bone resorption (Yin *et al.,* 1999). In view of the fact that MDA-MB-231 breast cancer cells inoculated into nude mice by intracardiac injection will rapidly metastasize to bone and produce PTHrP, mice inoculated with tumor were treated with EB1089. Continuous infusion with EB1089 from the time of implantation caused a reduction of the tumor burden in bone, which resulted from a decrease in both the number and the size of metastatic lesions, compared with control mice that were infused with vehicle alone (El Abdaimi *et al.,* 2000). It appears, therefore, that the low-calcemic analog of $1,25(OH)_2D_3$ might also prove to be an effective therapeutic modality to reduce the skeletal tumor burden of patients with late-stage breast cancer.

The presence of PTHrP in breast cancer patients has been associated with rapid progression of disease and with a poor outcome (Pecherstorfer *et al.,* 1994). Early work showed elevated circulating levels of amino-terminal PTHrP in a large percentage of hypercalcemic breast cancer patients (Henderson *et al.,* 1990) and that the vast majority of breast cancer metastases in bone expressed high levels of PTHrP (Powell *et al.,* 1991; Southby *et al.,* 1990; Vargas *et al.,* 1992). Another recent study used MCF-7 breast cancer cells transfected with PTHrP as a model system to explore the potential role of PTHrP in breast cancer metastases to bone (Thomas *et al.,* 1999). PTHrP-producing MCF-7 cells not only supported osteoclast formation and activity via the RANKL/RANK/OPG system *in vitro* but also enhanced the potential of these tumor cells to metastasize to bone *in vivo.* Taken together these observations predict that tumor-derived PTHrP acts at both the systemic (primary tumor) and local (skeletal matastases) levels to stimulate osteoclastic bone resorption, at least in part, through the RANKL/RANK/OPG system.

Inhibition of Osteolysis by Bisphosphonates

The bisphosphonates, which are nonhydrolyzable analogs of pyrophosphate, are highly effective inhibitors of osteoclastic bone resorption (see Chapter 78). They are now considered to be the modality of choice in the treatment of MAH and have been advocated as well in the treatment of focal osteolysis caused by breast cancer (Hortobagyi *et al.,* 1996) and multiple myeloma (Berenson *et al.,* 1996). Their efficacy as antiresorptive agents is a function of their metabolic stability, their capacity to bind avidly to hydroxyapatite and to stabilize crystal structure, and their ability to directly inhibit osteoclast activity and apparently to promote osteoclast apoptosis. This is accomplished by inhibition of prenylation of small GTP binding proteins or incorporation of the compounds into toxic forms of ATP (Rodan, 1998). Unlike calcitonin, which elicits a mild and short-lived hypocalcemic response, a single dose of a bisphosphonate such as pamidronate can result in a steady decline in serum calcium over several days.

Using metastatic breast cancer as an example of PTHrP-driven MAH, evidence from long-term clinical trials suggest that bisphosphonate therapy may not, unfortunately, be effective in the long-term management of MAH. Despite the marked reduction in skeletal tumor burden seen in women treated with pamidronate during late stage disease, the prophylactic use of these agents at earlier time points has met with variable success (Lipton, 2000). This has been attributed variously to low tolerance for the drugs, to resistance to their action in bone, and, most importantly, to their failure to attack the source of the problem, which in large part appears to be overproduction of PTHrP. In one study serum calcium was normalized in 90% of patients with MAH after an initial treatment with pamidronate, whereas hypercalcemia was corrected in only 15% of those patients by the third consecutive treatment (Body *et al.,* 1998). Furthermore, circulating PTHrP continued to rise in association with enhaced renal calcium reabsorption. Resistance to ibandronate, another bisphosphonate therapy for recurrent hypercalcemia, was observed in a large cohort of patients with a variety of PTHrP-producing tumors (Rizzoli *et al.,* 1999). Taken together, the results suggest that although bisphosphonates are invaluable members of our armamentarium for inhibiting osteolysis, additional therapeutic modalities may need to be developed, to inhibit the action of PTHrP in both kidney and bone. This may require inhibitors of production, processing, and/or action of this systemic factor (Ogata, 2000).

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CHAPTER 61

Local Factors in Skeletal Malignancy

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Tumors have multiple effects on the skeleton, all of which are mediated by tumor cells altering the behavior of bone cells and causing disruptions or enhancement of the normal bone remodeling process. These effects on bone cells are caused by increased production of peptides in tumor-bearing patients, some of which act at short range and some at a distance as systemic or hormonal factors, which then modulate bone cell activity. This is certainly the major and possibly the only mechanism by which tumors affect bone, and in this chapter a number of the peptides that have been implicated will be discussed. It is possible that tumor cells also interact directly with bone cells by cell–cell contact, or directly with the mineralized bone matrix without the intervention of bone cells, but the biological or clinical significance of these effects is unclear.

The common skeletal syndromes that tumors cause are:

1. The humoral hypercalcemia of malignancy (HHM), due to circulating hormonal mediators of osteoclastic bone resorption and renal tubular calcium reabsorption, which are secreted by tumor cells from a primary (or secondary) site distant from bone.
2. Osteolytic bone metastasis (with or without hypercalcemia), due to solid tumors metastatic to the bone marrow cavity and causing localized bone destruction at those sites.
3. Osteoblastic bone metastases, caused by solid tumor metastasis to the bone marrow cavity and causing new bone formation by producing local growth regulatory factors which activate osteoblasts.
4. Myeloma bone disease, the characteristic and distinctive form of osteolysis caused frequently by myeloma cells.
5. Oncogenic osteomalacia, the syndrome of phosphate depletion caused by urinary phosphate loss and characterized by rickets or osteomalacia.

When tumor cells affect the skeleton, the results are often catastrophic for the patient. However, the skeletal effects are only part of the overall clinical picture. The patient may also suffer from other paraneoplastic syndromes which greatly affect quality of life. Some of these syndromes occur with increased frequency in patients with hypercalcemia, and will be described in more detail.

Each of these paraneoplastic endocrine syndromes has been associated with soluble mediators produced either by tumor cells or by other cells in response to the tumor. In none, however, can all of the features of the associated clinical syndrome be accounted for entirely by a single mediator so far discovered or described. In some of these syndromes, most notably humoral hypercalcemia of malignancy, parathyroid hormone-related peptide (PTH-rP) is a major mediator. It is the purpose of this chapter to review evidence for the role of some of the other factors produced in excess in malignancy and the potential role that these may have on bone.

Humoral Hypercalcemia of Malignancy Syndrome

Parathyroid Hormone-Related Peptide

The possibility that a factor with PTH-like effects may cause humoral hypercalcemia was first suggested by Albright (1941). This hypothesis was confirmed almost 50 years later when a parathyroid hormone-like peptide was purified from a number of cell lines as well as tumor

extracts and then eventually cloned (Moseley *et al.,* 1987; Stewart *et al.,* 1987; Strewler *et al.,* 1987). With a similar amino acid sequence to PTH at the N-terminal end of the molecule, this peptide mimics the biological effects of PTH on both bone resorption and renal tubular function. It does this by binding to and activating the PTH receptor. It causes increased nephrogenous cyclic adenosine monophosphate (cyclic AMP) generation, inhibition of renal tubular phosphate reabsorption, and enhanced reabsorption of calcium from the renal tubules (Yates *et al.,* 1988). These features are characteristic of patients with humoral hypercalcemia of malignancy, and in that syndrome PTH-rP is the presumed mediator. However, there are differences between the syndrome of humoral hypercalcemia of malignancy and primary hyperparathyroidism that are unaccounted for. Primary hyperparathyroidism is caused by excess parathyroid hormone produced by a parathyroid adenoma or parathyroid gland hyperplasia. If HHM was due entirely to a factor that binds to and interacts with the PTH receptor in an identical manner to PTH, then the two syndromes should be the same. Differences in the two syndromes however, are quite clear. In most patients with the humoral hypercalcemia of malignancy, there is a decrease in intestinal calcium absorption and 1,25-dihydroxyvitamin D synthesis (Stewart *et al.,* 1980), in contrast to that seen in patients with primary hyperparathyroidism. In patients with humoral hypercalcemia of malignancy bone formation is decreased (Stewart *et al.,* 1982), again in contrast to patients with primary hyperparathyroidism. In patients with humoral hypercalcemia of malignancy, there is no evidence of renal tubular bicarbonate wasting, which is characteristic of primary hyperparathyroidism. In view of the differences between these syndromes, it appears likely that other factors are responsible for the differences. Another alternative is that there are differences in the biological effects of PTH and PTH-rP that have not been described as yet, despite intense investigation. PTH-rP has been described in detail in other chapters and will not be discussed further here.

Parathyroid Hormone

Since the earliest observations of Albright, PTH has been sought for in tumors associated with hypercalcemia. There were early reports that PTH was produced frequently in patients with breast cancer and other malignancies associated with extensive osteolysis (Benson *et al.,* 1974), but in retrospect it now appears likely that these were due to insensitivity in the immunoassays that were used to detect PTH-like activity. Nevertheless, PTH is a rare cause of hypercalcemia associated with cancer (Simpson *et al.,* 1983). Due to ectopic PTH production by nonparathyroid tumors (Yoshimoto *et al.,* 1989; Nussbaum *et al.,* 1990; Strewler *et al.,* 1990). In the case described by Nussbaum *et al.,* there was a potential molecular explanation for the aberrant production of PTH by the tumor cells. In this tumor, there was a DNA rearrangement in the region of the promoter which may have been responsible for the overexpression of PTH by the tumor.

Cytokines Which Have Been Associated with Malignancy

A number of cytokines have been associated with the humoral hypercalcemia of malignancy syndrome. These include interleukin-1, tumor necrosis factor and interleukin-6. We think it is likely that they may be involved in some other clinical manifestations in addition to hypercalcemia. As an example, interleukin-6 is frequently produced by either tumor cells or host cells in patients with malignancy, and it may be responsible for some of the other endocrine syndromes associated with malignancy such as cachexia, anemia, thrombocytosis, and acute phase reactions. Interleukin-6 markedly enhances the effects of PTH and PTH-rP on bone resorption and hypercalcemia (De la Mata *et al.,* 1995). It presumably does this because of its effects to increase pools of cells at earlier stages in the osteoclast lineage (De la Mata *et al.,* 1995). Interleukin-6 causes a marked increase in generation of CFU-GM and committed marrow precursors. It is in itself a weak bone resorbing factor (Black *et al.,* 1991), but may act permissively for the effects of other factors which act later on the osteoclast lineage such as PTH-rP (De la Mata *et al.,* 1995). This has been demonstrated using Chinese hamster ovarian cells that have been transfected with the interleukin-6 and PTH-rP genes (De la Mata *et al.,* 1995). On its own, however, interleukin-6 has been associated with the hypercalcemia–leukocytosis syndrome. In the MH-85 human squamous cell carcinoma of the maxila, there is marked production of interleukin-6 by the tumor cells, and Yoneda *et al.* (1991) have shown that hypercalcemia in this syndrome is abrogated by the use of neutralizing antibodies to human interleukin-6. These antibodies do not react with murine interleukin-6, and thus the interleukin-6 which is presumably responsible for the syndrome is produced by the human tumor.

Other cytokines have also been associated with the humoral hypercalcemia of malignancy syndrome. Interleukin-1α is frequently produced by tumors in association with PTH-rP (Sato *et al.,* 1989; Fried *et al.,* 1989; Nowak *et al.,* 1990). In fact, in the human tumor studied by Sato *et al.* (1989), PTH-rP was produced together with interleukin-1α and it was shown by these authors that the two factors together caused synergistic effects on hypercalcemia.

There have also been a number of other examples where solid tumors have been shown to stimulate normal host immune cells to produce osteotropic cytokines such as tumor necrosis factor (Sabatini *et al.,* 1990 a,b; Yoneda *et al.,* 1991). Examples are the human melanoma cells A375, the rat Leydig cell tumor and the MH-85 human squamous cell carcinoma of the maxilla. In each of these tumors, it has been shown that the tumor cells secrete soluble factors which can stimulate normal host immune cells and particularly cells in the monocyte-macrophage family, to produce cytokines such as tumor necrosis factor. Sabatini *et al.* (1990b) showed that the mediator responsible for tumor necrosis factor (TNF) production in the A375 tumor was granulocyte macrophage colony-stimulating

factor (GM-CSF). In the case of the MH-85 tumor, Yoneda *et al.* (1991) showed that another mediator was involved that was distinct from GM-CSF. In this latter case, the hypercalcemia could be reversed by treating tumor-bearing mice with antibodies to tumor necrosis factor. There was a fourfold increase in circulating tumor necrosis factor in tumor-bearing animals. Removal of the spleens also caused a reduction in the hypercalcemia, presumably because the spleen is the major source of host immune cells producing TNF. There was no production of TNF by the tumor cells themselves.

The production of these cytokines by host immune cells may be part of the normal humoral defense mechanism designed to protect the host from the presence of a tumor. However, at the same time these cytokines are having a beneficial effect, they also may be causing some of the paraneoplastic endocrine syndromes associated with malignancy. In the examples described here, they were likely to be responsible for cachexia, leukocytosis, anemia, and hypertriglyceridemia, which occurred in the tumor-bearing host. Reduction of the cytokine levels with the use of neutralizing antibodies relieved some of these syndromes in the above models, and particularly in the rat Leydig tumor and in the MH-85 tumor (Yoneda *et al.,* 1991; Sabatini *et al.,* 1990a,b).

Prostaglandins of the E Series

It has been known for 25 years that prostaglandins of the E series stimulate osteoclastic bone resorption in organ cultures of fetal rat long bones and neonatal mouse calvariae (Klein and Raisz 1970). Following these seminal observations, it was for some time thought that prostaglandins may be important mediators in the bone destruction associated with metastatic breast cancer and also possibly in the humoral hypercalcemia of malignancy (Powles *et al.,* 1976; Bennett *et al.,* 1975; Seyberth *et al.,* 1975). The coculture of breast cancer cells together with bone results in osteoclastic bone resorption which could be reversed by inhibitors of prostaglandin synthesis such as aspirin and indomethacin (Powles *et al.,* 1973, 1976; Bennett *et al.,* 1975; Greaves *et al.,* 1980). However, this may have been an artifact of the culture conditions, and studies using prostaglandin synthesis inhibitors in patients with breast cancer metastatic to bone have been uniformly disappointing (Powles *et al.,* 1982). It now appears more likely that prostaglandins of the E series are minor pathogenetic factors in the humoral hypercalcemia of malignancy. They are occasionally found in patients in which it is clear that the increase in bone resorption can be reversed by drugs such as indomethacin, but these patients are unusual (Mundy *et al.,* 1983). Moreover, it is also possible that in the animal models which are associated with increased prostaglandin production (Tashjian *et al.,* 1972), other mediators as well may be involved. Prostaglandins are probably rarely if ever sole causes of the humoral hypercalcemia of malignancy syndrome.

Myeloma Bone Disease

The malignant disorder, myeloma, is characterized by extensive bone destruction. It is the major clinical hallmark of the disease. Bone destruction is manifested by a number of important clinical features including intractable bone pain, fractures that occur either spontaneously or following trivial injury, and hypercalcemia, which can be life-threatening. The majority of patients, probably more than 80%, have bone pain as a presenting symptom (Snapper and Kahn 1971). The bone lesions which occur in myeloma occur in several patterns (Mundy and Bertolini 1986), the most common of which are multiple, discrete lytic lesions which occur at the site of deposits or nests of myeloma cells. These are apparent in the axial skeleton, skull, vertebrae, ribs, and proximal ends of long bones. The characteristic lesions show discrete lysis without evidence of new bone formation. Some patients develop diffuse osteopenia, which can be readily confused with osteoporosis. However, this osteopenia is extremely severe, aggressive, of rapid onset, and associated with vertebral body compression and collapse. In some patients, there may be pressure on the spinal cord with associated symptoms of spinal cord compression. Some patients with myeloma have solitary plasmacytomas with single discrete osteolytic lesions associated. Even more rarely, patients with myeloma have no lytic lesions or bone loss, but rather demonstrate an increase in new bone formation around collections of myeloma cells. These patients with osteosclerotic myeloma often have the so-called POEM's syndrome (severe progressive sensory and mode of polyneuropathy, organomegaly, endocrine dysfunction, and papilloedema associated with their myeloma) (Bardwick *et al.,* 1980).

The bone destruction which is associated with myeloma is mediated by osteoclasts. Osteoclasts in myeloma always occur adjacent to collections of myeloma cells, so it is likely that the mechanism for the bone destruction in myeloma is locally mediated, presumably by cytokines released by the myeloma cells in the microenvironment of the osteoclast (Mundy *et al.,* 1974a,b). The major area of dispute in myeloma is the identity of the responsible cytokine or cytokines.

About one-third of patients with myeloma develop hypercalcemia at some time during the course of the disease (Mundy and Bertolini, 1986). Hypercalcemia is almost always accompanied by an impairment in glomerular filtration. This impairment in glomerular filtration may be due to multiple causes in patients with myeloma, including the production of free light chains (or Bence Jones proteins) by the myeloma cells, by uric acid nephropathy, by chronic infections, or by the hypercalcemia itself.

Bone formation is usually markedly impaired in patients with myeloma for reasons that are totally unclear. This can be manifested clinically by measurement of serum alkaline phosphatase which is not increased, unlike most other patients with osteolytic bone lesions, and radionuclide bone scans which show no evidence of increased skeletal uptake

of the isotope, which indicates impaired osteoblastic responses to the increase in bone resorption.

Although the observation that local cellular mechanisms were largely responsible for the bone destruction in myeloma was first made 25 years ago (Mundy *et al.,* 1974a), there are still unresolved questions as to the nature of the local mediator(s) responsible for the increased osteoclast recruitment and activation. Numerous cytokines have been implicated including interleukin-1, interleukin-6, and tumor necrosis factor β (lymphotoxin) and hepatocyte growth factor, all of which can stimulate osteoclast formation and activity. Independently or together, each of these cytokines has been associated with the bone destruction of myeloma (Cozzolino *et al.,* 1989; Garrett *et al.,* 1987; Kawano *et al.,* 1989; Hjertner *et al.,* 1999). The evidence for a role of these cytokines comes from the different modes of studying the mechanisms of bone resorption. Part of the problem is the lack until recently of reliable animal models of the disease. As a consequence, it has not been possible in myeloma to confirm that production of any single cytokine *in vitro* is also relevant *in vivo,* as it has been in patients with the humoral hypercalcemia of malignancy due to solid tumors. The evidence for lymphotoxin as a possible mediator comes from cultures of established human and animal myeloma cell lines (Garrett *et al.,* 1987). In these cultures, it has been shown that the bone resorbing activity produced by the myeloma cells can be mostly accounted for by lymphotoxin, and that this bone resorbing activity can be largely neutralized by antibodies to lymphotoxin. However, freshly isolated cells from patients with myeloma give a different picture. In this situation, the cultured cells, which are not homogeneous, produce interleukin-1β (Cozzolino *et al.,* 1989; Kawano *et al.,* 1989). Whether this is artifact or whether this is due to the conditions of the culture remains unknown. For example, for a number of years the field was misled by the production of prostaglandins *in vitro* from cultured freshly isolated cells which turned out to be most likely a culture artifact.

Interleukin-6 plays an important role in myeloma, independent of its effects on bone (Klein *et al.,* 1989; Bataille *et al.,* 1989). Interleukin-6 is the major growth factor which is produced by myeloma cells and is responsible for the neoplastic phenotype in myeloma cells. Myeloma cells produce small amounts of interleukin-6, as do the stromal cells in the myeloma cell microenvironment. It is a point of controversy over whether myeloma cell growth *in vitro* is due to autocrine production of interleukin-6 by myeloma cells or paracrine production by the host cells. Nevertheless, interleukin-6 is likely to be an important factor in bone resorption. Although interleukin-6 shows weak bone resorbing factor activity (Black *et al.,* 1991), it is more likely to be acting in a permissive role rather than as the major bone resorbing factor (De la Mata *et al.,* 1995).

HGF has also been reported to be involved in myeloma bone disease. It is secreted by freshly isolated myeloma cells and myeloma cell lines. Moreover, high-serum HGF has been reported to be associated with poor prognosis in myeloma patients (Seidel *et al.,* 1998). Although there is presently no direct evidence that HGF mediates myeloma-associated bone disease in patients *in vivo,* HGF has been shown to be chemotactic for osteoclasts *in vitro* and to stimulate osteoclastogenesis and bone resorption *in vitro* (Sato *et al.,* 1995; Fuller *et al.,* 1995). This effect of HGF is via a paracrine effect on marrow stromal/osteoblastic cells (Sato *et al.,* 1995; Grano *et al.,* 1996). It is interesting to note that serum HGF levels have also been shown to be elevated in breast cancer patients (Taniguchi *et al.,* 1995).

As mentioned above, neither human nor murine myeloma cells, which cause extensive bone resorption *in vivo,* produce significant amounts of bone resorbing cytokines *in vitro.* Data now emerging suggest that the dichotomy between *in vitro* and *in vivo* observations may be related to the fact that *in vivo,* myeloma cells exist tightly bound to various elements of the marrow stroma. It is now apparent that myeloma cells behave differently in the marrow microenvironment and that cell–cell and/or cell–extracellular matrix (ECM) interactions between tumor cells and marrow stroma are critical, not only for the growth and survival of the tumor cells, but also for initiating and maintaining the aggressive bone destruction.

Cell–cell and cell–ECM adhesion events are increasingly being implicated in the etiology and pathogenesis of many diseases including cancer. Human and murine myeloma cells express very late antigen 4 (VLA4) (Michigami *et al.,* 2000; Teoh and Anderson, 1997; Vanderkerken *et al.,* 1997), a heterodimeric complex of α4 and β1 integrin subunits. All the three known VLA4 ligands, VCAM-1, fibronectin, and osteopontin, are present in the marrow, and of particular significance for myeloma, VCAM-1 is expressed by bone marrow stromal cells constitutively. There is evidence that indicates that myeloma cells use VLA4 not only to home to bone, but also to preferentially adhere to bone marrow stromal cells through VCAM-1 as well as the ECM (Faid *et al.,* 1996; Teoh and Anderson 1997; Michigami *et al.,* 2000). Data from other studies including ours provide compelling evidence implicating these cellular interactions, not only in the growth of myeloma in bone, but also in initiating the sequence of events that lead to bone destruction. For example, data from our studies indicate a link between VCAM/1/VLA4 and osteoclast formation via induction of RANKL following direct cell–cell contact between bone marrow stromal cells and myeloma cells. RANKL is a member of the TNF ligand superfamily whose expression by marrow stromal osteoblastic cells has been shown to be obligatory for osteoclast formation and activity. 5TGM1 myeloma cells exhibit tight adherence to the mouse marrow stromal cell line ST2 *in vitro,* and contact between the two cell types increased RANKL mRNA expression in comparison to either cell type alone. This interaction also causes production of a soluble factor(s) by the myeloma cells capable of enhancing RANKL expression in ST2 cells and inducing resorption in fetal rat long bones (Michigami *et al.,* 2000; Oyajobi *et al.,* 1998). The effect of these cell–cell interactions was mimicked by treating the myeloma cells with recombinant soluble VCAM-1 (Oyajobi *et al.,* 1998) and, conversely, the production of the

soluble activity was blocked by neutralizing antibodies to either α4 or to VCAM-1 (Michigami *et al.,* 2000).

Recent evidence suggests a role for MIP-1α in myeloma bone disease. MIP-1α belongs to a family of small (8–12 kDa) proteins that are chemoattractive for leukocytes (Opdenakker and Van Damme, 1999; Rollins, 1997; Zlotnik *et al.,* 1999). These proteins called chemokines are classified into two major groups, cysteine–cysteine (CC) or cysteine–X–cysteine (CXC), depending on the positions of the first two invariant cysteine residues. Using competitive PCR, mRNA for MIP-1α, a CC chemokine, was shown to be elevated in bone marrow plasma of myeloma patients compared to normal controls (Choi *et al.,* 2000). Furthermore, elevated levels of MIP-1α were detected in marrow supernatants from a majority of myeloma patients with active disease (8/12) by ELISA but not in normal controls (0/9). An anti-MIP-1α neutralizing antibody blocked the stimulatory effect of bone marrow supernatants from myeloma patients on osteoclast formation in human bone marrow cultures, but had no effect on control levels of osteoclast formation (Choi *et al.,* 2000). Other workers have also recently reported elevated MIP-1α levels in four of eight aspirates from myeloma patients (Abe *et al.,* 1999). MIP-1α-producing myeloma cell lines markedly enhanced formation of $TRAP^+$ multinucleated cells when cocultured with rabbit bone marrow cells, and this was abrogated by an anti-VLA4 antibody (Abe *et al.,* 1999). We have found also that MIP-1α is expressed by human and murine myeloma cell lines including 5TGM1 cells. Importantly, MIP-1α level is increased in a 5TGM1/ST2 coculture system, implying a role for VLA4–VCAM-1 interaction in regulating MIP-1α expression. Since MIP-1α is known to promote cellular adhesion by upregulating expression of integrins such as α4 (Rollins, 1997; Zlotnik *et al.,* 1999), MIP-1α could potentially increase adherence of myeloma cells to marrow stromal cells by upregulating α4 and consequently activating VLA4. This increased cellular adhesion would, in turn, augment MIP-1α production as previously described (Steinhauser *et al.,* 1998), resulting in recruitment of monocytes/macrophages, which are precursors of osteoclasts.

Thus, there is a growing body of evidence linking the growth of myeloma cells in the bone marrow microenvironment, their capacity to stimulate bone resorption, and the cytokines that they produce. Myeloma seems to have a special predilection for bone, unlike other hematologic malignancies. We believe that this special predilection may be related to the capacity of bone, and in particular of osteoclasts, to produce the major growth factor for myeloma cells, namely interleukin-6. Osteoclasts are the most prodigious cell source of interleukin-6 that is known (Bonewald, unpublished observation). Other cells in the bone marrow microenvironment also produce interleukin-6 and in particular cells from the osteoblast lineage in response to cytokines such as interleukin-1 and tumor necrosis factor. This production of interleukin-6 in this microenvironment may be responsible for enhancing the growth of myeloma cells and for promoting their aggressive behavior (Garrett *et al.,* 1995). Thus, we propose that there may be a vicious cycle in the bone marrow microenvironment between the myeloma cell and osteoclastic bone destruction—the more aggressive the behavior of the myeloma cells, the greater the bone destruction, which in turn causes excess production of interleukin-6 which makes the myeloma cell growth even more aggressive.

The implications of these findings may be very important. For example, if bone destruction can be reduced in patients with myeloma, this may have beneficial effects not just on the bone disease and the capacity to reduce skeletal complications, but also on the growth of the myeloma cells in that microenvironment. The results of extensive studies with the use of drugs such as the bisphosphonates in myeloma is eagerly awaited, particularly if their use is accompanied by a beneficial effect on tumor burden.

Osteoblastic Bone Metastases

Some tumors affect the skeleton by causing osteoblastic metastases. This situation is much less common than that of the osteolytic metastasis, probably representing no more than 5–10% of all metastatic bone lesions. However, it is very common in some tumors, and particularly solid tumors of the male urogenital tract. Prostate cancer is the most notable example of a human tumor that is associated with increased bone formation. In prostate cancer, almost all patients will eventually develop osteoblastic bone metastases if they live long enough (Galasko, 1986; Mundy and Martin 1993). The osteoblastic metastasis is due to the production by the tumor cells of growth regulatory factors which enhance the activity of osteoblasts to proliferate and differentiate to form bone nodules (Charhon *et al.,* 1983).

The mechanism of formation of the osteoblastic metastasis of course has been of great interest to everyone in the field of bone biology. The early histomorphometric studies suggested that osteoblastic metastases were due to soluble factors which were produced by the tumor cells which had metastasized to the marrow cavity (Charhon *et al.,* 1983). This new bone formation occurred without prior bone resorption and therefore does not represent an example of enhancement of the coupling process (Charhon *et al.,* 1983). Extensive studies have been carried out to identify some of the mediators that may be produced by prostate cancer cells which could be responsible for this effect. Jacobs and Lawson (1980) identified mitogenic factors for fibroblasts which were produced by prostate cancer cells that were partially characterized. Following that, workers such as Simpson *et al.* (1985) and Koutsilieris *et al.* (1987) found that there was activity that stimulated osteoblasts also produced by prostatic cancer tissue.

We have studied several human and animal tumors which are associated with extensive new bone formation *in vivo.* These include the PA-III rat adenocarcinoma of the male urogenital tract and the WISH human amniotic tumor. The WISH tumor has been noted for almost 25 years to

stimulate bone formation (Wlodarski *et al.,* 1970). We examined extracts and conditioned media from the WISH tumor and identified a mitogenic activity for osteoblasts (Izbicka *et al.,* 1993). This was purified to homogeneity and we identified an extended form of basic fibroblast growth factor. The PA-III rat prostate adenocarcinoma produces a number of bone growth stimulatory factors *in vitro.* These factors belong to the bone morphogenic protein (BMP)-like family. One of these factors in preliminary studies has been neutralized by using antisense to BMP-3 (Harris, unpublished observation). Identification of the precise nature of these factors is awaited with considerable interest.

The factors which have been associated as potential mediators of osteoblastic metastases associated with prostate cancer are transforming growth factor β, fibroblast growth factors, plasminogen activator sequences, and the bone morphogenetic proteins.

Transforming Growth Factor β

Transforming growth factor β is abundant in PC3 human prostate cancer cells (Marquardt *et al.,* 1987; Wilding, 1991), one of the common human models of prostate cancer. Because transforming growth factor (TGF) βI and -II have profound effects on bone cells, including enhancement of bone formation *in vivo* and stimulation and proliferation of osteoblast precursors as well as chemotaxis of osteoblast precursors, this TGFβ production could in fact be part of the osteoblastic response to prostate cancer. However, much work would need to be done in determining the precise role and its relationship to binding proteins which mask its biological activity.

Fibroblast Growth Factors

Prostate cancer cells frequently produce large amounts of acidic and basic fibroblast growth factors. Fibroblast growth factors are powerful stimulators of new bone formation (Dunstan *et al.,* 1999). Recently, we have found that the human WISH tumor causes bone formation by an increase in the extended form of basic fibroblast growth factor (Izbicka *et al.,* 1993). There is now an extensive range of factors in the fibroblast growth factor family that could be responsible for increased bone formation.

Fibroblast growth factors are powerful stimulators of bone formation *in vivo.* They cause bone formation without any evidence of previous bone resorption (Dunstan *et al.,* 1999). They are also critical in normal skeletal development and limb bud formation (Cohn *et al.,* 1995). Point mutations in receptors for the fibroblast growth factor family cause skeletal defects such as achondroplasia (Rousseau *et al.,* 1994).

Bone Morphogenetic Proteins

The bone morphogenetic proteins are polypeptide members of the extended transforming growth factor β family. They are powerful stimulators of bone formation *in vivo.* They have different effects on bone cells from TGFβ, since their primary effect is to promote differentiation and new bone formation *in vitro.* Their effects on bone cell proliferation are probably indirect. These peptides are frequently produced by prostate cancer cells (Harris *et al.,* 1994) and are likely mediators of at least part of the osteoblastic response associated with prostate cancer (Harris *et al.,* 1992).

Plasminogen Activator Sequences

Recently, purification of mitogenic activity from the human prostate cancer cell line PC3 has identified amino acid sequences of plasminogen activator (Rabbani *et al.,* 1990 a,b). Plasminogen activator sequences do have growth regulatory effects. These peptides are similar or identical to the urokinase-type plasminogen activator. It is possible that they have mitogenic effects in their own right, and they also may be responsible for activation of other growth factors which could cause stimulation of the bone formation process.

Endothelin-1

Endothelin-1 has been implicated as a potential mediator of the osteoblastic metastases that are associated with prostate cancer and possibly with other solid tumors that cause osteoblastic metastasis. Endothelin-1 has been found in increased amounts in the serum of patients with prostate cancer (Nelson *et al.,* 1995) and has been shown to increase bone cell proliferation. We have also found that it increases osteoblast accumulation, maturation, and bone formation in organ cultures of neonatal calvaria (Yin *et al.,* 1999). We have found that endothelin-1 expression is enhanced in cultured breast cancer cells that are associated with osteoblastic metastasis when these cells are inoculated into the left cardiac ventricle of nude mice (Yin *et al.,* 1999). Moreover, we have found that the effects to form osteoblastic metastasis are alleviated *in vivo* by a specific receptor antagonist to the endothelin-A receptor, and the bone forming activity which is produced by the cultured tumor cells *in vitro* is also inhibited by this receptor antagonist (Yin *et al.,* 2000).

Platelet-Derived Growth Factor

Recently we have found evidence that platelet-derived growth factor (PDGF) is also a potential mediator of osteoblastic metastasis. It has been known for many years that PDGF is a powerful stimulator of osteoblast proliferation (Canalis *et al.,* 1980, 1989). We have identified PDGF in the conditioned media of a human breast cancer associated with osteoblastic metastasis, namely the MCF-7 tumor (Yi *et al.,* 1999). The conditioned media from cultured tumor cells stimulated bone formation in murine calvarial bones, and was blocked by neutralizing antibodies to PDGF (Yi *et al.,* 1999).

Osteolytic Bone Metastases

Our understanding of the mechanisms involved in tumor cell metastasis to distant organ sites has improved considerably during the past 15 years. Most studies have examined tumor cell metastasis to common sites such as liver and lung. However, until recently, there have been few studies on the mechanisms of tumor cell metastasis to bone. Tumor cell metastasis to bone initially involves the same steps involved in tumor cell metastasis to any organ, but in addition there are special extra features of the bone microenvironment which render some tumors more likely to grow in this site. Our understanding has increased recently with the development of animal models for studying human tumor cell spread to bone (Nakai *et al.,* 1992; Mundy and Yoneda, 1995), techniques with the features that the tumor cells can be manipulated as well as the bone microenvironment to study important interactions which take place at the metastatic bone site.

Some tumor cells have a particular predilection to spread to bone. The commonest examples among human tumors are carcinomas of the lung, breast, and prostate (Mundy and Martin, 1993). For these tumor cells to spread to bone, they clearly have functional properties which enable them to travel from the tissue of origin to the site of metastasis (Fig. 1), occupy a niche at that site, and then grow avidly. Clarifying the special properties which are important in metastasis of tumor cells to the bone site will hopefully lead to potential therapeutic targets for either prevention or reversal of this process.

Several cellular mechanisms have been suggested for the destruction of bone which occurs at osteolytic sites. First, it is possible that tumor cells themselves can cause bone destruction. They certainly have the capability of doing this *in vitro.* We have previously shown that human breast cancer cells, when cultured on devitalized bone matrices, can cause resorption of bone (Eilon and Mundy 1978). However, *in vivo,* it seems likely that the major mechanism for bone destruction is osteoclast-mediated. Meticulous scanning electron microscopy studies of bone surfaces always reveal evidence of resorption pits made by osteoclasts, but no evidence of resorption pits made by tumor cells (Boyde *et al.,* 1986). Although scanning electron microscopy cannot exclude the possibility that tumor cells cause some bone destruction, it is obvious that osteoclastic bone resorption is probably the major mechanism whereby osteolysis occurs in patients with tumors.

The mechanisms that tumor cells utilize to stimulate osteoclast activity are probably similar to those used in the humoral hypercalcemia of malignancy. The tumor cells or host cells that are activated by the presence of the tumor release osteoclast stimulating factors which cause new osteoclast formation and subsequent bone resorption, in the absence of a normal osteoblastic response to prior resorption. The factors which are responsible for osteoclast activation

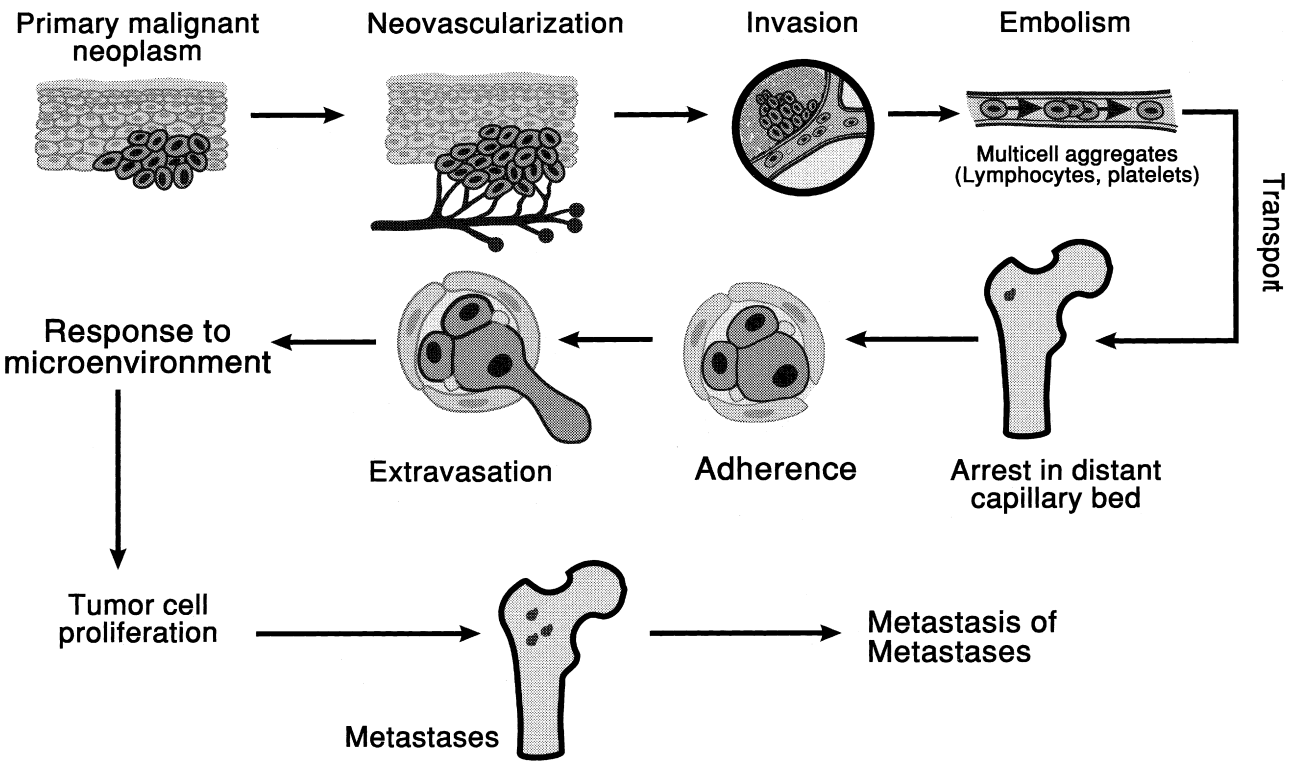

Figure 1 Multiple steps that are utilized by tumor cells that metastasize to bone. Note that each step represents a potential therapeutic target for development of pharmacologic agents to prevent or inhibit the bone metastatic process.

include PTH-rP. This may occur even in the absence of significant PTH-rP production at the primary site. For example, clinical studies have shown that although only about 50% of primary human breast cancers express PTH-rP and that PTH-rP production is not common in nonbone metastatic sites, when breast cancer cells are grown in bone PTH-rP expression is enhanced in over 90% of cases (Powell *et al.,* 1991). We have examined the potential pathophysiologic mechanisms which are responsible for enhanced PTH-rP production by breast cancer cells in metastatic bone sites. We have utilized the human breast cancer cell line MDA-231 which in culture produce low concentrations of PTH-rP (3–5 pmol). When these tumor cells are inoculated into the left ventricle of nude mice, they cause characteristic osteolytic metastases associated with increased osteoclastic bone resorption. In tumor-bearing nude mice, plasma PTH-rP is not detectable, and the mice do not develop hypercalcemia. However, bone marrow plasma at the sites of osteolytic lesions reveal markedly increased PTH-rP, paralleling what is seen in the clinical studies in patients, and suggesting that MDA-231 cells in the bone microenvironment produce increased amounts of PTH-rP. We have treated these tumor-bearing mice with a murine monoclonal antibody direct against PTH-rP 1–34, and found that the antibodies prevent the development of bone metastases, decrease the area of osteolytic metastases which are already established, and moreover decrease tumor bulk in bone (Guise *et al.,* 1996). The antibodies also cause a significant increase in survival of the tumor-bearing nude mice. These data suggest not only that PTH-rP is playing an important pathophysiological role in the bone metastasis, but that enhanced local PTH-rP expression can occur without hypercalcemia or increased plasma PTH-rP and indicates that PTH-rP can mediate the effects of tumors on the skeleton without causing hypercalcemia.

We have been interested in the pathophysiologic mechanisms that may be responsible for PTH-rP production by breast cancer cells at the bone site. From the studies of Paget of over a century ago, it was recognized that tumor cells have a special predilection to grow at some sites. Paget described the environment of favorable metastatic sites as "congenial soil." The bone matrix may well be such a congenial soil. Bone matrix is the most abundant source of growth regulatory factors in the body and these are released when bone is resorbed. Presumably they could alter the behavior of tumor cells in the bone microenvironment. This notion suggests that rates of bone remodeling may be related to tumor cell growth for certain susceptible tumors. To investigate this hypothesis, we have treated nude mice which were inoculated with human breast cancer cells with the bisphosphonate risedronate, which impairs bone remodeling (Sasaki *et al.,* 1995). Under these circumstances, we found complete inhibition of the capacity of tumor cells not only to cause osteolytic bone lesions but also to grow in the bone microenvironment (Sasaki *et al.,* 1995). One of the possible mechanisms by which this occurs is through excess production of factors such as PTH-rP. We found that TGFβ, which is one of the most abundant and biologically important growth factors in the bone matrix, enhances PTH-rP production by metastatic breast cancer cells (Guise *et al.,* 1994). Similar results have previously been described by others, as well as us (Firek *et al.,* 1994; Guise *et al.,* 1994). When neutralizing antibodies of PTH-rP administered to mice inoculated with the breast cancer cells, PTH-rP production is suppressed and bone metastasis is inhibited.

To further test this hypothesis, we have enhanced bone resorption at sites where bone metastasis rarely occurs. This was done over the calvaria of mice by local injections of interleukin-1. Following interleukin-1 injections, and then inoculation into the left ventricle of the heart with human MDA-231 cells, we found marked enhancement of tumor cell metastasis to calvarial sites (Yoneda *et al.,* 1995). This also indicates that tumor cell metastasis is dependent on local rates of bone remodeling and that rapidly remodeling bone provides a very fertile soil for tumor cell growth.

Further studies have emphasized the importance of the bone microenvironment in the behavior of human breast cancer cells once they have metastasized to bone. We have found that tumor cells which are made unresponsive to TGFβ by stably transfecting them with mutant truncated receptors to TGFβ caused decreased PTH-rP production and less osteolysis when inoculated into the left cardiac ventricle (Yin *et al.,* 1999). In contrast, when these same tumor cells are stably transfected with constitutively active TGFβ receptors, this effect is reversed. Under these circumstances, the tumor cells produce excess PTH-rP and more osteolysis. This effect of these tumor cells to cause osteolysis is blocked by neutralizing antibodies to PTH-rP. These data confirm that there is an important vicious cycle in the bone microenvironment which is mediated on one hand by TGF produced in bone as a consequence of bone remodeling and on the other by PTH-rP, which is expressed by the tumor cells in the bone microenvironment and enhances bone turnover, bone remodeling, and TGFβ production. It is important to note that TGFβ may not be the only factor produced by bone in the bone microenvironment as a consequence of remodeling that affects breast cancer cells. For example, IGF-1 is also present in bone in abundant amounts and is released in active form when bone is resorbed and can enhance breast cancer cell proliferation.

The implications of these findings for the treatment of patients with cancer may be very important. For example, it is certainly possible to manipulate rates of bone remodeling *in vivo* by the use of resorption inhibitors such as the bisphosphonates. The use of these compounds may be very important not only in preventing the skeletal complications of cancer such as intractable bone pain, fractures, and hypercalcemia, but also in preventing tumor cell growth in the metastatic site. In other words, inhibitors of bone resorption may turn out to be useful adjuvant therapy in association with cytotoxic drugs for the treatment of metastatic cancer. In particular, for those tumors which have a special predilection to grow in bone such as breast cancer, it may be advisable to use relatively

nontoxic drugs early in the disease if the patient has a high risk of developing bone metastases.

Associated Syndromes

The hypercalcemia of malignancy, whether occurring in association with osteolytic bone disease or as a consequence of the production of humoral mediators of bone resorption by tumor cells, is caused by soluble factors acting as humoral agents. These mediators are also responsible for other paraneoplastic syndromes which frequently accompany hypercalcemia. These include cachexia, hypertriglyceridemia, and anemia.

One paraneoplastic syndrome that is particularly common in hypercalcemia is leukocytosis. Yoneda *et al.* (1991a,b) examined a series of patients with squamous cell carcinomas of the head and neck. They found that the hypercalcemia–leukocytosis syndrome was extremely common. We believe that this syndrome is frequently overlooked or the leukocytosis is ascribed to other factors such as infection. It is most likely that the leukocytosis is due to cytokines produced either by the tumor cells or by host immune cells which are activated by the presence of the tumor (Fig. 2). Tumor cells frequently produce colony-stimulating factors in association with PTH-rP. Colony-stimulating factors which have been shown to be produced by tumors and which could be responsible for leukocytosis include GM-CSF, G-CSF, and CSF-1. However, other cytokines are frequently overproduced in patients with cancer and could be responsible for leukocytosis. These include tumor necrosis factor, interleukin-6, and interleukin-1α. Yoneda and colleagues have shown that in several human tumors inoculated in nude mice, the hypercalcemia-leukocytosis syndrome occurs, and that the features of this syndrome can be reversed by neutralizing antibodies to cytokines such as tumor necrosis factor and interleukin-6. Not only can these paraneoplastic syndromes be reversed, but these antibodies also reverse other paraneoplastic syndromes which frequently occur such as cachexia, hypertriglyceridemia and anemia.

Some of the well-described animal models of humoral hypercalcemia of malignancy in which PTH-rP is implicated also produce excess amounts of these cytokines and are associated with the hypercalcemia–leukocytosis syndrome. Examples include the rat Leydig tumor and Walker tumor in the rat. A model which was studied extensively by Yoneda *et al.* (1991a), the MH-85 human squamous cell carcinoma, is also associated with hypercalcemia and leukocytosis in tumor-bearing nude mice. In this model, the hypercalcemia–leukocytosis can be reversed by neutralizing antibodies to interleukin-6 and tumor necrosis factor (Yoneda *et al.,* 1991a; 1993). A similar model is the murine breast cancer model studied by Lee and colleagues (Lee and Baylink, 1983). In this model, hypercalcemia and leukocytosis also occur and this model has also been associated with increased production of colony-stimulating factors.

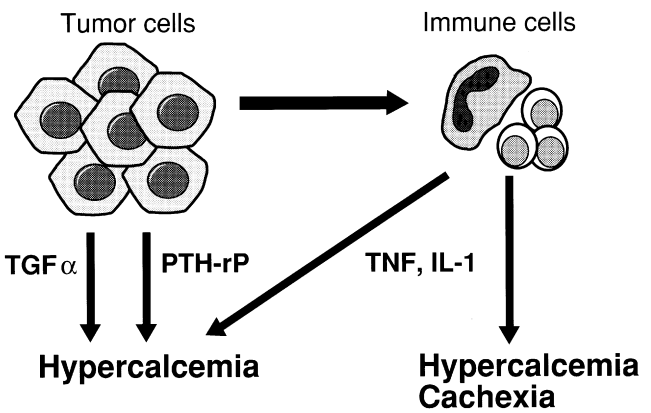

Figure 2 Hypothesis for production of bone-resorbing cytokines by normal host immune cells in hypercalcemia of malignancy, confirmed now in several cases (see text for details).

Conclusions

Tumors frequently, possibly always, produce aberrant amounts of multiple growth factors and cytokines which work in conjunction to cause paraneoplastic syndromes associated with malignancy, of which hypercalcemia is one. Moreover, the effects of these factors, which may originate not only from the tumor cells but also from normal host cells, may be synergistic rather than additive. Under these circumstances, inhibition of the effects of one factor by neutralizing antibodies, peptide antagonists, or inhibitors may abrogate the syndrome. Studies on the multiple mechanisms which tumors utilize to alter the behavior of normal cells such as bone cells has increased our understanding not only of the pathophysiology of the effects of tumors on the skeleton, but also of normal bone development and bone remodeling.

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CHAPTER 62

Molecular Basis of PTH Underexpression

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Hypoparathyroidism is an endocrine disorder in which hypocalcemia and hyperphosphatemia are the result of a deficiency in parathyroid hormone (PTH) secretion or action (Thakker, 1993; Bilezikian and Thakker, 1998). There are a variety of causes of hypoparathyroidism (Table I) and the disorder may occur as part of a pluriglandular autoimmune disorder or as a complex congenital defect, as for example in the DiGeorge syndrome or in association with other developmental anomalies involving dysmorphic features, nephropathy, sensorineural deafness, lymphoedema, and cortical thickening of tubular bones. In addition, hypoparathyroidism may develop as a solitary endocrinopathy and this form has been called *isolated* or *idiopathic* hypoparathyroidism. Familial occurrences of *isolated* hypoparathyroidism have been reported and autosomal dominant (Barr *et al.,* 1971; Ahn *et al.,* 1986), autosomal-recessive (Bronsky *et al.,* 1968; Parkinson and Thakker, 1992), and X-linked recessive (Peden, 1960; Whyte and Weldon, 1981; Thakker *et al.,* 1990) inheritances have been established (McKusick, 1988). The molecular genetic basis for each of these forms of hypoparathyroidism has been investigated and this has helped to elucidate further the mechanisms involved in the control of extracellular calcium homeostasis (Fig. 1).

Extracellular calcium ion concentration is tightly regulated through the actions of PTH on kidney and bone. The intact peptide is secreted by the parathyroid glands at a rate that is appropriate to, and dependent upon the prevailing extracellular calcium ion concentration. Hypocalcemic disorders can be classified according to whether they arise from a deficiency of PTH, a defect in the PTH-receptor (i.e., the PTH/PTHrP receptor), or an insensitivity to PTH caused by defects downstream of the PTH/PTHrP receptor (Fig. 1). The application of the recent developments in molecular biology to the study of the hypoparathyroid disorders has enabled the characterization of some of the mechanisms involved in the regulation of parathyroid gland development, of PTH secretion, and of PTH-mediated actions in target tissues (Thakker, 1993; Bilezikian and Thakker, 1998). Thus, mutations in the calcium-sensing receptor (CaSR) gene have been reported in patients with autosomal dominant hypocalcaemia with hypercalciuria. In addition, mutations in the PTH gene, the transcriptional factor GATA3, and the mitochondrial genome have been demonstrated to be associated with some forms of hypoparathyroidism; defects in the PTH/PTHrP receptor gene have been identified in patients with Blomstrand's chondrodysplasia; and mutations in the stimuatory G protein ($G_s\alpha$) have been found in individuals with pseudohypoparathyroidism type Ia and pseudo-pseudohypoparathyroidism. Furthermore, the gene causing the polyglandular autoimmune syndrome (APECED) has been characterized and candidate genes have been identified for the DiGeorge syndrome and for pseudohypoparathyroidism type Ib. These molecular genetic studies have provided unique opportunities for elucidating the pathogenesis of the hypoparathyroid disorders and these advances together with the structure and function of the PTH gene, will be reviewed in this chapter.

Isolated Hypoparathyroidism

Isolated hypoparathyroidism may be inherited either as an autosomal-dominant, autosomal-recessive, or X-linked recessive disorder. Some autosomal forms of hypoparathyroidism have been shown to be due to abnormalities of the PTH, CaSR, and Gcm2 genes.

Table I Inherited Forms of Hypoparathyroidism and Their Chromosomal Locations

Disease	Inheritance	Gene product	Chromosomal location
Isolated hypoparathyroidism	Autosomal dominant	PTH[a]	11*p*15
	Autosomal recessive	PTH[a]	11*p*15
	X-Linked recessive	Unknown	X*q*26–*q*27
Hypocalcaemic hypercalciuria	Autosomal dominant	CaSR	3*q*13–*q*21
Hypoparathyroidism associated with complex congenital syndromes			
DiGeorge type 1 (DGS1)	Autosomal dominant	*rnex40*[b] *nex2.2–nex3*[b] UDF1L[b]	22*q*11
DiGeorge type 2 (DGS2)	Autosomal dominant		10*p*13–*p*14
HDR	Autosomal dominant	GATA3	10*p*15
Hypoparathyroidism associated with KSS, MELAS, and MTPDS	Maternal	Mitochondrial genome	
Blomstrand lethal chondrodysplasia	Autosomal recessive	PTH/PTHrPR	3*p*21.1–*p*22
Kenney–Caffey	Autosomal dominant[c]	Unknown	?
Barakat	Autosomal recessive[c]	Unknown	?
Lymphoedema	Autosomal recessive	Unknown	?
Nephropathy, nerve deafness	Autosomal dominant[c]	Unknown	?
Nerve deafness without renal dysplasia	Autosomal dominant	Unknown	?
Dysmorphology, growth failure	Autosomal recessive	Unknown	1*q*42–*q*43
Hypoparathyroidism associated with polyglandular autoimmune syndrome (APECED)	Autosomal recessive	AIRE	21*q*22.3
Pseudohypoparathyroidism (type Ia)	Autosomal dominant parentally imprinted	GNAS1	20*q*13.2–*q*13.3
Pseudohypoparathyroidism (type Ib)	Autosomal dominant parentally imprinted	GNAS1	20*q*13.3–*q*13.3

Note HDR, hypoparathyroidism, deafness and renal anomalies; MELAS, mitochondrial encephalopathy, stroke like episodes and lactic acidosis. KSS, Kearns–Sayre syndrome; MTPDS, mitochondrial trifunctional protein deficiency syndrome.?, Location not known.

[a] Mutations of PTH gene identified only in some families.

[b] Most likely candidate genes.

[c] Most likely inheritance shown.

PTH Gene Abnormalities

PTH Gene Structure and Function

The PTH gene is located on chromosome 11*p*15 and consists of three exons, which are separated by two introns (Naylor *et al.,* 1983). Exon 1 of the PTH gene is 85 bp in length and is untranslated (Fig. 2), whereas exons 2 and 3 encode the 115-amino-acid pre-proPTH peptide. Exon 2 is 90 bp in length and encodes the initiation (ATG) codon, the prehormone sequence, and part of the prohormone sequence. Exon 3 is 612 bp in size and encodes the remainder of the prohormone sequence, the mature PTH peptide, and the 3′ untranslated region (Vasicek *et al.,* 1983). The 5′ regulatory sequence of the human PTH gene contains a vitamin D response element 125 bp upstream of the transcription start site, which downregulates PTH mRNA transcription in response to vitamin D receptor binding (Okazaki *et al.,* 1988; Demay *et al.,* 1992). PTH gene transcription (as well as PTH peptide secretion) is also dependent upon the extracellular calcium and phosphate (Naveh-Many *et al.,* 1995; Slatopolsky *et al.,* 1996, Almoden *et al.,* 1996) concentrations, although the presence of specific upstream "calcium or phosphate response element(s)" have not yet been demonstrated (Russell *et al.,* 1983, Naveh-Many *et al.,* 1989). The mature PTH peptide is secreted from the parathyroid chief cell as an 84-amino-acid peptide and this is regulated through a G protein-coupled CaSR which is also expressed in renal tubules. However, when the PTH mRNA is first translated it is as a pre-proPTH peptide. The "pre" sequence consists of a 25-amino-acid signal peptide (leader sequence) which is responsible for directing the nascent peptide into the endoplasmic reticulum to be packaged for secretion from the cell (Kemper *et al.,* 1974). The "pro" sequence is 6 amino acids in length and, although its function is less well defined than that of the "pre" sequence, it is also essential for correct PTH processing and secretion (Kemper *et al.,* 1974). After the 84-amino-acid mature PTH peptide is secreted from the parathyroid cell, it is cleared from the circulation with a short half-life of about 2 min, via nonsaturable hepatic uptake and renal excretion. PTH shares a receptor with PTH-related peptide (PTHrP also known as PTHrH, PTH-related hormone) (Jüppner *et al.,* 1991), and

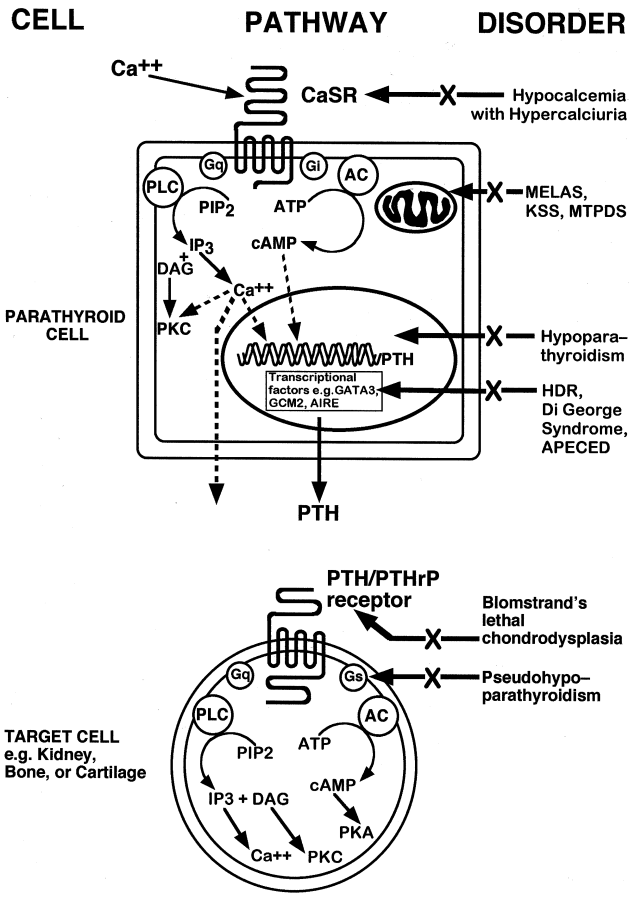

Figure 1 Schematic representation of some of the components involved in calcium homeostasis. Alterations in extracellular calcium are detected by the calcium-sensing receptor (CaSR), which is a 1078-amino-acid G-protein coupled receptor. The PTH/PTHrP-receptor is also a G-protein coupled receptor. Thus, Ca^{2+}, PTH, and PTHrP involve G protein-coupled signaling pathways, and interaction with their specific receptors can lead to activation of Gs, Gi, and Gq. Gs stimulates adenylyl cyclase (AC) which catalyzes the formation of cAMP from ATP. Gi inhibits AC activity. cAMP stimulates PKA which phosphorylates cell-specific substrates. Activation of Gq stimulates PLC, which catalyzes the hydrolysis of phosphoinositide (PIP_2) to inositol triphosphate (IP_3), which increases intracellular calcium, and diacylglycerol (DAG), which activates PKC. These proximal signals modulate downstream pathways, which result in specific physiological effects. Abnormalities in several genes and encoded proteins in these pathways, have been identified in patients with hypoparathyroid disorders (Table I). Adapted from Thakker (1999).

this receptor (Fig. 1) is a member of a subgroup of G protein-coupled receptors. The PTH/PTHrP receptor gene is located on chromosome 3*p*21–*p*24 (Gelbert *et al.,* 1994) and is highly expressed in kidney and bone, where PTH is its predominant agonist (Abou-Samra *et al.,* 1992). However, the most abundant expression of the PTH/PTHrH receptor occurs in chondrocytes of the metaphyseal growth plate where it mediates predominantly the autocrine/paracrine actions of PTHrP (Segré, 1996; Potts and Jüppner, 1997). Five polymorphisms of the PTH gene have been reported and two of these are associated with restriction fragment length polymorphisms (RFLPs) (Schmidtke *et al.,* 1984), another two are the result of single base changes that are not associated with RFLPs (Miric and Levine, 1992), and one is due to a variation in the length of a microsatellite repetitive sequence in intron 1 (Parkinson *et al.,* 1993). These polymorphisms are inherited in a Mendelian manner and are thus useful as genetic markers in family studies. Mutations involving the PTH gene affect the regulation of calcium homeostasis (Fig. 1) and are associated with hypoparathyroidism (Table I).

AUTOSOMAL DOMINANT HYPOPARATHYROIDISM

DNA sequence analysis of the PTH gene (Fig. 2) from one patient with autosomal dominant isolated hypoparathyroidism has revealed a single base substitution (T→C) in codon 18 of exon 2 (Arnold *et al.,* 1990), which resulted in the substitution of arginine (**C**GT) for the normal cysteine (**T**GT) in the signal peptide. The presence of this charged amino acid in the midst of the hydrophobic core of the signal peptide impeded the processing of the mutant preproPTH, as demonstrated by *in vitro* studies. These revealed that the mutation impaired the interaction with the nascent protein and the translocation machinery, and that cleavage of the mutant signal sequence by solubilized signal peptidase was ineffective (Arnold *et al.,* 1990; Karaplis *et al.,* 1995). Ineffective cleavage of the prepro PTH sequence results in a molecule that does not proceed successfully through the subsequent intracellular steps required for ultimate delivery of PTH to secretary granules. The parathyroid cell, therefore, cannot respond to hypocalcaemia with the secretion of native, biologically active PTH.

AUTOSOMAL RECESSIVE HYPOPARATHYROIDISM

Autosomal recessive hypoparathyroidism has usually arisen in families with consanguineous marriages (Parkinson and Thakker, 1992; Parkinson *et al.,* 1993). Abnormalities in the PTH gene have been sought (Parkinson *et al.,* 1993) and mutations identified in two unrelated families (Parkinson and Thakker, 1992; Sunthorneprarakul *et al.,* 1999). In one such family a donor splice site at the exon 2-intron 2 boundary has been identified (Parkinson and Thakker, 1992). This mutation involved a single base transition (**g→c**) at position 1 of intron 2 and an assessment of the effects of this alteration in the invariant **gt** dinucleotide of the 5′ donor splice site consensus on mRNA processing were assessed by an analysis of the non-tissue-specific transcription of the normal and mutant PTH genes. This non-tissue-specific expression of genes has been estimated to be at the rate of one molecule of correctly spliced mRNA per 1000 cells (van Heijne, 1983; Chelly *et al.,* 1989). Although the physiological relevance of this low level of non-tissue-specific, or illegitimate transcription is not known, it is of clinical importance. Easily accessible peripheral blood lymphocytes can be used to detect abnormalities in mRNA processing, thereby avoiding the requirement for tissue that may only be obtainable by biopsy. Use of these methods revealed that the donor splice site mutation resulted in exon skipping, in which exon 2 of the PTH gene was lost and exon 1 was spliced to exon 3.

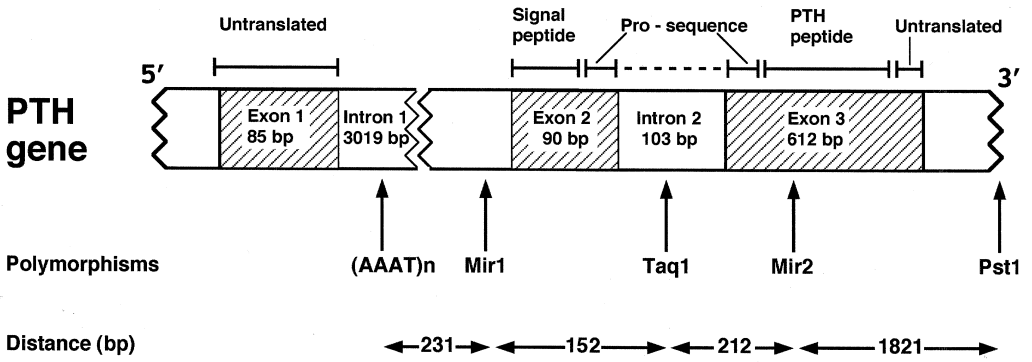

Figure 2 Schematic representation of the PTH gene. The PTH gene consists of three exons and two introns; the peptide is encoded by exons 2 and 3. The PTH peptide is synthesized as a precursor which contains a pre- and a pro-sequence. The mature PTH peptide, which contains 84 amino acids, and larger carboxy-terminal PTH fragments are secreted from the parathyroid cell. The polymorphic sites associated with the PTH gene are indicated. Two restriction fragment length polymorphisms (RFLPs) are associated with the PTH gene, and the *Taq*I polymorphic site is within intron 2 and the *Pst*I polymorphic site is 1.7 kbp downstream in the 3' direction of the gene (Schmidtke *et al.,* 1984). Two other polymorphisms (Miric and Levine, 1992) of the PTH gene designated Mir1 and Mir2 are located in intron 1 and exon 3, respectively, and the tetranucleotide $(AAAT)_n$ polymorphism is in intron 1 (Parkinson *et al.,* 1993). The distance between the tetranucleotide $(AAAT)_n$ polymorphism and the Mir1 polymorphism is 231 base pairs (bp), that between the Mir1 polymorphism and the *Taq*1 RFLP is 152 bp, that between the *Taq*I RFLP site and the Mir2 polymorphism is 212 bp, and that between the Mir2 polymorphism and the *Pst*I RFLP is 1821 bp. Linkage disquilibrium between the $(AAAT)_n$, *Taq*I, and *Pst*I polymorphic sites has been established (Parkinson *et al.,* 1993). Adapted from Parkinson and Thakker (1992).

The lack of exon 2 would lead to a loss of the initiation codon (ATG) and the signal peptide sequence (Fig. 2) which are required respectively for the commencement of PTH mRNA translation and for the translocation of the PTH peptide. Thus, the patients' parathyroid cells would not contain any translated PTH products. In the other family a single base substitution (**T→C**) involving codon 23 of exon 2 was detected. This resulted in the substitution of proline (**C**CG) for the normal serine (**T**CG) in the signal peptide (Sunthornepvarakul *et al.,* 1999). This mutation alters the −3 position of the pre-pro-PTH protein cleavage site (van Heijne, 1983). Indeed, amino acid residues at the −3 and −1 positions of the signal peptidase recognition site have to conform to certain criteria for correct processing through the rough endoplasmic reticulum (RER), and one of these is an absence of proline in the region −3 and +1 of the site (van Heijne, 1983). Thus, the presence of a proline, which is a strong helix-breaking residue, at the −3 position is likely to disrupt cleavage of the mutant pre-pro-PTH that would be subsequently degraded in the RER, and PTH would not be available (Sunthornepvarakul *et al.,* 1999).

Calcium-Sensing Receptor Gene Abnormalities

Mutations of the PTH gene have been detected in only a minority of autosomal forms of isolated hypoparathyroidism and this indicates that other genes are likely to be involved (Table I). One of these is the CaSR gene, and another is the Gcm2 (Glial Cells Missing 2) gene (see below).

The CaSR, which is located in the plasma membrane of the cell (Fig. 1), is at a critical site to enable the cell to recognize changes in extracellular calcium concentration. Thus, an increase in extracellular calcium leads to CaSR activation of the G-protein signaling pathway, which in turn increases the free intracellular calcium concentration and leads to a reduction in transcription of the PTH gene. CaSR mutations that result in a loss of function are associated with familial benign (hypocalciuric) hypercalcemia (FHH) (Pollak *et al.,* 1993; Chou *et al.,* 1995; Pearce *et al.,* 1995; Lanicic *et al.,* 1995; Aida *et al.,* 1995; Heath *et al.,* 1996). However, CaSR missense mutations that result in a gain of function (or added sensitivity to extracellular calcium) lead to hypocalcemia with hypercalciuria (Pollak *et al.,* 1994; Finegold *et al.,* 1994; Perry *et al.,* 1994; Pearce *et al.,* 1996; Baron *et al.,* 1996). These hypocalcemic individuals are generally assymptomatic and have serum PTH concentrations that are in the low-normal range, and because of the insensitivities of previous PTH assays in this range, such patients have often been diagnosed to be hypoparathyroid. In addition, such patients may have hypomagnesemia. Treatment with vitamin D or its active metabolites to correct the hypocalcemia in these patients results in marked hypercalciuria, nephrocalcinosis, nephrolithiasis, and renal impairment. Thus, these patients need to be distinguished from those with other forms of hypoparathyroidism, and in order to further emphasize this, it is probably best to refer to this disorder due to CaSR mutations as autosomal dominant hypocalcemic hypercalciuria (ADHH).

Gcm2 Gene Abnormalities

Gcm2, which is the mouse homolog of the Drosphilia gene Gcm, is expressed exclusively in the parathyroid glands, suggesting that it may be a specific regulator of parathyroid gland development (Günther *et al.,* 2000; Kim *et al.,* 1998). In order to investigate this, mice deleted for Gcm2

were generated by the method of homologous recombination using embryonic stem cells. Mice that were heterozygous (+/−) for the deletion were normal, whereas mice that were homozygous (−/−) for the deletion lacked parathyroid glands and developed the hypocalcemia and hyperphosphatemia observed in hypoparathyroidism (Günther *et al.,* 2000). However, despite their lack of parathyroid glands, Gcm2-deficient (−/−) mice did not have undetectable serum PTH levels, but instead had PTH levels identical to those of normal (+/+, wild-type) mice. This endogenous level of PTH in the Gcm2-deficient (−/−) mice was too low to correct the hypocalcaemia, but exogenous continuous PTH infusion could correct the hypocalcemia (Günther *et al.,* 2000). Interestingly, there were no compensatory increases in PTHrP or $1,25(OH)_2$ vitamin D_3. These findings indicate that Gcm2 mice have a normal response (and not resistance) to PTH and that the PTH in the serum of Gcm2-deficient mice was active. The auxillary source of PTH was determined by combined expression and ablation studies (Günther *et al.,* 2000). These revealed a cluster of PTH-expressing cells under the thymic capsule in both the Gcm2-deficient (−/−) and wild-type (+/+) mice. These thymic PTH-producing cells also expressed the CaSR, and long-term treatment of the Gcm2-deficient mice with $1,25(OH)_2$ vitamin D_3 restored the serum calcium concentrations to normal and reduced the serum PTH levels, thereby indicating that the thymic production of PTH can be downregulated (Günther *et al.,* 2000). However, it appears that this thymic production of PTH cannot be upregulated as serum PTH levels are not high despite the hypocalcaemia in the Gcm2-deficient mice. This absence of upregulation would be consistent with the very small size of the thymic PTH-producing cell cluster, when compared to the size of normal parathyroid glands. The development of the thymic PTH-producing cells likely involves Gcm1, which is the other mouse homolog of Drosophilia Gcm (Kim *et al.,* 1998). Gcm1 expression, which could not be detected in parathyroid glands, colocalized with PTH expression in the thymus (Günther *et al.,* 2000). Thus, Gcm2 specifically controls the differentiation of the cells of third pharyngeal pouch into parathyroid glands, and Gcm1 regulates an auxillary developmental pathway that involves differentiation of PTH-producing cells in the thymus. Gcm genes are likely to have similar roles in human parathyroid development, as a recent report indicates that an intragenic deletion of Gcm2 in a patient is associated with hypoparathyroidism. These findings may also help to explain the high incidence of PTH-producing tumors in the thymus.

X-Linked Recessive Hypoparathyroidism

Hypoparathyroidism with an X-linked recessive transmission pattern has been reported in two multigenerational kindreds (Peden, 1960; Whyte and Weldon, 1981). Only males were affected, and they suffered from infantile epilepsy and hypocalcemia. The hypoparathyroidism is due to a defect in parathyroid gland development. Linkage studies utilizing X-linked RFLPs in these two families assigned the mutant gene to chromosome X*q*26–*q*27 (Thakker *et al.,* 1990). A novel approach utilizing mitochondrial DNA analysis established a common ancestry in these two X-linked hypoparathyroid kindreds (Mumm *et al.,* 1997). A common ancestry for these two kindreds from Eastern Missouri had been suspected, but it could not be established despite five generations of extensive genealogical records (Whyte and Weldon, 1981). The mitochondrial genes are transmitted through the maternal line exclusively. If relatedness among the two kindreds involved the maternal lines, analysis of mitochondrial genetic markers would reveal common features. The DNA sequence of the mitochondrial (mt) D-loop was compared among individuals in both kindreds. The mt DNA sequence was identical among affected males and their maternal lineage for individuals in both kindreds, but differed at 3 to 6 positions when compared with the mitochondrial DNA of the fathers. These results demonstrated that the two kindreds with X-linked recessive hypoparathyroidism are indeed related and that an identical gene defect is likely to be responsible for the disease. Additional studies have refined the location of this gene to be between factor IX (FIX) and DXS1205 (Trump *et al.,* 1998). Analysis of a yeast artificial chromosome (YAC) contig of this region (Zucchi *et al.,* 1996) indicates that the region is 1.5 million bp in size and contains at least three candidate genes. The specific gene defect responsible for this form of X-linked hypoparathyroidism has yet to be identified. The results of this study also demonstrate that the mitochondrial genetic approach may be of importance in detecting common ancestry in other X-linked diseases.

Complex Syndromes Associated with Hypoparathyroidism

Hypoparathyroidism may occur as part of a complex syndrome which may either be associated with a congenital development anomaly or with an autoimmune syndrome. The congenital developmental anomalies associated with hypoparathyroidism include the DiGeorge, the HDR (Hypoparathyroidism, Deafness, and Renal anomalies), the Kenney–Caffey, and the Barakat syndromes and also syndromes associated with either lymphoedema or dysmorphic features and growth failure (Table I).

DiGeorge Syndrome

Patients with the DiGeorge syndrome (DGS) typically suffer from hypoparathyroidism, immunodeficiency, congenital heart defects, and deformities of the ear, nose, and mouth (Bilezikian and Thakker, 1998). The disorder arises from a congenital failure in the development of the derivatives of the third and fourth pharyngeal pouches with resulting absence or hypoplasia of the parathyroids and thymus. Most cases of DGS are sporadic but an autosomal dominant inheritance of DGS has been observed and an association between the syndrome and an unbalanced translocation and

deletions involving 22*q*11.2 have also been reported (Scambler *et al.,* 1991), and this is referred to as DGS type 1 (DGS1). In some patients, deletions of another locus on chromosome 10*p* have been observed in association with DGS (Monaco *et al.,* 1991), and this is referred to as DGS type 2 (DGS2). Mapping studies of the DGS1 deleted region on chromosome 22*q*11.2 have defined a 250 kb minimal critical region (Gong *et al.,* 1996), and cloning of the translocation breakpoint on 22*q*11.21 from a DGS1 patient (Augusseau *et al.,* 1986) has revealed that there are probably 2 genes (rnex40 and nex2.2–nex3), transcribed in opposite directions, which are disrupted by this breakpoint (Budarf *et al.,* 1995). The coding region of one of these genes, designated *rnex40,* has homology to the mouse and rat androgen receptors and contains a leucine zipper motif, suggesting that the DGS1 candidate gene may be a DNA-binding protein. Eleven nucleotides of the rnex40 gene are deleted at the translocation junction, making it likely that loss of function of this gene is responsible for at least part of the DiGeorge phenotype (Budarf *et al.,* 1995). Another partial transcript, referred to as nex2.2–nex3, was also identified from this breakpoint. Both rnex40 and nex2.2–nex3 are deleted in all DGS1 patients with 22*q*11 deletions and studies aimed at assessing the presence of hemizygosity and mutations in these 2 genes in DGS1 patients who do not have detectable 22*q*11 deletions are required to demonstrate the role of these genes in the etiology of the DGS1. Such studies have been performed for a human homolog of a yeast gene that encodes a protein involved in the degradation of ubiquinated proteins, referred to as UDF1L (Yamagishi *et al.,* 1999). UDF1L is located on 22*q*11 and has been found to be deleted in all of 182 patients with the 22*q*11 deletion syndrome (Yamagishi *et al.,* 1999), which includes patients with the DGS1, the velo-cardio-facial (VCFS), and conotruncal anomaly face (CAFS) syndromes (Scambler *et al.,* 1991; Gong *et al.,* 1996). However, a smaller deletion of approximately 20 kb that removed exons 1 to 3 of UDF1L was detected in one patient (Yamagishi *et al.,* 1999). This patient, who had a *de novo* deletion resulting in haplo-insufficiency of UDF1L, suffered from neonatal onset cleft palate, small mouth, low-set ears, broad nasal bridge, an interrupted aortic arch, a persistent truncus arteriosus, hypocalcemia, T lymphocyte deficiency, and syndactyly of her toes (Yamagishi *et al.,* 1999). These results indicate that abnormalities of the UDF1L gene may contribute to the etiology of early-onset DGS1 in man. However, heterozygous mice that are deleted for one udf1l allele (Udf1l+/−) do not have cardiac defects (Lindsag *et al.,* 1999). This is in contrast to the situation in heterozygous mice (Df1/+) that are deleted (Magnaghi *et al.,* 1998) for the region that is homologous to the human 22*q*11 segment. The Df1/+ mice develop cardiovascular abnormalities, which are due to defective development of the fourth pharyngeal arch arteries, and are of the same type as DGS1 patients (Lindsay *et al.,* 1999). However, the Df1/+ mice have normal serum calcium, phosphate and PTH concentrations and also have normally functioning B and T cells (Lindsay *et al.,* 1999). These findings indicate that the genes responsible for hypoparathyroidism and immune deficiency may either lie outside the Df1 region, or that heterozygous mutations may be insufficient to produce these defects in mice, or that the genetic background may alter the expression of the mutation. Interestingly, another candidate gene for DGS1 does lie outside the Df1 region, and this is Hira, which is a transcription factor that interacts with Pax3, that in turn is expressed in cardiac and neural crest cells (Magnaghi *et al.,* 1998). Thus, there may be more than one gene involved in the development of the complex manifestations of early onset DGS1. However, it is important to note that patients with late-onset DGS1 (Scire *et al.,* 1994; Sykes *et al.,* 1997) develop symptomatic hypocalcemia in childhood or during adolescence with only subtle phenotypic abnormalities. These late-onset DGS1 patients have similar microdeletions in the 22*q*11 region, and the molecular definition of these variants of the DiGeorge syndrome may well provide additional insights into the regulation of PTH secretion and/or parathyroid gland development.

Hypoparathyroidism, Deafness, and Renal Anomalies Syndrome

The combined inheritance of hypoparathyroidism, deafness and renal dysplasia as an autosomal dominant trait was reported in one family in 1992 (Bilous *et al.,* 1992). Patients had asymptomatic hypocalcaemia with undetectable or inappropriately normal serum concentrations of PTH, and normal brisk increases in plasma cAMP in response to the infusion of PTH. The patients also had bilateral, symmetrical, sensorineural deafness involving all frequencies. The renal abnormalities consisted mainly of bilateral cysts that compressed the glomeruli and tubules and lead to renal impairment in some patients. Cytogenetic abnormalities were not detected and abnormalities of the PTH gene were excluded (Bilous *et al.,* 1992). However, cytogenetic abnormalities involving chromosome 10*p*14–10*p*ter were identified in two unrelated patients with features that were consistent with HDR. These two patients suffered from hypoparathyroidism, deafness and growth and mental retardation; one patient also had a solitary dysplastic kidney with vesico-ureteric reflux and a uterus bicornis unicollis (Fryns *et al.,* 1981) and the other patient, who had a complex reciprocal, insertional translocation of chromosomes 10*p* and 8*q,* had cartilaginous exostoses (Van Esch *et al.,* 1999). Neither of these patients had immunodeficiency or heart defects, which are key features of DGS2 (see above), and further studies defined two non-overlapping regions; thus, the DGS2 region was located on 10*p*13–*p*14 and HDR on 10*p*14–10*p*ter. Deletion mapping studies in two other HDR patients further defined a critical 200-kb region that contained GATA3 (Van Esch *et al.,* 2000), which belongs to a family of zinc-finger transcription factors that are involved in vertebrate embryonic development. DNA sequence analysis in other HDR patients identified mutations that resulted in a haploinsufficiency and loss of GATA3 function (Van Esch *et al.,* 2000). The HDR phenotype is consistent

with the expression pattern of GATA3 during human and mouse embryogenesis in the developing kidney, otic vesicle, and parathyroids. However, GATA3 is also expressed in the developing central nervous systen (CNS) and the hematopoietic organs in man and mice, and this suggests that GATA3 may have a more complex role. Indeed, homozygous GATA3 knockout mice have defects of the CNS and a lack of T-cell development, although the heterozygous GATA3 knockout mice appear to have no abnormalities (Pandolfi *et al.,* 1995). It is important to note that HDR patients with GATA3 haploinsufficiency do not have immune deficiency, and this suggests that the immune abnormalities observed in some patients with 10*p* deletions are most likely to be caused by other genes on 10*p*. Similarly, the facial dysmorphism, growth and development delay, commonly seen in patients with larger 10*p* deletions were absent in the HDR patients with GATA3 mutations, further indicating that these features were likely due to other genes on 10*p* (Van Esch *et al.,* 2000). These studies of HDR patients clearly indicate an important role for GATA3 in parathyroid development and in the etiology of hypoparathyroidism.

Mitochondrial Disorders Associated with Hypoparathyroidism

Hypoparathyroidism has been reported to occur in three disorders associated with mitochondrial dysfunction: the Kearns-Sayre syndrome (KSS), the MELAS syndrome and a mitochondrial trifunctional protein deficiency syndrome (MTPDS). KSS is characterized by progressive external ophthalmoplegia and pigmentary retinopathy before the age of 20 years and is often associated with heart block or cardiomyopathy. The MELAS syndrome consists of a childhood onset of mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes. In addition, varying degrees of proximal myopathy can be seen in both conditions. Both the KSS and MELAS syndromes have been reported to occur with insulin-dependent diabetes mellitus and hypoparathyroidism (Moraes *et al.,* 1989; Zupanc *et al.,* 1991). A point mutation in the mitochondrial gene tRNA leucine (UUR) has been reported in one patient with the MELAS syndrome who also suffered from hypoparathyroidism and diabetes mellitus (Morten *et al.,* 1993). Large deletions, consisting of 6741 and 6903 bp and involving >38% of the mitochondrial genome, have been reported in other patients who suffered from KSS, hypoparathyroidism, and sensorineural deafness (Zupanc *et al.,* 1991; Isotani *et al.,* 1996). Rearrangements (Wilichowski *et al.,* 1997) and duplication (Abramowicz *et al.,* 1996) of mitochondrial DNA have also been reported in KSS. Mitochondrial trifunctional protein deficiency is a disorder of fatty-acid oxidation that is associated with peripherial neuropathy, pigmentary retinopathy, and acute fatty liver degeneration in pregnant women who carry an affected fetus. Hypoparathyroidism has been observed in one patient with trifunctional protein deficiency (Dionisi-Via *et al.,* 1996). The role of these mitochondrial mutations in the etiology of hypoparathyroidism remains to be further elucidated.

Kenney–Caffey Syndrome

Hypoparathyroidism has been reported to occur in over 50% of patients with the Kenney–Caffey syndrome, which is associated with short stature, osteosclerosis and cortical thickening of the long bones, delayed closure of the anterior fontanel, basal ganglia calcification, nanophthalmos, and hyperopia (Fanconi *et al.,* 1986; Franceschini *et al.,* 1992). Parathyroid tissue could not be found in a detailed postmortem examination of one patient (Boynton *et al.,* 1979) and this suggests that hypoparathyroidism may be due to an embryological defect of parathyroid development. A molecular genetic analysis using PTH gene RFLP analysis revealed no abnormalities (Bergada *et al.,* 1988) and mutations at other loci, for example in developmental genes, need to be investigated.

Additional Familial Syndromes

Single familial syndromes in which hypoparathyroidism is a component have been reported (Table 1). The inheritance of the disorder in some instances has been established and molecular genetic analysis of the PTH gene has revealed no abnormalities. Thus, an association of hypoparathyroidism, renal insufficiency and developmental delay has been reported in one Asian family in whom autosomal recessive inheritance of the disorder was established (Shaw *et al.,* 1991). An analysis of the PTH gene in this family revealed no abnormalities (Parkinson *et al.,* 1993). The occurrence of hypoparathyroidism, nerve deafness and a steroid-resistant nephrosis leading to renal failure, which has been referred to as the *Barakat syndrome* (Barakat *et al.,* 1977), has been reported in four brothers from one family, and an association of hypoparathyroidism with congenital lymphoedema, nephropathy, mitral valve prolapse and brachytelephalangy has been observed in two brothers from another family (Dahlberg *et al.,* 1983). Molecular genetic studies have not been reported from these two families. A syndrome in which hypoparathyroidism was associated with severe growth failure and dysmorphic features has been reported in 12 patients from Saudi Arabia (Sanjad *et al.,* 1991). Consanguinity was noted in 11 of the 12 patients' families, the majority of which originated from the Western province of Saudi Arabia. This syndrome, which is inherited as an autosomal recessive disorder has also been identified in families of Bedonin origin and homozygosity and linkage disequilibrium studies have located this gene to chromosome 1*q*42–*q*43 (Parvari *et al.,* 1998). Molecular genetic investigations of these disorders will help to identify additional genes that regulate the development of the parathyroid glands.

Blomstrand's Disease

Blomstrand's chondrodysplasia is an autosomal recessive human disorder characterized by early lethality, dramatically advanced bone maturation and accelerated chondrocyte differentiation (Blomstrand *et al.,* 1985). Affected infants, who

usually have consanguineous unaffected parents (Young *et al.,* 1993; Leroy *et al.,* 1996; Loshkajian *et al.,* 1997; den Hollander *et al.,* 1997; Oostra *et al.,* 1998), develop pronounced hyperdensity of the entire skeleton with markedly advanced ossification that results in extremely short and poorly modeled long bones. Mutations of the PTH/PTHrP receptor that impair its function are associated with Blomstrand's disease (Jobert *et al.,* 1998; Zhang *et al.,* 1998; Karaplis *et al.,* 1998). Thus it seems likely that affected infants will in addition to the skeletal defects, also have abnormalities in other organs, which will include secondary hyperplasia of the parathyroid glands, presumably due to hypocalcemia.

Pluriglandular Autoimmune Hypoparathyroidism

Hypoparathyroidism may occur in association with candidiasis, pernicious anemia, allopecia, vitiligo, and autoimmune Addison's disease, and the disorder has been referred to as either the autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) syndrome or the polyglandular autoimmune type 1 syndrome (Ahonen *et al.,* 1990). This disorder has a high incidence in Finland, and a genetic analysis of 58 patients from 42 Finnish families indicated autosomal recessive inheritance of the disorder (Ahonen, 1985). In addition, the disorder has been reported to have a high incidence among Iranian Jews, although the occurrence of candidiasis was less common in this population (Zlotogora and Shapiro, 1992). Linkage studies of 14 Finnish families mapped the APECED gene to chromosome 21*q*22.3 (Aaltonen *et al.,* 1994). Further positional cloning studies led to the isolation of a novel gene from chromosome 21*q*22.3. This gene, referred to as AIRE (autoimmune regulator), encodes a 545-amino-acid protein that contains motifs suggestive of a transcriptional factor and includes a nuclear localization signal, two zinc-finger motifs, a proline-rich region and three LXXLL motifs (Nagamine *et al.,* 1997; Finnish-German APECED Consortium, 1997). Six AIRE mutations have been reported in the APECED families and a codon 257 (Arg→Stop) mutation was the predominant abnormality in 82% of the Finnish families (Nagamine *et al.,* 1997; Finnish-German APECED Consortium, 1997). The identification of the genetic defect causing APECED will not only facilitate genetic diagnosis but will also enhance the elucidation of the mechanisms causing autoimmune disease.

Pseudohypoparathyroidism (PHP)

Patients with pseudohypoparathyroidism are characterised by hypocalcemia and hyperphosphatemia due to PTH resistance (Bilezikian and Thakker, 1998; Yu *et al.,* 1995; van Dop, 1989; Weinstein, 1998). Instead of PTH deficiency these patients have elevated levels of serum PTH which is biochemically and biologically normal. Complete resistance to PTH, demonstrated by no increase in urinary cyclic AMP and urinary phosphate excretion after PTH infusion, is referred to as PHP type I. The occurrence of PHP type I with the somatic features of Albright's hereditary osteodystrophy (AHO) is referred to as PHP type Ia, whereas the presence of biochemical features without somatic features is referred to as PHP type Ib. The occurrence of somatic features (AHO) without the biochemical abnormalities is referred to as pseudo-pseudohypoparathyroidism (PPHP). The absence of a normal rise in urinary excretion of cyclic AMP excretion after an infusion of PTH in PHP type Ia and PPHP indicated a defect at some site of the PTH receptor-adenylyl cyclase system. This receptor system is regulated by at least two G proteins, one of which stimulates (Gsα) and another of which inhibits (Giα) the activity of the membrane-bound enzyme that catalyzes the formation of the intracellular second messenger cyclic AMP.

Inactivating mutations of the Gsα gene (referred to as GNAS1), which is located on chromosome 20*q*13.2, have been identified in PHP type Ia and PPHP patients (Yu *et al.,* 1995; van Dop, 1989; Weinstein, 1998). A mutational 'hotspot' involving a 4-bp deletion (Δ GACT) of codon 189 and the first nucleotide of codon 190 has been identified (Yu *et al.,* 1995). These mutations, which are heterozygous, lead to an ≈50% reduction in $G_s\alpha$ activity/protein, and are thought to explain, at least partially, the resistance toward PTH and other hormones (e.g., thyroid stimulating hormone (TSH), gonadotrophins and glucagon) that mediate their actions through G protein-coupled receptors (Yu *et al.,* 1995; van Dop, 1989; Weinstein, 1998). However, a similar reduction in $G_s\alpha$ activity/protein is also found in patients with PPHP, who show the same physical features as individuals with PHP-Ia, but lack the endocrine abnormalities. Thus, *GNAS1* mutations do not fully explain the PHP-Ia or PPHP phenotypes (Yu *et al.,* 1995; van Dop, 1989; Weinstein, 1998; Schuster *et al.,* 1993; Miric *et al.,* 1993; Weinstein *et al.,* 1990), and studies of PHP-Ia and PPHP that occurred within the same kindred revealed that the hormonal resistance is parentally imprinted. Thus, PHP-Ia occurred in a child only when the mutation was inherited from a mother affected with either PHP-Ia or PPHP (Davies and Hughes, 1993; Wilson *et al.,* 1994). These findings in man gained support by observations in heterozygous *Gnas* knockout mice that lacked exon 2. Mice that had inherited the mutant allele from a female showed undetectable $G_s\alpha$ protein in the renal cortex and decreased blood calcium concentration due to PTH resistance. In contrast, offspring that had inherited the mutant allele lacking exon 2 from a male, showed no evidence of endocrine abnormalities (Yu *et al.,* 1998). Tissue- or cell-specific $G_s\alpha$ expression is thus likely to be involved in the pathogenesis of PHP-Ia and PPHP, and this may also help to explain the dominant phenotype that arises from heterozygous *GNAS1* mutations. Expression of the *GNASI* gene has been shown to be further complicated by alternative splicing which results in several different mRNAs, some of which are derived only from either the paternal or the maternal allele. This complexity of the *GNASI* gene may contribute to the unique phenotypic

abnormalities observed in patients with PHP as the alternative splicing gives rise to at least three different gene products which are transcribed either from the maternal or the paternal allele, or from both alleles (Hayward *et al.,* 1998a, b; Peters *et al.,* 1999). The first of these products is $G_s\alpha$ which is encoded by exons 1 through 13 of the *GNASI* gene and mediates the biological functions of a large variety of G protein-coupled receptors, including the PTH/PTHrP receptor. The second product is $XL_{\alpha s}$ which comprises of a novel first exon (XL) spliced onto exons 2 through 13. The encoded XLαs, which is an ≈92-kDa protein, that shares considerable amino acid sequence identity with the carboxyl-terminal portion of $G_s\alpha$ (Kehlenbach *et al.,* 1994), may not function as a stimulatory G protein. $XL_{\alpha s}$ expression occurs at numerous sites and is particularly high in endocrine and neuroendocrine cells (Kehlenbach *et al.,* 1994). Furthermore XLαs appears to be transcribed only from the paternal allele (Hayward *et al.,* 1998a,b; Peters *et al.,* 1999). The third product is NESP55 (Hayward *et al.,* 1998a), which is transcribed only from the maternal allele (Hayward *et al.,* 1998b; Peters *et al.,* 1999), is encoded by yet another exon of the *GNASI* gene that is located upstream of exon XL and the $G_s\alpha$-specific exons 1 through 13. NESP55, which is thought to act as a neuroendocrine secretory protein (Ischia *et al.,* 1997), shares no amino acid sequence homology with either $XL_{\alpha s}$ or $G_s\alpha$, but its mRNA does contain $G_s\alpha$-specific exons within its 3′ noncoding region. The complexity of the *GNASI* gene and its use of different, allele-specific promoters, makes it plausible to postulate that mutations in the $G_s\alpha$-specific exons 1 to 13 (Yu *et al.,* 1995; van Dop, 1989; Weinstein, 1998; Schuster *et al.,* 1993; Miric *et al.,* 1993; Weinstein *et al.,* 1990), will affect not only the functional properties of $G_s\alpha$, but also those of $XL_{\alpha s}$ and of NESP55.

GNAS1 mutations have not been detected in PHP type Ib (PHP-Ib), which has been considered to be due to a defect of the PTH/PTHrP receptor. However, it is important to note that PHP-Ib patients generally have a normal or increased osseous response to PTH, as assessed by bone turnover and osteoclastic resorption (Yu *et al.,* 1995; ven Dop, 1989; Murray *et al.,* 1993; Farfel, 1999), and the normal growth plate development in these patients is consistent with a normal chondrocyte response to PTHrP. These observations made it unlikely that defects in the PTH/PTHrP receptor were the cause of PHP-Ib, and indeed studies of the PTH/PTHrP receptor gene and mRNA in PHP-Ib patients have failed to identify mutations (Schipani *et al.,* 1995; Bettoun *et al.,* 1997; Suarez *et al.,* 1995; Fukumoto *et al.,* 1996). In order to identify the location of the PHP-Ib gene a genome-wide search was therefore undertaken in four unrelated kindreds and this mapped the PHP-Ib locus to chromosome 20*q*13.3, a location that also contains the GNAS1 gene (Jüppner *et al.,* 1998). In addition, parental imprinting of the genetic defect was observed and this is similar to the findings in kindreds with PHP-type Ia and/or PPHP. Two possible explanations for these observations have been proposed. First, PHP-Ib maybe due to a defect in a tissue- or cell-specific enhancer, or promoter of the *GNAS1* gene and this may affect, directly or indirectly, the expression levels of the $G_s\alpha$-specific transcripts and/or the transcripts encoding $XL_{\alpha s}$ and NESP55; or second, PHP-Ib may be caused by a defect in a gene close to the GNAS1 gene which is transcribed only from the maternal allele and affects PTH/PTHrP receptor or Gsα expression and/or function in some renal cells.

Conclusions

Application of the methods of molecular genetics to the study of the hypoparathyroid disorders has resulted in considerable advances that have identified some genes and their encoded proteins that are involved in the regulation of PTH synthesis and secretion, and in mediating its actions in different target issues. In addition, the identification of mutations has helped to provide molecular explanations and insights into a variety of familial and sporadic disorders of calcium homeostasis and bone development. Genetic mapping studies have also helped to identify the chromosomal locations of some hypoparathyroid disorders, and future positional cloning approaches, which will progress with greater rapidity owing to the Human Genome Sequencing project, will provide additional insights into the mechanisms that regulate PTH action and calcium homeostasis.

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CHAPTER 63

Jansen's Metaphyseal Chondrodysplasia and Blomstrand's Lethal Chondrodysplasia

Two Genetic Disorders Caused by PTH/PTHrP Receptor Mutations

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Introduction

The PTH/PTHrP receptor (also referred to as type I PTH/PTHrP receptor or PTH1R) mediates the actions of two ligands, parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP), and stimulates at least two distinct second messenger pathways, cAMP/PKA and IP_3/Ca^{2+}/PKC (Jüppner *et al.,* 1991; Abou-Samra *et al.,* 1992; Schipani *et al.,* 1993). This receptor is most abundantly expressed in kidney, bone, and growth plates and at lower levels in a large variety of other tissues (Tian *et al.,* 1993; Urena *et al.,* 1993). The PTH-dependent endocrine actions that are mediated through the PTH/PTHrP receptor, i.e., the regulation of mineral ion homeostasis, had been explored through studies in intact and parathyroidectomized animals. The role of the PTH/PTHrP receptor in mediating the PTHrP-dependent autocrine/paracrine regulation of chondrocyte growth and differentiation became apparent only through the analysis of genetically manipulated mice. For example, animals that are "null" for PTHrP or the PTH/PTHrP receptor die *in utero* or shortly thereafter and show a profound acceleration of growth plate mineralization. In contrast, mice overexpressing PTHrP under the control of a growth plate-specific promoter are viable, but showed a severe delay in chondrocyte maturation, which leads to impaired bone growth and elongation (Karaplis *et al.,* 1994; Amizuka *et al.,* 1994; Weir *et al.,* 1996; Lanske *et al.,* 1996; Vortkamp *et al.,* 1996). These studies in mice thus provided important clues regarding the phenotypic abnormalities that were to be expected in humans with PTH/PTHrP receptor mutations and consequently led to the identification of different activating and inactivating PTH/PTHrP receptor mutations in two genetic disorders, Jansen's metaphyseal chondrodysplasia (JMC) and Blomstrand's lethal chondrodysplasia (BLC). Since the biological consequences of these PTH/PTHrP receptor

mutations require a detailed understanding of the PTH- and PTHrP-dependent actions that are mediated through this G protein-coupled receptor, we will first review the physiological roles of these two peptide hormones.

Parathyroid Hormone

Besides 1,25$(OH)_2$ vitamin D_3 (1,25$(OH)_2D_3$), PTH is the most important endocrine regulator of extracellular calcium homeostasis in mammals (Kronenberg *et al.,* 1993; Jüppner *et al.,* 1999). PTH is predominantly expressed in the parathyroid glands. However, lower protein and mRNA levels were recently identified in the hypothalamus and the thymus of *glial cells missing 2* (*gcm2*)-ablated mice (Günther *et al.,* 2000). These findings confirmed earlier studies in rats (Nutley *et al.,* 1995), but it remains unlikely that PTH derived from tissues other than the parathyroid glands is normally involved in the regulation of mineral ion homeostasis. Its synthesis and secretion by the parathyroid glands is dependent predominantly on the extracellular concentration of calcium (Brown, 1983; Silver and Kronenberg, 1996), which is monitored by a calcium-sensing receptor (Brown *et al.,* 1993, 1999) and to a lesser extent by 1,25$(OH)_2D_3$ and phosphate (Silver and Kronenberg, 1996; Almaden *et al.,* 1996; Slatopolsky *et al.,* 1996; Moallem *et al.,* 1998).

PTH acts primarily on kidney and bone, where it binds to cells expressing the PTH/PTHrP receptor and thereby initiates a series of processes that serve to maintain blood calcium and phosphate concentrations within narrow limits (Fig. 1). In kidney, the mRNA encoding the PTH/PTHrP receptor is expressed primarily in the convoluted and straight proximal tubules, the cortical portion of thick ascending limb, and the distal convoluted renal tubules (Riccardi *et al.,* 1996; Lee *et al.,* 1996; Yang *et al.,* 1997), i.e., in those renal segments which respond to PTH with an increase in cAMP accumulation (Chabardes *et al.,* 1975, 1980; Morel *et al.,* 1981).

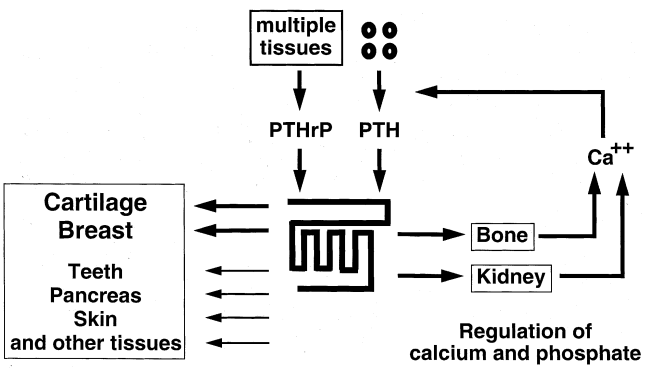

Figure 1 The PTH/PTHrP receptor is abundantly expressed in kidney and bone were it mediates the PTH-dependent regulation of calcium and phosphate homeostasis. It is also expressed in numerous other tissues, particularly in the growth plate chondrocytes, where it mediates the regulation of cellular proliferation and differentiation during development.

The most important PTH-mediated actions in the kidney affect the synthesis of 1,25$(OH)_2D_3$ from its precursor 25OHD_3, the excretion of phosphate, and the reabsorption of calcium. The stimulation of 1α-hydroxylase activity, an action which appears to be at least partially cAMP-dependent (Garabedian *et al.,* 1972; Fraser and Kodicek, 1973; Horiuchi *et al.,* 1977; Kong *et al.,* 1999; Fu *et al.,* 1997; Takeyama *et al.,* 1997), is largely restricted to the proximal convoluted tubule. The resulting increase in 1,25$(OH)_2D_3$ production enhances the absorption of calcium and phosphate from the intestine. The PTH-dependent inhibition of renal tubular phosphate reabsorption has been extensively documented in a variety of *in vivo* and *in vitro* studies (for reviews, see Stewart and Broadus, 1987; Amiel *et al.,* 1998; Murer *et al.,* 1999; Silve and Friedlander, 2000). Thus to increase renal phosphate excretion, PTH reduces the abundance of the type II sodium-phosphate cotransporter (Npt-2, also referred to as NaPi-2a) on the apical surface of proximal tubules, particularly of the proximal straight tubules (pars recta). This effect of PTH is mediated by both cAMP-dependent and -independent mechanisms and is associated with an increased internalization and subsequent lysosomal degradation of Npt-2 (Bell *et al.,* 1972; Goldfarb *et al.,* 1978; Pfister *et al.,* 1998, 1999; Lotscher *et al.,* 1999; for review, see Murer *et al.,* 2000). PTH/PTHrP receptor protein expression has been demonstrated by immunohistochemical and immunoelectron microscopic analysis, and by functional studies, on both basolateral and luminal membranes in proximal tubular cells *in vitro* and in intact proximal tubules (Reshkin *et al.,* 1991; Kaufmann *et al.,* 1994; Amizuka *et al.,* 1997; Traebert *et al.,* 2000). Apical receptors may be preferentially coupled to cAMP-independent signaling pathway, while basolateral receptor activation initiates both cAMP-dependent and cAMP-independent effects. Recently, megalin, a multifunctional clearance receptor expressed on the apical surface of proximal tubular cells has been shown to regulate the renal catabolism of PTH and to potentially antagonize PTH/PTHrP receptor activity (Hilpert *et al.,* 1999). In the distal convoluted tubule, PTH stimulates, possibly through second messengers other than cAMP, the reabsorption of calcium (Friedman *et al.,* 1996, 1999). PTH also decreases the glomerular filtration, inhibits the proximal reabsorption of bicarbonate and of amino acids, and stimulates gluconeogenesis (Amiel *et al.,* 1998; Jaeger *et al.,* 1987). Consistent with the findings regarding the second messenger systems that mediate the proximal and distal actions of PTH, recently established clonal cell lines derived from proximal segments of rat renal tubules showed a PTH-dependent accumulation of cAMP accumulation and a rapid increase in intracellular free calcium. In contrast to the characteristics of these "proximal" tubular cells, where PTH stimulation led to a rapid release of intracellular calcium from intracellular stores, the increase in this second messenger in the "distal" tubular cells was dependent largely on extracellular calcium (Friedman *et al.,* 1996, 1999). These findings indicated that distinct portions of the nephron respond differently to challenge with PTH.

Similar to its renal tubular effects, the PTH-dependent actions on bone are complex and often difficult to study. As outlined below in more detail, the hormone can influence, either directly or indirectly, the proliferation and differentiation of several bone cell precursors. Furthermore, the effects resulting from PTH stimulation of mature osteoblasts appear to be different depending on the intensity and duration of the stimulus, the type of bone (trabecular versus cortical), and the hormonal impregnation of bone. As a result, the hormonal effects observed *in vitro* often fail to reflect the conditions *in vivo.* For example, PTH stimulates both bone formation and osteoclastic bone resorption; however, the continuous administration of PTH *in vivo* is thought to favor bone resorption over bone formation, whereas intermittent doses of the hormone results in net anabolic effects (Tam *et al.,* 1982; Lane *et al.,* 1998; for review, see Finkelstein, 1996; Neer *et al.,* 2001). Rapid changes in blood PTH concentration may occur *in vivo,* since small changes in ionized calcium can generate significant variations in PTH secretion (Brown *et al.,* 1999) and these may contribute to the observed circadian rhythm of PTH concentration in the circulation (Kripke *et al.,* 1978; Harms *et al.,* 1989, 1994). Along with other endocrine factors, notably $1,25(OH)_2D_3$, PTH modulates the responsiveness of bone cells (Crowell *et al.,* 1981; Turner *et al.,* 1995).

The initial, PTH-dependent effect on bone is a rapid release of calcium from areas of the matrix that allow rapid exchange with the extracellular fluid (ECF). These events are followed, after a delay of several hours, by PTH-induced changes in bone cell metabolism. After stimulating osteoblast activity and thus bone formation (Silve *et al.,* 1982), activated osteoblasts increase the activity of already present osteoclasts as well as the differentiation of osteoclast precursors into mature bone-resorbing cells. The coupling between bone-forming osteoblasts and bone-resorbing osteoclasts is thought to depend, at least in part, on the ability of PTH to induce changes in the synthesis and/or activity of several different osteoblast-specific proteins, including insulin-like growth factor 1 (IGF1) (Canalis *et al.,* 1989). However, the most important of these "coupling" factors is RANKL (also referred to as osteoclast-differentiation factor (ODF), TRANCE, or osteoprotegerin ligand), a tumor necrosis factor (TNF)-related protein which is anchored on the surface of osteoblasts. RANKL interacts with the receptor RANK, which is expressed on preosteoclasts and mature osteoclasts, and facilitates the differentiation of precursor cells into osteoclasts and stimulates the activity of these bone-resorbing cells (Quinn *et al.,* 1998; Yasuda *et al.,* 1998). PTH thus acts on two distinct tissues, kidney and bone, to increase through different mechanisms the blood concentration of calcium and to thereby prevent significant hypocalcemia.

Parathyroid Hormone-Related Peptide

PTHrP was first discovered as the major cause of the humoral hypercalcemia of malignancy syndrome (Stewart *et al.,* 1980; Moseley *et al.,* 1987; Suva *et al.,* 1987; Strewler *et al.,* 1987; Mangin *et al.,* 1988). Within its amino-terminal portion, PTHrP shares partial amino acid sequence homology with PTH, and as a result of these limited structural similarities, amino-terminal fragments of both peptides have largely indistinguishable biological properties, at least with regard to the regulation of mineral ion homeostasis (Kemp *et al.,* 1987; Horiuchi *et al.,* 1987; Fraher *et al.,* 1992; Everhart-Caye *et al.,* 1996). Shortly after its initial isolation from several different tumors, PTHrP and its mRNA were found in large variety of fetal and adult tissues, suggesting that this peptide has an important biological role throughout life (Ikeda *et al.,* 1988; Broadus and Stewart, 1994; Yang and Stewart, 1996). However, it was not until the generation of genetically manipulated mice that the major physiological roles of PTHrP became apparent. These roles include the regulation of chondrocyte proliferation and differentiation during the process of endochondral bone formation (Karaplis *et al.,* 1994; Amizuka *et al.,* 1994; Weir *et al.,* 1996; Lanske *et al.,* 1996; Vortkamp *et al.,* 1996) and epithelial–mesenchymal interactions during organogenesis of certain epithelial organs, including skin, mammary gland, and teeth (Wysolmerski *et al.,* 1994, 1996, 1998; Philbrick *et al.,* 1998) (for review, see Wysolmerski and Stewart, 1998; Strewler, 2000). During fetal development, the expression of mRNA transcripts encoding PTHrP or the PTH/PTHrP receptor is closely linked, both spatially and temporally, implying that the ligand and its receptor are involved in paracrine/autocrine signaling events at these sites (Karaplis *et al.,* 1994; Wysolmerski *et al.,* 1998; Philbrick *et al.,* 1998; Lee *et al.,* 1995). These observations are furthermore consistent with the hypothesis that the PTH/PTHrP receptor mediates most of the actions of PTHrP.

Other recently described PTHrP-dependent effects are likely to involve mid-/carboxyl-terminal peptide fragments that are generated through alternative splicing and/or posttranslational processing and involve distinct, only incompletely characterized cell surface receptors and/or direct interactions with the nucleus (Wu *et al.,* 1996; Kovacs *et al.,* 1996; Lanske *et al.,* 1999; Henderson *et al.,* 1995; Nguyen and Karaplis, 1998; Massfelder *et al.,* 1997). While these non-amino-terminal PTHrP fragments are unlikely to be of biological importance for adult mineral ion metabolism, recent evidence suggests that large carboxyl-terminal fragments of PTH such as PTH(7–84) act, directly or indirectly, as antagonists of the calcemic actions of PTH(1–84) (Slatopolsky *et al.,* 2000; Nguyen-Yamamoto *et al.,* 2001). Through yet undefined mechanisms, carboxyl-terminal fragments of PTH may thus contribute to the regulation of calcium homeostasis.

The PTH/PTHrP Receptor: A Receptor for Two Distinct Ligands

The isolation of cDNAs encoding the PTH/PTHrP receptor from several different species, including humans, and subsequent expression of these cDNAs in various mammalian cell lines has confirmed and extended three key

observations: (1) the recombinant PTH/PTHrP receptor binds amino-terminal fragments of PTH and PTHrP with similar or indistinguishable affinity; (2) both ligands stimulate with similar potency the formation of at least two second messengers, cAMP and inositol phosphate, and (3) identical receptors are expressed in renal tubular cells and in osteoblasts (Jüppner *et al.,* 1991; Abou-Samra *et al.,* 1992; Schipani *et al.,* 1993). Furthermore, similar to the widely expressed PTHrP, the mRNA encoding the PTH/PTHrP receptor is found in a large variety of fetal and adult tissues (Tian *et al.,* 1993; Urena *et al.,* 1993; Riccardi *et al.,* 1996; Lee *et al.,* 1994, 1995, 1996) and at particularly abundant concentrations in proximal tubular cells, in osteoblasts, and in prehypertrophic chondrocytes of growth plates (Karaplis *et al.,* 1994; Weir *et al.,* 1996; Lanske *et al.,* 1996; Vortkamp *et al.,* 1996).

PTH/PTHrP receptor belongs to the class B family of heptahelical G protein-coupled receptors (GPCR) which also comprises the receptors for secretin, calcitonin, glucagon, and several other peptide hormones (Jüppner, 1994; Mannstadt *et al.,* 1999; Gardella and Jüppner, 2000). These hormone receptors share no homology with other G protein-coupled receptors, for example the members of the class A and class C family of receptors, and the organization of genes those encoding the latter receptors are distinctly different from encoding the class B receptors. All class B receptors are characterized by an amino-terminal, extracellular domain that comprises approximately 150 amino acids and by eight conserved extracellular cysteine residues, as well as several other conserved amino acids which are dispersed throughout the amino-terminal domain, the membrane-spanning helices, and the connecting loops.

The organization of the PTH/PTHrP receptor gene which comprises in mammals 14 coding exons, appears to be similar in all vertebrates (Kong *et al.,* 1994; McCuaig *et al.,* 1994). The ~2.5-kb transcript encoding the full-length PTH/PTHrP receptor is the predominant mRNA species in most tissues (Tian *et al.,* 1993; Urena *et al.,* 1993), but several larger and smaller sized transcripts have also been detected, suggesting the presence of splice variants of the PTH/PTHrP receptor. In contrast to alternatively spliced mRNAs that encode functional calcitonin and CRF receptors lacking portions of the seventh membrane-spanning helix (Shyu *et al.,* 1996; Grammatopoulos *et al.,* 1999), no alternatively spliced, functionally active PTH/PTHrP receptors have yet been identified (Jobert *et al.,* 1996; Joun *et al.,* 1997).

Transcripts encoding identical PTH/PTHrP receptors are derived from at least three different promoters that located upstream of one of three different untranslated exons (Kong *et al.,* 1994; McCuaig *et al.,* 1994; Bettoun *et al.,* 1997; Manen *et al.,* 1998). Little is known about the factors that control the activity of these promoters, some of which appear to be species- and tissue-specific (Amizuka *et al.,* 1997; Bettoun *et al.,* 1998), and their activity may be different throughout development and adult life (Bettoun *et al.,* 1998; Amizuka *et al.,* 1999). Transcripts derived from the P1 and the P2 promoter have been observed in rodents and humans (McCuaig *et al.,* 1994; Joun *et al.,* 1997; McCuaig *et al.,* 1995). P1-derived transcripts are found mainly, at least in rodents, in vascular smooth muscles and in peritubular endothelial cells of the adult kidney (Amizuka *et al.,* 1997). In contrast, transcripts derived from the ubiquitous P2 promoter can be detected in numerous fetal and adult tissues, including cartilage and bone (Amizuka *et al.,* 1999). The P2 promoter activity is equivalent in humans and mice, while the activity of the P1 promoter is prominent only in rodents and appears to be weak or absent in humans. A third promoter, P3, has been identified thus far only in humans, where it is thought to control PTH/PTHrP receptor expression in several different tissues, including kidney and bone (Manen *et al.,* 1998; Bettoun *et al.,* 1998). Methylation appears to play a role in controlling human PTH/PTHrP receptor gene promoter activity (Bettoun *et al.,* 2000). A differential regulation of P2 activity in osteoblasts and chondrocytes following vitamin D3 administration has been demonstrated in rodents (Amizuka *et al.,* 1999).

A second PTH-receptor, termed type 2 PTH receptor or PTH2-receptor, which belongs to the same family of G protein-coupled receptors as the PTH/PTHrP receptor, is less broadly expressed, with little if any expression in kidney and bone (Usdin *et al.,* 1996, 1999). The human PTH2-receptor is efficiently activated by PTH, but not by PTHrP (Usdin *et al.,* 1995; Gardella *et al.,* 1996; Behar *et al.,* 1996), while the rat receptor homolog is not activated by either of these two ligands (Hoare *et al.,* 1999). Instead, both PTH2-receptor isoforms are efficiently and equally activated by TIP39, a recently identified hypothalamic peptide, which shows limited amino acid sequence homology with PTH and PTHrP. It is therefore likely that TIP39 represents the primary agonist for the PTH2-receptor (Usdin *et al.,* 1999; Usdin, 1997). Although TIP39 does not activate the PTH/PTHrP receptor, the full-length peptide as well as several fragments that are truncated at the amino-terminus can bind to the latter receptor and furthermore inhibit PTH- and PTHrP-stimulated cAMP accumulation (Hoare *et al.,* 2000a,b; Jonsson *et al.,* 2001). It appears unlikely, however, that TIP39 has a role in the regulation of mineral ion homeostasis.

Role of PTHrP and the PTH/PTHrP Receptor in Endochondral Bone Formation

PTHrP was initially discovered as the cause of the humoral hypercalcemia of malignancy syndrome. However, its most prominent physiological role was revealed only through the homologous ablation of its gene in mice (Karaplis *et al.,* 1994) and through the development of transgenic animals which express PTHrP under the control of a growth plate-specific promoter (Weir *et al.,* 1996).

Homozygous PTHrP gene-ablated animals die during the perinatal period and show striking skeletal changes, which include domed skulls, short snouts, and mandibles

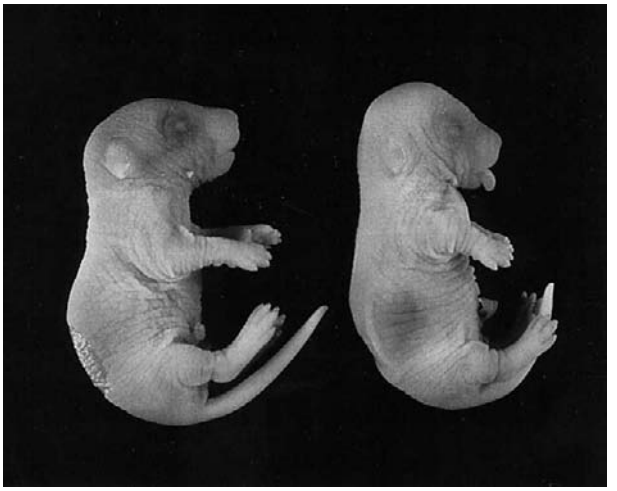

Figure 2 Phenotype of a homozygous PTHrP-ablated mouse (day 18.5 of embryonic development). Note the chondrodysplasia characterized by a domed skull, short snout and mandible, protruding tongue, narrow thorax, and disproportionately short limbs. From Karaplis *et al.* (1994), with permission. (See also color plate.)

and disproportionately short extremities, yet no obvious developmental defects in other organs (Fig. 2, see also color plate). These skeletal changes are caused by a dramatic acceleration of chondrocyte differentiation that leads to premature growth plate mineralization (Karaplis *et al.,* 1994) (Fig. 3). Heterozygous animals, lacking only one copy of the PTHrP gene, show normal growth and development, and are fertile, but develop, despite apparently normal calcium and phosphorus homeostasis, mild osteopenia later in life (Amizuka *et al.,* 1996). Growth plate abnormalities that are, in many aspects, the opposite of those found in PTHrP-ablated mice are observed in animals that overexpress PTHrP under the control of the α1(II) collagen promoter (Weir *et al.,* 1996). Throughout life these animals are smaller in size than their wild-type litter mates, and show a disproportionate foreshortening of limbs and tail, which is most likely due to a severe delay in chondrocyte differentiation and endochondral ossification. Thus, too little or too much PTHrP expression in the growth plate leads to short-limbed dwarfism, although through entirely different mechanisms.

From these and other studies, it is now well established that PTHrP facilitates the continuous proliferation of chondrocytes in the growth plate, and that it postpones their programmed differentiation into hypertrophic chondrocytes. Consistent with this role of PTHrP in endochondral bone formation, earlier *in vitro* studies had shown that PTH (used in these studies instead of PTHrP) affects chondrocyte maturation and activity (Lebovitz and Eisenbarth, 1975; Smith *et al.,* 1976). More recent studies confirmed these findings by showing that PTH and PTHrP stimulate, presumably through cAMP-dependent mechanisms (Jikko *et al.,* 1996), the proliferation of fetal growth plate chondrocytes, inhibit the differentiation of these cells into hypertrophic chondrocytes, and stimulate the accumulation of cartilage-specific proteoglycans that are thought to act as inhibitors of mineralization (Takano *et al.,* 1985; Koike *et al.,* 1990; Iwamoto *et al.,* 1994). In the absence of these cartilage-specific PTHrP effects, growth plates of homozygous PTHrP gene-ablated mice have a thinner layer of proliferating chondrocytes, while the layer of

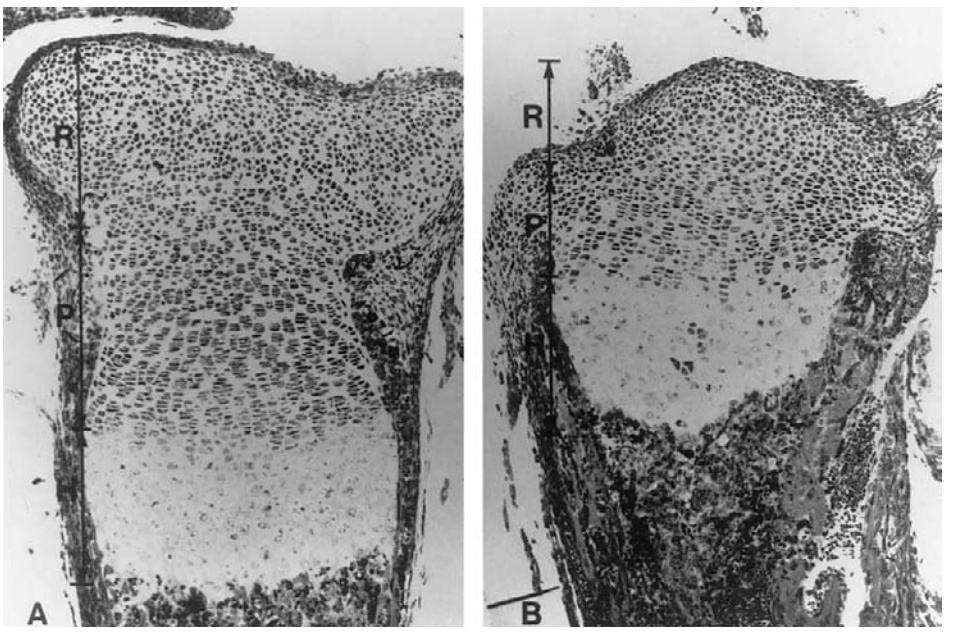

Figure 3 Low-magnification photomicrographs of the proximal tibiae of a wild-type mouse (A) and a homozygous PTHrP-ablated mouse (B) (E 18.5). Note that the tibial epiphysis of the PTHrP-ablated mouse is shortened, the zones of reserve and proliferative chondrocytes are markedly reduced. From Amizuka *et al.* (1994), with permission.

hypertrophic chondrocytes is relatively normal in thickness, but somewhat disorganized. Taken together these findings suggested that the lack of PTHrP accelerates the normal differentiation process of growth plate chondrocytes; i.e., resting and proliferating chondrocytes undergo fewer cycles of cell division and differentiate prematurely into hypertrophic cells, which then undergo apoptosis before being replaced by invading osteoblasts.

The phenotypic changes in mice which are "null" for either PTHrP or the PTH/PTHrP receptor are similar, and current evidence indicates that the autocrine/paracrine actions of PTHrP within the growth plate are mediated through the PTH/PTHrP receptor (Karaplis *et al.,* 1994; Lanske *et al.,* 1996; Chung *et al.,* 1998). Furthermore, mice missing either PTHrP or its receptor are resistant to the actions of *Indian Hedgehog (Ihh),* a developmentally important protein which is most abundantly expressed in growth plate chondrocytes that are about to differentiate into hypertrophic cells. *Ihh* binds directly to *patched,* a membrane receptor, which interacts with *smoothened* and thereby suppresses the constitutive activity of the latter protein (Stone *et al.,* 1996; Marigo *et al.,* 1996). The ectopic expression of *Ihh* in the chicken wing cartilage stimulates the production of PTHrP and thereby blocks the normal chondrocyte differentiation program (Vortkamp *et al.,* 1996); whether PTHrP represses, as part of a feedback loop, the expression of *Ihh* remains to be established. PTHrP and *Ihh* are thus critically important components of normal bone growth and elongation (Lanske *et al.,* 1996; Vortkamp *et al.,* 1996). However, not all actions of PTHrP appear to be mediated through the PTH/PTHrP receptor, since the ablation of the PTHrP gene or the PTH/PTHrP receptor gene leads to subtle, but distinctly different, abnormalities in early bone development (Lanske *et al.,* 1999). This suggests that some actions of PTHrP in bone involve either distinct receptors or the peptide's direct nuclear actions (Henderson *et al.,* 1995; Nguyen and Karaplis, 1998; Massfelder *et al.,* 1997).

Role of PTHrP in Regulating Epithelial–Mesenchymal Interactions

Studies with transgenic mice, in which PTHrP expression is targeted through a human keratinocyte-specific promoter (K14) to the developing epidermis and mammary gland, demonstrated that PTHrP plays also a critical role in hair follicle development and branching morphogenesis of the mammary gland (Wysolmerski *et al.,* 1994, 1996). These conclusions were further supported by findings in PTHrP-null mice that had been rescued from neonatal death by targeting PTHrP expression to chondrocytes through the α1(II) collagen promoter (Weir *et al.,* 1996). These rescued mice lack mammary epithelial ducts, due to a failure of the initial round of branching growth that is required for transforming the mammary bud into the primary duct system; ablation of both copies of the PTH/PTHrP receptor gene recapitulated the phenotype of PTHrP-ablated animals (Wysolmerski *et al.,* 1998).

Using similar approaches, it was furthermore demonstrated that PTHrP is required for normal tooth eruption. Teeth appeared to develop normally in rescued PTHrP knockout mice, but became trapped by the surrounding bone and underwent progressive impactation (Philbrick *et al.,* 1998). In these tissues, PTHrP mRNA was identified by *in situ* hybridization in epithelial cells, while the PTH/PTHrP receptor mRNA was found on mesenchymal or stromal cells. These observations led to the concept that the communication between epithelium and mesenchyme involves the PTH/PTHrP receptor, and that PTHrP-signaling is essential for normal development of these tissues (Wysolmerski and Stewart, 1998).

Jansen's Metaphyseal Chondrodysplasia (JMC)

JMC, first described in 1934 (Jansen, 1934), is a rare autosomal dominant form of short-limbed dwarfism associated with laboratory abnormalities that are typically observed only in patients with either primary hyperparathyroidism or with the humoral hypercalcemia of malignancy syndrome (reviewed in Jüppner, 1996; Parfitt *et al.,* 1996). These biochemical changes, i.e., hypercalcemia, renal phosphate wasting, and increased urinary cAMP excretion, occur despite low or undetectable concentrations of PTH in the circulation and of PTHrP concentrations that are not elevated (Frame and Poznanski, 1980; Holt, 1969; Kessel *et al.,* 1992; Rao *et al.,* 1979; Silverthorn *et al.,* 1983; Schipani *et al.,* 1999; Kruse and Schütz, 1993). Severe hypercalcemia, which is often asymptomatic, and hypophosphatemia had been noted in Jansen's first patient (De Haas *et al.,* 1969) and in a subsequently described child with the same disorder (Cameron *et al.,* 1954). It was not until the description of a third patient, however, that the association between the abnormalities in endochondral bone formation and in mineral ion homeostasis was formally considered (Gram *et al.,* 1959). At that time the biochemical abnormalities could not be readily distinguished from those observed in primary hyperparathyroidism, but the surgical exploration of the patient revealed no obvious abnormalities of the parathyroid glands. It was therefore concluded that the changes in mineral metabolism were either "secondary to the underlying bone defect" or "related to an undefined metabolic disorder that gave rise to both metaphyseal and biochemical changes" (Gram *et al.,* 1959). Most reported cases of JMC are sporadic, but the description of two unrelated affected females that gave birth to affected daughters (Lenz, 1969; Holthusen *et al.,* 1975; Charrow and Poznanski, 1984) suggested an autosomal dominant mode of inheritance; this conclusion was subsequently confirmed for one of these families at the molecular level (Schipani *et al.,* 1996).

At birth some patients with JMC have dysmorphic features, which can include high skull vault, flattening of the of the nose and forehead-low set ears, hypertelorism, high-arched palate, and micro- or retrognathia (for a review, see

Jüppner, 1996). Although body length is within normal limits at birth, growth becomes increasingly abnormal, eventually leading to the development of short stature. Additional signs may include kyphoscoliosis with a bell-shaped thorax and widened costochondral junctions, metaphyseal enlargement of the joints, waddling gait, prominent supraorbital ridges, and frontonasal hyperplasia. The legs are usually bowed and short, while the arms are relatively long.

Radiological studies have shown considerable, age-dependent differences in the osseous manifestations of JMC. In younger patients, severe metaphyseal changes, especially of the long bones are present (Fig. 4). The metaphyses are enlarged and expanded, giving a club-like appearance to the ends of the long bones with a wide zone of irregular calcifications. Patches of partially calcified cartilage that protrude into the diaphyses are also present and appear relatively radiolucent. These findings, which are characteristically observed throughout early childhood, are similar to the lesions observed in rickets. However, distinct from the findings in rickets, metacarpal and metatarsal bones are also involved.

Later in childhood, the changes are no longer reminiscent of rickets. Until the onset of puberty, almost all tubular bones show irregular patches of partially calcified cartilage that protrude into the diaphyses; the spine and vertebral bodies show no obvious abnormalities (Frame and Poznanski, 1980; Kessel *et al.,* 1992; Kao *et al.,* 1979; Silverthorn *et al.,* 1983; Schipani *et al.,* 1999; Kruse and Schütz, 1993; Cameron *et al.,* 1954; Charrow and Poznanski, 1984). After adolescence, the cartilaginous tissue in the metaphyses gradually disappears and turns into bone, leading to bulbous deformities (see Fig. 4). The ends of most tubular bones remain expanded, deformed, and radiolucent, but a more normal trabecular pattern gradually emerges (Frame and Poznanski, 1980; Kessel *et al.,* 1992; Kao *et al.,* 1979; Silverthorn *et al.,* 1983; Schipani *et al.,* 1999; Kruse and Schütz, 1993; Cameron *et al.,* 1954; Charrow and Poznanski, 1984).

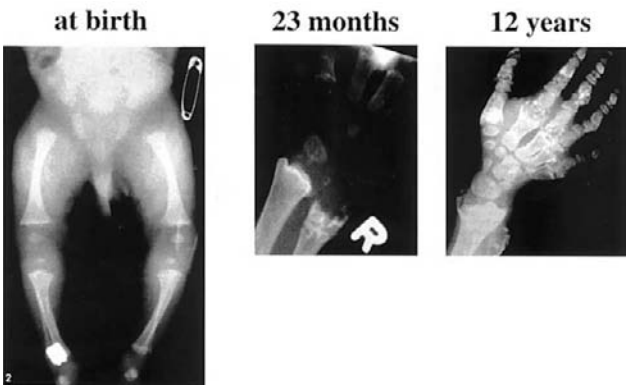

Figure 4 A patient with Jansen's disease and his radiological findings at different ages. (A) Patients with Jansen's metaphyseal chondrodysplasia (from Jansen, 1934, De Haas *et al.,* 1969; Silverthorn *et al.,* 1983); photograph of the latter patient kindly provided by Drs. Kooh and Cole, Hospital for Sick Children, Toronto, Canada). (B) X-rays from a patients with Jansen's disease at birth, 23 months, and 12 years of age. From Silverthorn *et al.* (1983), with permission.

In addition, sclerosis and thickening of the base of the skull and of the calvaria is noted in most cases. The former changes are thought to be the cause of cranial auditory and optical nerve compression, which has been observed later in life in some affected individuals. Loss of the normal cortical outline, areas of subperiosteal bone resorption, and generalized osteopenia are reminiscent of the changes seen in hyperparathyroidism. Furthermore, there is an increased in trabecular bone volume and a thinning of cortical bone (Parfott *et al.,* 1996). The two only reports that investigated the histological changes in the growth plates, described a severe delay in endochondral ossification of the metaphyses, including a lack of the regular columnar arrangement of the maturing cartilage cells, a lack of excess osteoid (which is usually indicative of active rickets or osteomalacia), little or no vascularization of cartilage, and no evidence for osteitis fibrosa (Cameron *et al.,* 1954; Jaffe, 1972).

One female patient with JMC was reported to be unable to breast-feed and to have, similar to her affected daughter, a dry and scaly skin (Schipani *et al.,* 1995). As discussed above, PTHrP and the PTH/PTHrP receptor are expressed in breast and skin, and this ligand/receptor system appears to have an important role in these two tissues. Tooth development and enamel formation appear normal in patients with JMC. Intelligence appears to be normal in all reported cases.

Most laboratory findings in JMC are reminiscent of those observed in patients with primary hyperparathyroidism or with the syndrome of humoral hypercalcemia of malignancy. In the newborn, blood phosphorus levels are typically at the lower end of the normal range, while alkaline phosphatase activity is almost invariably elevated. Hypercalcemia is usually absent at birth, but develops during the first months of life, and persists throughout life, but is more pronounced during infancy and childhood. Hypercalciuria is usually present and can be associated with an increased incidence of nephrocalcinosis (Kessel *et al.,* 1992). $1,25(OH)_2D_3$ levels have been reported to be normal or at the upper end of the normal range. Serum alkaline phosphatase activity and osteocalcin concentration are elevated throughout life, indicating that osteoblast activity is increased; compatible with an increased osteoclastic activity, urinary hydroxyproline excretion is elevated (Schipani *et al.,* 1999; Kruse and Schütz, 1993).

Jansen's Disease Is Caused by Activating PTH/PTHrP Receptor Mutations

Because of the findings in the various genetically manipulated mice described above, and because of the abundant expression of the PTH/PTHrP receptor in the three organs that are most obviously affected in JMC; i.e., kidney, bone, and metaphyseal growth plates, activating receptor mutations were considered as a cause of this rare disease. Indeed, in

several unrelated patients with this disorder, a heterozygous nucleotide exchange, which changes a histidine at position 223 to arginine, was identified in exon M2 of the PTH/PTHrP receptor gene (Schipani *et al.,* 1995; 1996, 1999; Minagawa *et al.,* 1997). In other patients, two additional heterozygous nucleotide exchanges were identified which change either a threonine at position 410 to proline (exon M5), or isoleucine at position 458 to arginine (exon M7) (Schipani *et al.,* 1996, 1999) (Fig. 5). The three mutated residues are predicted to be located at or close to the intracellular surface of the cell membrane and are strictly conserved in all mammalian members of this receptor family (Jüppner, 1994; Mannstadt *et al.,* 1999), suggesting an important functional role for these three residues. With the exception of one family where a mother-to-daughter transmission of the H223R mutation was documented (Schipani *et al.,* 1996), each of the three mutations was excluded in the healthy parents and siblings, and in genomic DNA from a significant number of unrelated healthy individuals. This suggests that JMC is usually caused by *de novo* mutations. To date, the T410P and the 1458R mutation was found in one patient each, while the H223R mutation was identified in eight patients, and is thus the most frequent PTH/PTHrP receptor mutation in JMC.

To test *in vitro* the functional consequences of the identified missense mutations in JMC, each of the three different nucleotide exchanges was introduced into the cDNA encoding the wild-type human PTH/PTHrP receptor (Schipani *et al.,* 1995, 1996, 1999; Minagawa *et al.,* 1997). COS-7 cells transiently expressing PTH/PTHrP receptors with either the H223R, the T410P, or the 1458R mutation showed significantly higher basal accumulation of cAMP, then cells expressing the wild-type PTH/PTHrP receptor (see Fig. 5). Cells expressing PTH/PTHrP receptors with either of the three point mutations showed no evidence for increased basal accumulation of IP3, indicating that this signaling pathway is not constitutively activated (Schipani *et al.,* 1995, 1996, 1999). Interestingly, the D578H mutation in the luteinizing hormone receptor, which is at a position equivalent to the T410P mutation in the PTH/PTHrP receptor, led to constitutive activity of both signaling pathways, cAMP and IP3 (Liu *et al.,* 1999). It is therefore plausible that the lack of constitutive IP3 generation by three activating mutations in the PTH/PTHrP receptor could be related to insufficient sensitivity of the methods that were used to explore this second messenger system.

When challenged with increasing concentrations of either PTH or PTHrP, cells expressing the mutant H223R and T410P receptors showed, in comparison to cells expressing the wild-type PTH/PTHrP receptor, reduced maximal cAMP accumulation. In contrast, cells expressing the 1458R mutant showed the same maximal cAMP accumulation as cells transfected with the wild-type receptor (Schipani *et al.,* 1999). Agonist-dependent IP accumulation was observed with COS-7 cells expressing the I458R and the T410P mutant, but not with cells expressing the H223R mutant. Despite the differences in the *in vitro* response to PTH or PTHrP, patients with either of the three PTH/PTHrP receptor mutations showed no obvious differences in their clinical and/or biochemical presentation.

Activating mutations in other G protein-coupled receptors have been implicated in several other human diseases, but none of these involve members of the class B receptor family. These disorders include rare forms of retinitis pigmentosa or congenital stationary blindness (activating mutations in rhodopsin) (Robinson *et al.,* 1992; Dryja *et al.,* 1993), thyroid adenomas or non-autoimmune hyperthyroidism (activating TSH receptor mutations) (Parma *et al.,* 1993; Duprez *et al.,* 1994; Paschke *et al.,* 1994; Kopp *et al.,* 1995; Tonacchera *et al.,* 1996; Grüters *et al.,* 1998; Khoo *et al.,* 1999; Nogueira *et al.,* 1999; Russo *et al.,* 1999; Trultzsch *et al.,* 1999), gonadotropin-independent male precocious puberty (Shenker *et al.,* 1993; Latronico *et al.,* 1995; Kraalj *et al.,* 1995;

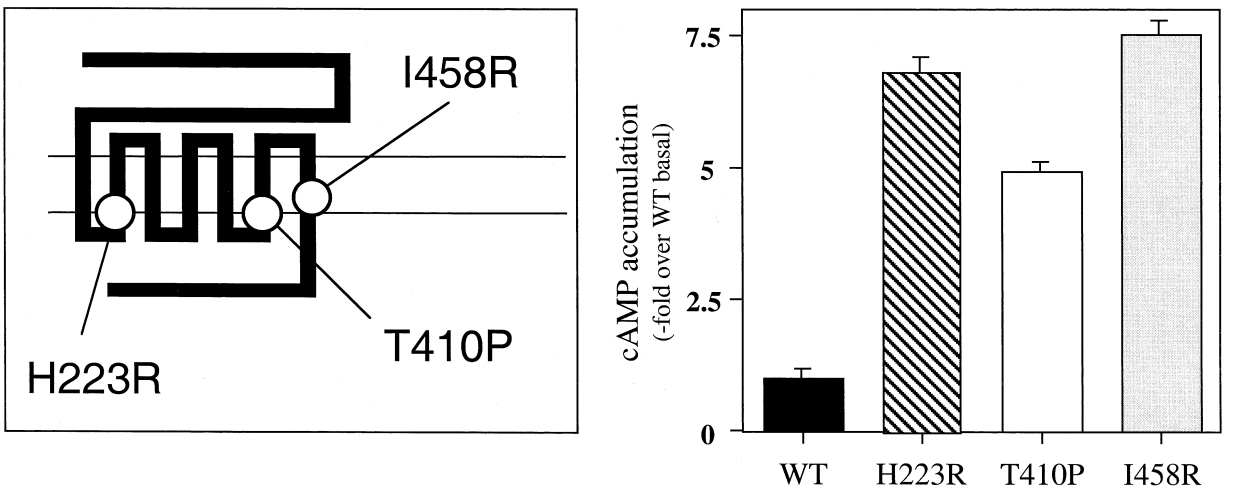

Figure 5 Schematic representation of the PTH/PTHrP receptor and basal, agonist-indepenent cAMP accumulation of wild-type and mutant receptors. The approximate location of the three different missense mutations that were identified in patients with Jansen's disease are indicated (left). Basal cAMP accumulation of COS-7 cells expressing wild-type ■ and mutant PTH/PTHrP receptors (H223R, ▨; T410P, ▭; 1458R, ▭ (right). Modified from Schipani *et al.* (1996, 1999).

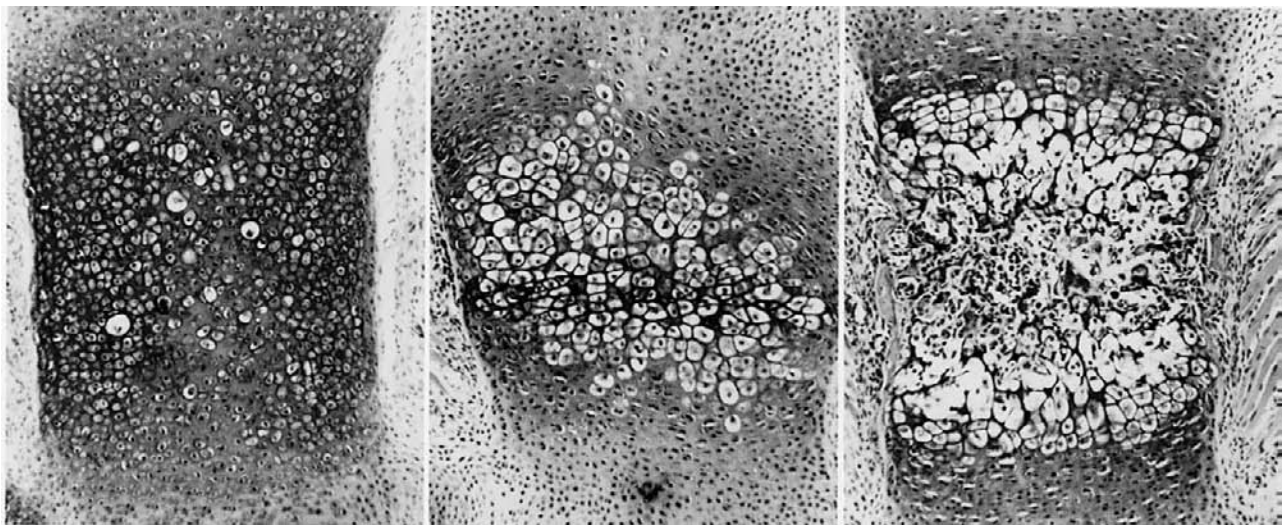

Figure 6 Histological sections, stained with hematoxylin and eosin, of decalcified sternum from a newborn wild-type mouse (left) and transgenic littermates that were heterozygous (middle) or homozygous (right) for expression of a constitutively active PTH/PTHrP receptor under the control of the type II collagen promoter. From Schipani *et al.* (1997). (See also color plate.)

Shenker, 1998), and Leydig-cell tumors (activating mutations in the luteinizing hormone receptor) (Liu *et al.,* 1999), and autosomal dominant forms of familial hypocalcemia (activating calcium-sensing receptor mutations) (Pollak *et al.,* 1994; Pearce, 1995; Pearce and Brown, 1996; Baron *et al.,* 1996; Rearce *et al.,* 1997; Brown *et al.,* 1998; Watanabe *et al.,* 1998; Okazaki *et al.,* 1999). Cell membrane receptors exhibiting constitutive signaling have also been described in the pathogenesis of Kaposi's sarcoma and primary effusion lymphomas (constitutive signaling of the Kaposi's sarcoma herpesvirus-G protein-coupled receptor (KSHV-GPCR) via activation of phosphoinositide-specific phospholipase) (J. A. Burger *et al.,* 1999; M. Burger *et al.,* 1999).

To prove that the growth plate abnormalities in Jansen's disease are indeed caused by constitutively active PTH/PTHrP receptors, transgenic mice were generated that express the H223R mutant under the control of the rat α1(II) collagen promoter, thereby targeting receptor expression to proliferating chondrocytes (Schipani *et al.,* 1997). Two transgenic mouse lines were established, both of which showed delayed mineralization and decelerated differentiation of proliferative chondrocytes into hypertrophic chondrocytes, a delay in vascular invasion and a prolonged presence of hypertrophic chondrocytes (Fig. 6, see also color plate). In one of these mouse lines, the defect in endochondral bone formation was apparent only at the microscopic level, while the second line showed shortened and deformed limbs that are reminiscent of the findings in patients with Jansen's disease. Based on these results in transgenic mice it appears likely that the growth abnormalities in JMC are caused by the expression of mutant, constitutively active PTH/PTHrP receptor in growth plate chondrocytes.

In an attempt to better understand how the PTH/PTHrP receptor can modulate bone development and turnover, transgenic mice were recently generated in which the human PTH/PTHrP receptor with the H223R mutation is expressed under the control of the type I collagen promoter. Constitutive activity of this receptor was thus targeted to mature osteoblasts and osteoblast precursors. When compared to control littermates, long bones of transgenic mice were reduced in length. The histological analysis of these bones revealed findings that are reminiscent of the skeletal abnormalities in patients with hyperparathyroidism and with Jansen's disease (Parfitt *et al.,* 1996), i.e., a thinner and more porous cortex in the diaphysis, an increased trabeculation of the metaphysis, and a reduction in bone marrow space (Fig. 7, see also color plate). Besides increased osteoblast function in trabecular bone and at the endosteal surface of cortical bone, osteoblastic activity in the periosteum was inhibited. Furthermore, mature osteoblasts as well as a heterogeneous population of preosteoblasts were increased in the trabecular compartment by a mechanism of increased proliferation and decreased apoptosis, and expression of the constitutively active PTH/PTHrP receptor in osteoblasts resulted in a dramatic increase in osteoclast number. The net effect of these actions was a substantial increase in trabecular bone volume and a decrease in cortical bone mass. These studies identified the PTH/PTHrP receptor as an important mediator of both bone forming and bone resorbing actions of PTH, and point out the complexity and heterogeneity of the osteoblast population and/or their regulatory microenvironment (Calvi *et al.,* 2001).

Blomstrand's Lethal Chondrodysplasia

Blomstrand's lethal chondrodysplasia is a recessive human disorder characterized by early lethality, advanced bone maturation and accelerated chondrocyte differentiation, and most likely severe abnormalities in mineral ion homeostasis. The first patient was described by Blomstrand and

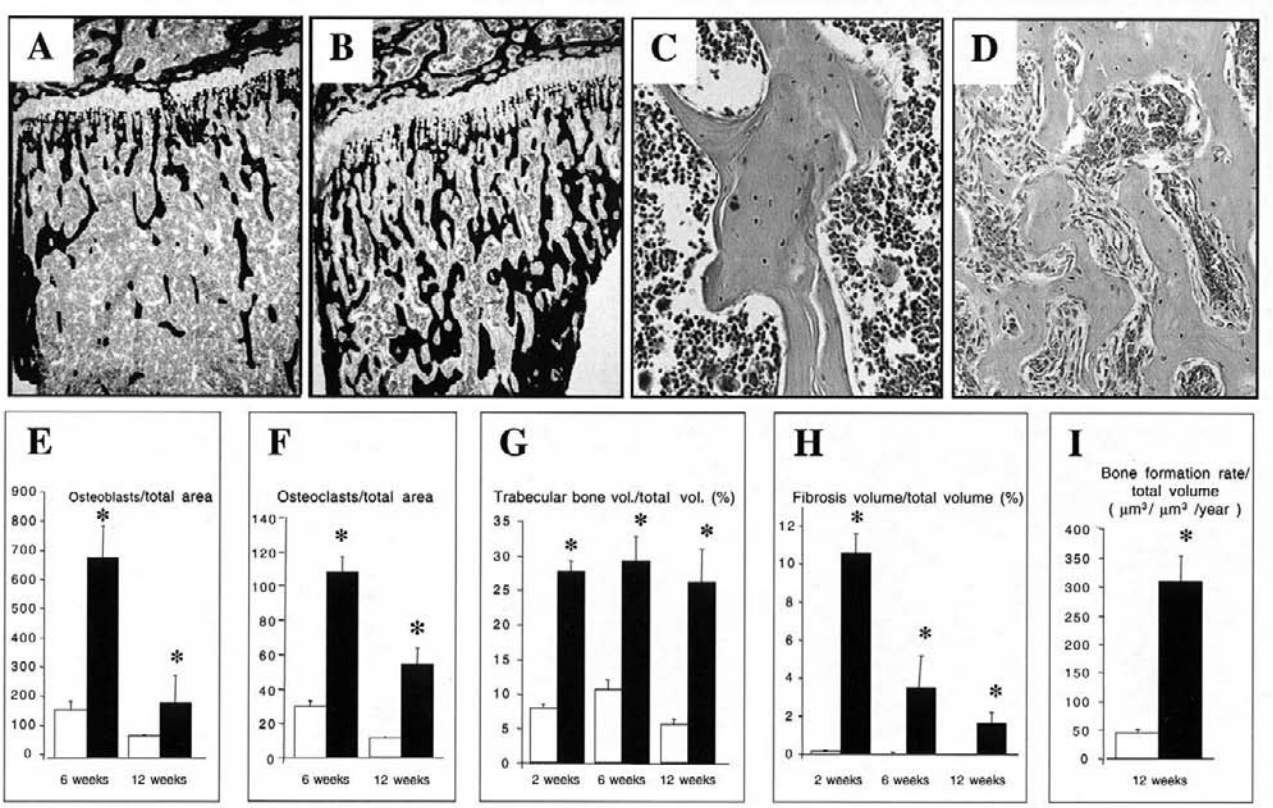

Figure 7 Histology and histomorphometric analysis of trabecular bone from wild-type and CL2 transgenic mice. Histologic sections of tibiae, stained by the method of von Kossa, from 12-week-old wild-type mice (A) and CL2 transgenic littermates (B). High-power light microscopy of decalcified sections, stained with hematoxylin and eosin, of the metaphyseal area from the proximal tibia of 12-week-old wild-type (C) and CL2 transgenic littermates (D). Histomorphometric analysis performed in wild-type (white bars) and CL1 transgenic littermates (black bars) (E–I). Ages of animals are indicated on the *x*-axis; asterisks indicate a statistically significant difference between two groups of mice ($P < 0.05$); error bars represent the SEM. Modified from Calvi *et al.* (2001). (See also color plate.)

colleagues in 1985; descriptions of several other patients followed (Young *et al.,* 1993; Leroy *et al.,* 1996; Loshkajian *et al.,* 1997; den Hollander *et al.,* 1997; Oostra *et al.,* 1998, 2000; Galera *et al.,* 1999; Karperien *et al.,* 1999). The disorder was shown to occur in families of different ethnic backgrounds and appears to affect males and females equally. Most affected infants are born to consanguineous parents [only in one instance were unrelated parents reported to have two offspring that are both affected by Blomstrand's disease (Loshkajian *et al.,* 1997)], suggesting that BLC is an autosomal recessive disease. Infants with BLC are typically born prematurely and die shortly after birth. Birth weight, when corrected for gestational age, appears to be normal, but may be overestimated because most infants are hydroptic; also the placenta can be immature and edematous. Nasal, mandibular, and facial bones are hypoplastic; the base of the skull is short and narrow; the ears are low set; the thoracic cage is hypoplastic and narrow with short thick ribs and hypoplastic vertebrae. In contrast, the clavicles are relatively long and often abnormally shaped, the limbs are extremely short, and only the hands and feet are of relatively normal size and shape. Internal organs show no apparent structural or histological anomalies, but preductal aortic coarctation was observed in most published cases. The lungs are hypoplastic and the protruding eyes typically show cataracts. Defects in mammary gland and tooth development, previously overlooked, were demonstrated in two recently studied fetuses with BLC. In these fetuses, nipples were absent, and no subcutaneous ductal tissue could be identified by histochemical analysis. Tooth buds were present, but developping teeth were severely impacted within the surrounding alveolar bone, leading to distortions in their architecture and orientation (Wysolmerski *et al.,* 2001).

Radiological studies of patients with BLC reveal pronounced hyperdensity of the entire skeleton and markedly advanced ossification (Fig. 8). As mentioned above, the long bones are extremely short and poorly modeled, show markedly increased density, and lack metaphyseal growth plates. Endochondral bone formation is dramatically advanced and is associated with a major reduction in epiphyseal resting cartilage, preventing the development of epiphyseal ossification centers (Fig. 9, see also color plate). The zones of chondrocyte proliferation and of column formation are lacking, and the zone that normally comprises the layer of hypertrophic chondrocytes is poorly defined, narrow, and irregular (Oostra *et al.,* 2000). Cortical bone is thickened and

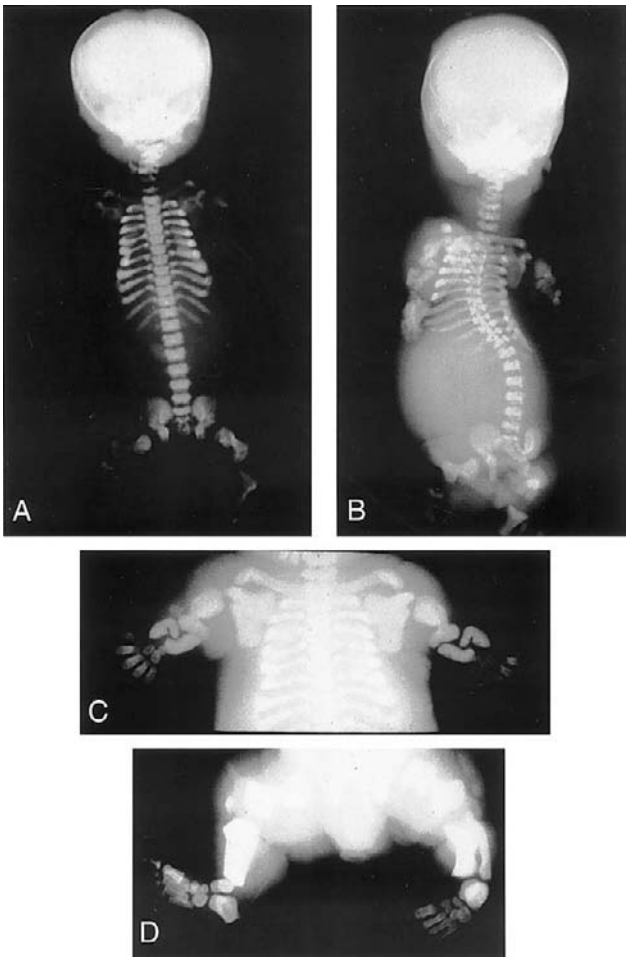

Figure 8 Radiological findings in two fetuses with Blomstrand's lethal chondrodysplasia (BLC). Anterio-posterior (A) and lateral (B) views of a male fetus at 26 weeks of gestation; upper (C) and lower (D) limbs of a female fetus with BLC at 33 weeks of gestation. Particularly striking is the dramatic acceleration of endochondral bone formation of all skeletal elements. No secondary ossification centers of ossification are seen in the long bones. The limbs are coarsely shaped and extremely short, while carpal and tarsal bones have a comparatively normal shape and size. Note also that the clavicles are relatively long, but show abnormal bending. From Loshkajian *et al.* (1997), with permission.

bone trabeculae are coarse with reduced diaphyseal marrow spaces. Capillary ingrowth, bone resorption, and bone formation are reported by some authors as being unaltered (Leroy *et al.,* 1996), while others describe these bone remodeling events as deficient (Loshkajian *et al.,* 1997).

Blomstrand's Disease Is Caused by Inactivating PTH/PTHrP Receptor Mutations

Recently, four different defects in the PTH/PTHrP receptor gene were described in genomic DNA from patients affected by BLC (Fig. 10). The first reported case, a product of non-consanguineous parents, was shown to have two distinct abnormalities in the PTH/PTHrP receptor gene (Jobert *et al.,* 1998). Through a nucleotide exchange in exon M5 of the maternal PTH/PTHrP receptor allele, a novel splice acceptor site was introduced which led to a mutant mRNA encoding an abnormal receptor that lacks a portion of the fifth membrane-spanning domain (amino acids 373 to 383 (Δ373–383)). This receptor mutant fails, despite seemingly normal cell surface expression, to respond to PTH or PTHrP with an accumulation of cAMP (Fig. 11) and inositol phosphate (data not shown). For yet unknown reasons, the paternal PTH/PTHrP receptor allele from this patient is very poorly expressed, suggesting an unidentified mutation in one of the different promoter regions or in a putative enhancer element.

A second patient with BLC, the product of a consanguineous marriage, was shown to have a nucleotide exchange that leads to a proline-to-leucine mutation at position 132 (P132L) (Zhang *et al.,* 1998; Karaplis *et al.,* 1998). This residue in the amino-terminal, extracellular domain of the PTH/PTHrP receptor is invariant in all mammalian members of this family of G protein-coupled receptors, indicating that the identified mutation is likely to have significant functional consequences. Indeed, COS-7 cells expressing this mutant PTH/PTHrP receptor showed, despite apparently normal cell surface expression, dramatically

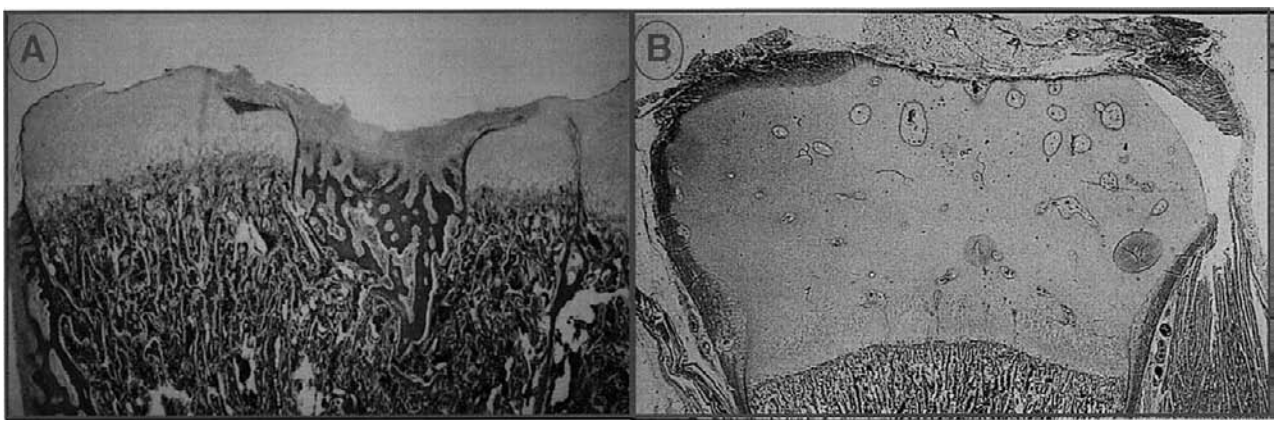

Figure 9 Section of the upper tibia end from a patient with BLC (A) and an age-matched control. Note the severely reduced size of the growth plate, the irregular boundary between the growth plate and the primary spongiosa, and the increased cortical bone thickness. From Loshkajian *et al.* (1997), with permission, and Anne-Lise Delezoide, personal collection. (See also color plate.)

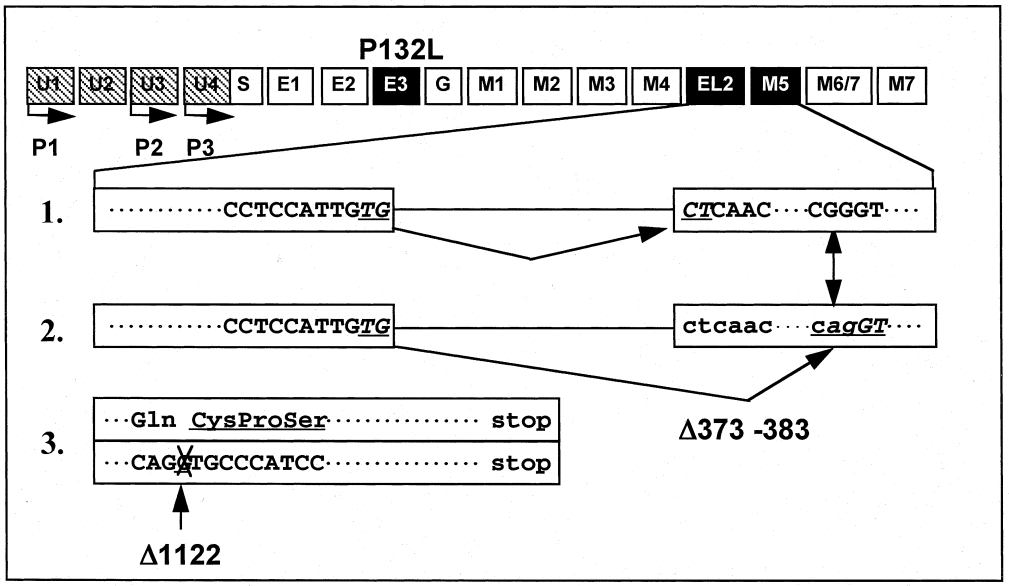

Figure 10 Schematic representation of the PTH/PTHrP receptor gene and location of the nucleotide changes identified in patients with Blomstrand's disease. Untranslated and translated exons gene (top) are indicated by stripped and open boxes respectively, and introns are indicated by solid lines. The mRNAs derived from the P1 or the P2 promoter are indicated by the solid and dotted lines, respectively, below the untranslated exons; the transcript derived from the P3 promoter starts within exon U4/S. The homozygous P132L mutation identified in one patient with Blomstrand's disease is indicated above exon M3 (Zhang *et al.,* 1998; Karaplis *et al.,* 1998). Three partial nucleotide sequences are shown (bottom): (1) wild-type exon EL2 and exon M5 (normal splice-donor and splice-acceptor sites at the junction between exon EL2/intron N11 and intron N11/exon M5, respectively, are underlined); (2) the mutation in exon M5 (nucleotide 1176 of the human cDNA is shown in bold) introduces a novel splice-acceptor site in the maternal allele which leads to the deletion of amino acid residues 373–383 of the human PTH/PTHrP receptor (Jobert et al., 1998); (3) the nucleotide deletion in exon EL2 (nucleotide 1122 of the human cDNA) was identified in another patient with Blomstrand's chondrodysplasia; the "arrow" indicates the amino acid (glutamine at position 364 of the human PTH/PTHrP receptor) after which the protein sequence is altered due to a shift in reading frame (Karperien *et al.,* 1999).

impaired binding of radiolabeled PTH and PTHrP analogs, greatly reduced agonist-stimulated cAMP accumulation (see Fig. 11), and showed no measurable inositol phosphate response. It is important to note, however, that cells expressing the P132L mutant receptor showed some agonist-induced second messenger response and showed little, but detectable, specific binding of radiolabeled PTHrP.

A homozygous deletion of G at position 1122 (exon EL2) was identified in a third case of BLC (Karperien *et al.,* 1999). This mutation led to a shift in the open reading frame, which

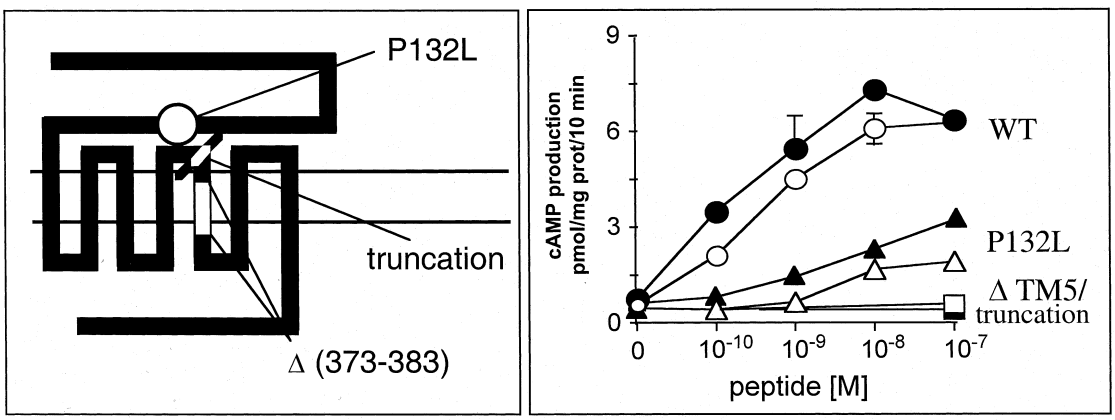

Figure 11 Schematic representation of the PTH/PTHrP receptor and functional evaluation of the wild-type and mutant receptors in COS-7 cells. Approximate location of loss-of-function mutation identified in patients with Blomstrand's disease (left) and cAMP accumulation in response to PTH (closed symbols) or PTHrP (open symbols) by wild-type and mutant PTH/PTHrP receptors. Modified from Karperien *et al.* (1999) and Zhang *et al.* (1998).

resulted in a truncated protein that completely diverged from the wild-type receptor sequence after amino acid 364, and thus lacked transmembrane domains 5, 6, and 7, the connecting intra- and extracellular loops, and the cytoplasmic tail (Δ365–593). Functional analysis of the D365–593 recombinant mutant receptor in COS-7 cells demonstrated a total absence of PTH-stimulated accumulation of intracellular cAMP, which was confirmed in studies performed with the patient's dermal fibroblasts (see Fig. 11).

As for the other cases of BLC, these findings provided a plausible explanation for the severe abnormalities in endochondral bone formation. The abnormalities in mammary gland and tooth development furthermore support the conclusion that the PTH/PTHrP receptor has in humans and mice identical roles in the development of these organs. Compatible with the role of PTH/PTHrP receptor and PTHrP in organogenesis, both were demonstrated to be expressed in the developing breast and tooth of human control fetuses (Wysolmerski *et al.,* 2001). It is also worth noting that abnormalities in skeletal development in the fetuses carrying the P132L mutation, which inactivates the PTH/PTHrP receptor incompletely, are less severe than those observed in most cases, particularly with regard to the bones of the lower limbs (Young *et al.,* 1993; Karperien *et al.,* 1999; Oostra *et al.,* 2000). This led to the proposal that two forms of BLC can be distinguished clinically and on the basis of the *in vitro* characteristics of the mutant PTH/PTHrP receptors (Oostra *et al.,* 2000). Taken together the findings in patients with BLC suggested that this rare human disease is the equivalent of the mouse PTH/PTHrP receptor "knockout" (Lanske *et al.,* 1996).

Inactivating mutations have been described in several other G protein-coupled receptors (reviewed in Spiegel, 1998, 1999). For example, genetic forms of growth hormone deficiency were shown to be caused by mutations in the growth hormone-releasing hormone receptor (Wajnrajch *et al.,* 1996; Godfrey *et al.,* 1993), mutations in the thyrotropin receptor are the cause of inherited hypothyroidism (Stein *et al.,* 1994; Gu *et al.,* 1995; Biebermann *et al.,* 1998), and mutations in the calcium-sensing receptor have been associated with familial hypocalciuric hypercalcemia and neonatal severe primary hyperparathyroidism (reviewed in Brown, 1999).

Conclusions

The findings in PTHrP- and PTH/PTHrP receptor-ablated mice, and in transgenic animals overexpressing PTHrP in the growth plate, predicted that human disorders caused by mutations in either of these two proteins would be associated with severe abnormalities in endochondral bone formation and in the regulation of mineral ion homeostasis. These insights from genetically manipulated animals led to the identification of activating and inactivating PTH/PTHrP receptor mutations in two rare genetic disorders, Jansen's and Blomstrand's diseases, respectively. In addition to resolving the pathogenesis of puzzling human disorders, these naturally occurring PTH/PTHrP receptor mutations have provided important new insights into the importance of this G protein-coupled receptor in mammalian development. The availability of mutant, constitutively active PTH/PTHrP receptors has furthermore provided novel tools for studying bone and cartilage development independent of PTH and PTHrP.

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CHAPTER 64

Pseudohypoparathyroidism

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Overview

The term *pseudohypoparathyroidism* (PHP) refers to a group of metabolic syndromes in which biochemical hypoparathyroidism (i.e., hypocalcemia and hyperphosphatemia) is due not to deficiency of parathyroid hormone (PTH) but rather to unresponsiveness of target tissues to the biological actions of PTH. Plasma levels of PTH are increased in patients with PHP, a feature that distinguishes these patients from those with true hypoparathyroidism, in whom parathyroid insufficiency is associated with low plasma levels of PTH. Thus PHP differs fundamentally from true hypoparathyroidism.

In their original report of PHP, Fuller Albright and his associates described the failure of patients with this syndrome to show a phosphaturic response to injected parathyroid extract (Albright *et al.,* 1942). These observations led to the speculation that biochemical hypoparathyroidism in PHP was due to an inability of the target organs, bone and kidney, to respond to PTH.

PTH Signal Transduction

PTH regulates mineral metabolism and skeletal homeostasis by modulating activity of specialized target cells that are present in bone and kidney. PTH action first requires binding of the hormone to specific receptors that are expressed on the plasma membrane of target cells. The classical PTH receptor is a ~75-kDa glycoprotein that is often referred to as the PTH/PTHrP or type 1 PTH receptor (PTH1R). Molecular cloning of cDNA's encoding PTH receptors from several species (Schipani *et al.,* 1993; Abou Samra *et al.,* 1992; Juppner *et al.,* 1991; Adams *et al.,* 1995) has indicated that the PTH1R expressed on bone and that expressed in kidney cells are identical. The PTH1R binds both PTH and parathyroid hormone-related protein (PTHrP), a factor made by diverse tumors that cause humorally mediated hypercalcemia, with equivalent affinity, which accounts for the similar activities of both hormones. In addition to the classical PTH receptor (PTH1R), two other receptor proteins, termed the type 2 and type 3 PTH receptors, with unique characteristics, have been identified. The human type 2 PTH receptor (PTH2R) has 52% amino-acid sequence identity to the human PTH1R, but is not expressed in conventional PTH target tissues (i.e., bone and kidney). The PTH2R interacts with PTH but not PTHrP (Behar *et al.,* 1996;Usdin *et al.,* 1995) and is most highly expressed in regions of the brain that lack PTH mRNA (Usdin *et al.,* 1995). The recently identified hypothalamic peptide, tuberoinfundibular peptide, of 39 amino acids (TIP39), binds the PTH2R with subnanomolar affinity (0.59 nM) and strongly activates adenylyl cyclase, suggesting that TIP39 may represent the natural ligand for this receptor. Although TIP39 binds binds to the PTH1R with moderate affinity (59 n*M*), it produces little or no stimulation of cAMP accumulation (Clark *et al.,* 1998; Hoare *et al.,* 2000a). The type 3 receptor (PTH3R) can interact with both PTH and PTHrP, and activates adenylyl cyclase but not phospholipase C (Hoare *et al.,* 2000b; Rubin and Juppner, 1999). However, as PTH does not appear to exist in fish, it is likely that a PTHrP-like peptide is the endogenous ligand for the PTH3R. All three PTH receptors are members of a large family of receptors that bind hormones, neurotransmitters, cytokines, light photons, taste, and odor molecules. These receptors consist of a single polypeptide chain that is predicted by hydrophobicity plots to span the plasma membrane with seven alpha helices (i.e., heptahelical), forming three extracellular and three or four intracellular loops and a cytoplasmic carboxyl-terminal tail. The heptahelical receptors are coupled

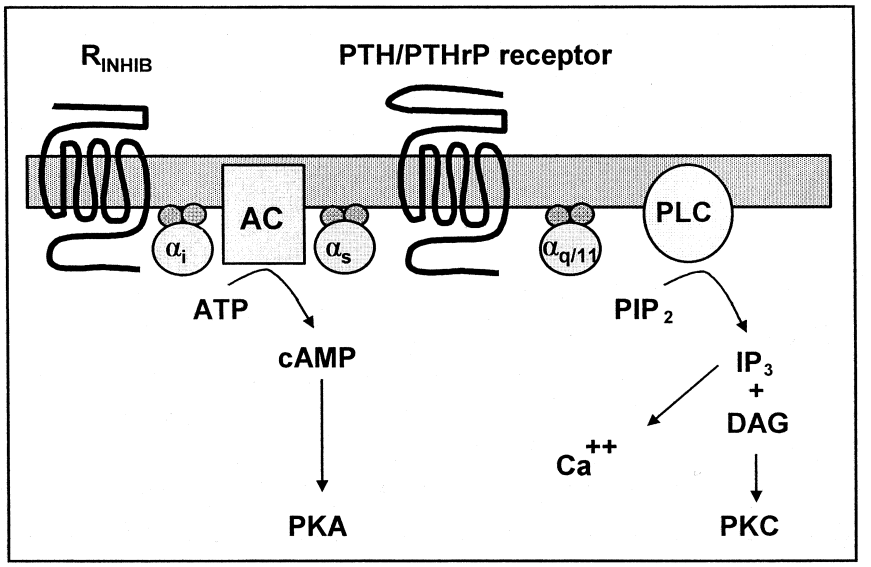

Figure 1 Cell surface receptors for PTH are coupled to two classes of G proteins. G_s mediates stimulation of adenylyl cyclase (AC) and the production of cAMP, which in turn activates protein kinase A (PKA). G_q stimulates phospholipase C (PLC) to form the second messengers inositol-(1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG) from membrane-bound phosphatidylinositol-(4,5)-bisphosphate. IP_3 increases intracellular calcium (Ca^{2+}) and DAG stimulates protein kinase C (PKC) activity. Each G protein consists of a unique a chain and a $\beta\gamma$ dimer.

by heterotrimeric ($\alpha\beta\gamma$) G proteins (Neer, 1995) to signal effector molecules localized to the inner surface of the plasma membrane (Fig. 1).

The heterotrimeric G proteins share a common structure consisting of an α subunit and a tightly coupled $\beta\gamma$ dimer. The α subunit interacts with detector and effector molecules, binds GTP, and possesses intrinsic GTPase activity (Bohm *et al.,* 1997). Mammals have over 20 different G protein α chains encoded by 16 genes; additional protein diversity results from the generation of alternatively spliced mRNAs. The mammalian α subunits are between 40 and 90% identical in amino acid sequence, and their overall length varies between 350 and 395 residues. The 16 α-subunits can be divided into four classes based both on their degree of primary sequence homology and on functional similarities (G_s, G_i, G_q, G_{12}). Within the past several years the crystal structures of various G protein α subunits bound to GDP, GTPγS, or aluminum fluoride and of G protein heterotrimers have been defined. G protein α subunits are composed of a GTPase domain, which is very similar to the structure of the smaller ras-like proteins and other members of the GTPase superfamily, and a variable helical domain, which is less conserved and present only in the α subunits of heterotrimeric G proteins. The GTPase domain contains the conserved guanine nucleotide-binding site and the residues necessary for effector activation. Guanine nucleotides sit within a cleft between the two domains. The helical residues appears to be important for maintaining guanine nucleotide in the binding pocket, as disruption of interactions between the two domains leads to an increased rate of GDP release in the basal state.

The α subunits associate with a smaller group of β (at least 5) and γ (more than 11) subunits (Clapham and Neer, 1997). The β and γ subunits combine tightly with one another (Schmidt and Neer, 1991; Schmidt *et al.,* 1992) and the resultant $\beta\gamma$ dimers demonstrate specific associations with different α subunits (Rahmatullah and Robishaw, 1994; Rahmatullah *et al.,* 1995). Combinatorial specificity in the associations between various G protein subunits provides the potential for enormous diversity and may allow distinct heterotrimers to interact selectively with only a limited number of the more than 1000 G protein-coupled receptors (Taussig and Zimmermann, 1998; Wess, 1998). At present it is unknown whether specific G protein subunit associations occur randomly or if there are regulated mechanisms that determine the subunit composition of heterotrimers.

The activation state of the heterotrimeric G protein is regulated by a mechanism in which the binding and hydrolysis of GTP acts a molecular timing switch (Fig. 2). In the basal (inactive) state, G proteins exist in the heterotrimeric form with GDP bound to the α chain. The association of α with $\beta\gamma$ occludes the sites of interaction of both of these molecules with downstream effector molecules, and the inactive state is maintained by an extremely slow rate of dissociation of GDP from the α chain ($K \approx 0.01$/min). The interaction of a ligand-bound receptor with a G protein facilitates the release of tightly bound GDP and the subsequent binding of cytosolic GTP. The binding of GTP to the α chain induces conformational changes that facilitate the dissociation of the α-GTP chain from the $\beta\gamma$ dimer and the receptor. The free α-GTP chain assumes an active conformation in which a new surface is formed that enables the α chain to interact with target

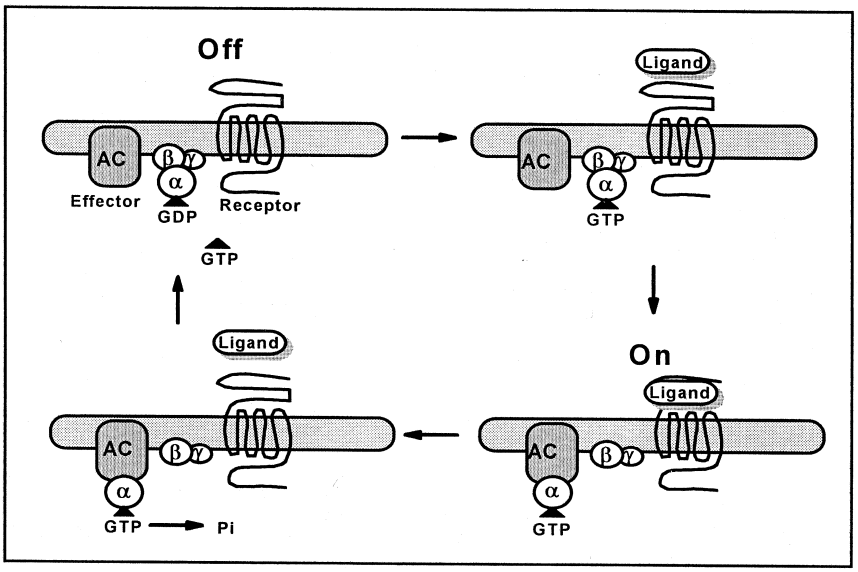

Figure 2 The cycle of hormone-dependent GTP binding and hydrolysis that regulates heterotrimeric G protein signal transduction. In the nonstimulated, basal (Off) state, GDP is tightly bound to the α chain of the heterotrimeric G protein. Binding of an agonist (Ligand) to its receptor (depicted with seven transmembrane spanning domains) induces a conformational change in the receptor and enables it to activate the G protein. The G protein now releases GDP and binds GTP present in the cytosol. The binding of GTP to the α chain leads to dissociation of the α-GTP from the $\beta\gamma$ dimer, and each of these molecules is now free to regulate downstream effector proteins. The hydrolysis of GTP to GDP by the intrinsic GTPase of the α chain promotes reassociation of α-GDP with $\beta\gamma$ and the inactive state is restored. The heterotrimeric G protein is ready for another cycle of hormone-induced activation.

enzymes and ion channels with 20- to 100-fold higher affinity than in the GDP bound state. Early studies had suggested that only the free α-GTP chain could regulate downstream signaling and that the $\beta\gamma$ dimers served only the passive role of localizing the α chain to the plasma membrane. However, more recent studies have shown that $\beta\gamma$ dimers also participate in the downstream signaling events through interaction with an ever-widening array of targets (Gautam *et al.,* 1998; Clapham and Neer, 1993); for example, $\beta\gamma$ dimers can influence activity of certain forms of adenylyl cyclase and phospholipase C, open potassium channels, participate in receptor desensitization, mediate mitogen-activated protein (MAP) kinase phosphorylation, and modulate leukocyte chemotaxis.

G protein signaling is terminated by the hydrolysis of α-GTP to α-GDP by a slow ($k_{cat} \approx 4$/min) GTPase that is characteristic of the α chain. This process promotes dissociation of the α chain from effector molecules and promotes reassociation with G protein $\beta\gamma$ dimers, thus preventing further interaction of these subunits with downstream effectors. The GTPase reaction is a high-energy transition state that requires association of the γ-phosphorous atom of GTP with the oxygen of a water molecule. Arginine^{-201} and glutamine^{-227} in Gα_s function as "fingers" to position the γ-phosphate of GTP. With hydrolysis of GTP to GDP, the α-GDP chain reassociates with the $\beta\gamma$ dimer and the beterotrimeric G protein is capable of participating in another cycle of receptor-activated signaling. Thus, the GTPase of the Gα chain provides a molecular "timing switch" that controls the duration, and thereby the intensity, of the signaling event. The intrinsic rates of GTP hydrolysis by G protein α chains differ widely, and interactions that influence the rate of the GTPase reaction can have profound consequences. Several factors can act as "GTPase-activating proteins" or GAPs (Ross *et al.,* 1998) to accelerate the slow intrinsic rate of GTP hydrolysis by Gα proteins. For example, RGS proteins (for "regulators of G protein signaling") act as GAPs for G_i, G_o G_z, and G_q by stabilizing the transition state of the GTPase reaction, thereby lowering the activation energy for this reaction. RGS proteins can stimulate a 40-fold increase in the catalytic rate of GTP hydrolysis of these α chains, and thus can markedly accelerate the termination of G protein signaling (see recent reviews (Siderovski *et al.,* 1999; Hepler, 1999) (Fig. 2)

Upon binding of either PTH or PTHrP, the PTH1R receptor activates G_s, leading to stimulation of adenylyl cyclase and generation of cAMP, and at about 10-fold higher concentration of ligand, the receptor also activates Gq, leading to stimulation of phospholipase C and generation of inositol-(1,4,5)-trisphosphate (IP3) and diacylglycerol (DAG) (Fig. 1). Thus, the PTH1R, as well as the calcitonin (Chabre *et al.,* 1992) and many other related receptors, can couple to both G_s and $G_{q/11}$. Each of these receptors has been shown to stimulate cAMP formation and phosphoinositide breakdown, the latter with an EC_{50} one order of magnitude higher than for adenylyl cyclase stimulation. One study examining the labeling of specific G proteins by GTP-γ-azidoanilide in response to PTH1R receptor stimulation

directly demonstrated coupling of PTH1R to G_s and G_q and minimal coupling to Gi_1 (Schwindinger *et al.,* 1998a). The best-characterized mediator of PTH action is cAMP, which rapidly activates protein kinase A (Bringhurst *et al.,* 1989). The relevant target proteins that are phosphorylated by protein kinase A and the precise mode(s) of action of these proteins remain uncharacterized, though proteins that activate genes responsive to cAMP and ion channel proteins are strong candidates. In contrast to the well-recognized biologic effects of cAMP in PTH target tissues, the physiological importance of metabolites of phosphotidylinositol hydrolysis and intracellular calcium as PTH-induced second messengers has not yet been established. A growing number of studies have revealed that the number of PTH receptors expressed, as well as the concentration of G protein and PTH, cooperates to determine the precise signal response.

General Pathophysiology

In addition to the clinical and biochemical features of hypoparathyroidism, the patients described by Albright exhibited a distinctive constellation of developmental and skeletal defects, subsequently referred to as Albright hereditary osteodystrophy (AHO), and including a round face; short, stocky physique; brachydactyly; heterotopic ossification; and mental retardation. The relationship between the biochemical abnormalities (hypocalcemia and hyperphosphatemia) and AHO could not be explained by Albright, and yet remains unclarified. Indeed, in certain families some affected members may show both AHO and PTH resistance whereas other family members may have AHO without evidence of any endocrine dysfunction, a disorder Albright termed "pseudo-pseudohypoparathyroidism" (pseudoPHP) to emphasize the physical similarities but biochemical differences between these patients and patients with PHP (Albright *et al.,* 1952).

The diagnostic classification of PHP is further extended by the existence of additional variants in which patients manifest PTH resistance and biochemical hypoparathyroidism but lack any of the features of AHO (Winter and Hughes, 1980; Drezner *et al.,* 1973). A classification of the many different forms of PHP is presented in Table I.

Characterization of the molecular basis for PHP commenced with the observations by Chase and Aurbach that cAMP mediates many of the actions of PTH on kidney and bone, and that administration of biologically active PTH to normal subjects leads to a significant increase in the urinary excretion of nephrogenous cAMP and phosphate (Chase *et al.,* 1969). The PTH infusion test remains the most reliable test available for the diagnosis of PHP, and enables distinction between several variants of the syndrome (Fig. 3). Patients with PHP type 1 fail to show an appropriate increase in urinary excretion of both nephrogenous cAMP and phosphate (Chase *et al.,* 1969), suggesting that an abnormality in the renal PTH receptor–adenylyl cyclase complex that produces cAMP is the basis for impaired PTH responsiveness. Subsequent studies by Bell *et al.,* in which administration of dibutyryl cAMP to patients with PHP type 1 produced a phosphaturic response, provided additional support for this hypothesis, and demonstrated that the renal response mechanism to cAMP was intact (Bell *et al.,* 1972). These studies have led to the conclusion that proximal renal tubule cells are unresponsive to PTH. By contrast, cells in other regions of the kidney appear responsive to PTH, as evidenced by the observation that urinary calcium excretion in patients with PHP type 1 is less than in patients with hormonopenic hypoparathyroidism after normalization of blood calcium levels in each group by vitamin D and oral calcium supplements (Mizunashi *et al.,* 1990; Shima *et al.,* 1988). Moreover, renal handling of calcium (and sodium) in response to exogenous PTH also appears to be normal in patients with PHP type 1 (Stone *et al.,* 1993). These results indicate that

Table I Characteristic Features of the Various Forms of Pseudohypoparathyroidism

	PHP type 1a	Pseudo-PHP	PHP type 1b	PHP type 1c	PHP type 2
Physical appearance	Albright hereditary, osteodystrophy may be subtle or (rarely) absent		Normal	Albright hereditary osteodystrophy	Normal
Response to PTH					
Urine cAMP	Defective	Normal	Defective	Defective	Normal
Urine phosphorous	Defective	Normal	Defective	Defective	Defective
Serum calcium level	Low or (rarely) normal	Normal	Low	Low	Low
Hormone resistance	Generalized	Absent	Limited to PTH target tissues	Generalized	Limited to tissues
$G\alpha_s$ activity	Reduced	Reduced	Normal	Normal	Normal
Inheritance	Autosomal dominant		Autosomal dominant (most cases)	Unknown	Unknown
Molecular defect	Heterozygous mutations in the *GNAS1* gene		Imprinting defect in *GNAS1* gene	Unknown	Unknown

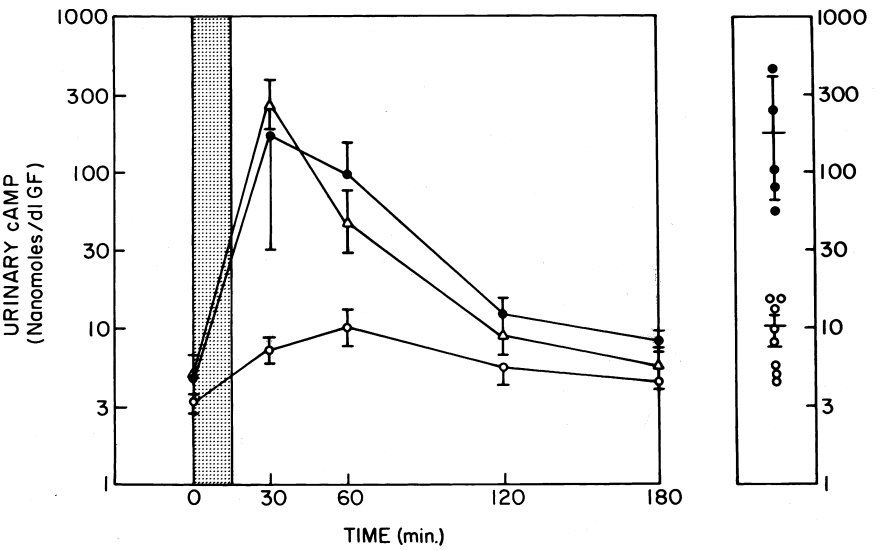

Figure 3 Urinary cAMP excretion in response to an infusion of bovine parathyroid extract (300 USP units). The peak response in normal subjects (△) as well as those with pseudo-PHP (●) is 50- to 100-fold times basal. Subjects with PHP type 1a (○) or PHP type 1b (not shown) show only a two- to five-fold increase. Urinary cAMP is expressed as nanomoles per 100 ml of GF, U_{cAMP} (nanomoles per 100 ml GF) = U_{cAMP} (nanomoles/dl) X S_{Cre} (mg/dl)/U_{Cre} (mg/dl). Reprinted with permission from Levine *et al.* (1986).

calcium reabsorption in the distal tubule is responsive to circulating PTH in subjects with PHP type 1, and imply that adequate amounts of cAMP are produced in these cells or that other second messengers (e.g., cytosolic calcium or diacylglycerol) may be responsible for PTH action (Fig. 1).

Administration of PTH to subjects with the less common form of the disorder, PHP type 2, produces a normal increase in urinary cAMP but fails to elicit an appropriate phosphaturic response (Drezner *et al.,* 1973). These observations have suggested that PTH resistance in PHP type 2 results from a biochemical defect that is either unrelated or distal to the PTH-stimulated generation of cAMP.

It has been generally assumed that bone cells in patients with PHP type 1 are innately resistant to PTH, but this remains unproved. In fact, cultured bone cells from a patient with PHP type 1 have been shown to increase intracellular cAMP normally in response to PTH treatment *in vitro* (Murray *et al.,* 1993). Evidence that bone cells are unresponsive to PTH is largely inferred from the observation that patients with PHP type 1 are hypocalcemic and that administration of PTH does not increase the plasma calcium level. However, clinical, radiological, or histological evidence of increased bone turnover and demineralization (Fig. 4) is common in patients with PHP type 1. One possible explanation for the variable bone responsiveness to PTH is the existence of two distinct cellular systems in bone upon which PTH exerts action: the remodeling system and the mineral mobilization or homeostatic system. The bone remodeling system appears to be more responsive to PTH in patients with PHP type 1 than the homeostatic system. This variability may reflect the lesser dependence of the remodeling system upon normal

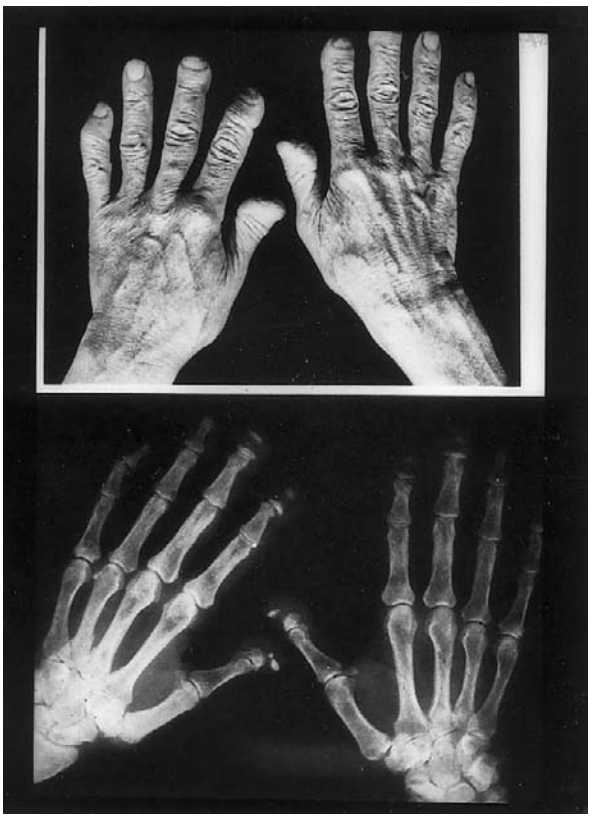

Figure 4 Photograph and radiograph of hands of a patient with marked hyperparathyroid bone disease. Marked periosteal bone erosion in terminal phalanges has resulted in "pseudoclubbing." From Levine *et al.,* (1982).

plasma levels of $1,25(OH)_2D$. Plasma levels of $1,25(OH)_2D$ are reduced in hypocalcemic patients with PHP type 1 (Drezner *et al.,* 1976) and could explain the concurrence of hypocalcemia and increased skeletal remodeling in many of these patients. Hypocalcemia leads to a compensatory overproduction of PTH, which could eventually overcome the $1,25(OH)_2D$ dependency for remodeling but not for PTH-stimulated calcium mobilization.

A role for $1,25(OH)_2D$ in modulating the responsiveness of the calcium homeostatic system to PTH is suggested by several observations. First, the calcemic response to PTH is deficient not only in patients with PHP type 1, but also in patients with other hypocalcemic disorders in which plasma levels of $1,25(OH)_2D$ are low. Moreover, normalization of the plasma calcium level in patients with PHP type 1 by administration of physiologic amounts of $1,25(OH)_2D$ or pharmacological amounts of vitamin D restores calcemic responsiveness (Drezner and Haussler, 1979). Second, patients with PHP type 1 who have normal serum levels of calcium and $1,25(OH)_2D_3$ without vitamin D treatment (so-called "normocalcemic" PHP) show a normal calcemic response to administered PTH (Drezner and Haussler, 1979). These findings suggest that $1,25(OH)_2D$ deficiency is the basis for the lack of a calcemic response to PTH in hypocalcemic patients with PHP type 1 and challenge the premise that bone cells are intrinsically resistant to the actions of PTH.

Subjects with PHP type 1 have increased serum levels of phosphate owing to an inability of PTH to decrease phosphate reabsorption in the kidney. Hypocalcemia *per se* may also contribute to the development of hyperphosphatemia, as very low levels of intracellular calcium impair renal phosphate clearance. Accordingly, restoration of plasma calcium levels to normal by chronic treatment with calcium and vitamin D can reduce elevated levels of serum phosphorous. Similar therapy has been shown to reverse the defective phosphaturic response to administered PTH in certain patients with PHP type 1, although the urinary cAMP response remains markedly deficient (Stogmann and Fischer, 1975). Therefore, persistence of a blunted urinary cAMP response to PTH in PHP type 1 patients in whom chronic vitamin D therapy has led to normalization of plasma calcium levels and restoration of a phosphaturic response need not imply, as has been at least suggested (Stogmann and Fischer, 1975), that there is no relationship between cAMP production and phosphate clearance.

Hyperphosphatemia has several important metabolic consequences. Elevated plasma phosphate levels can decrease plasma calcium levels both by increasing the deposition of extracellular calcium into bone and soft tissues and by decreasing the mobilization of calcium from the skeleton. Furthermore, elevated plasma phosphate concentrations can reduce 1-α-hydroxylase activity in renal tubular cells and decrease synthesis of $1,25(OH)_2D$. This effect may be of equal or greater significance than the defective renal cAMP response to PTH as a cause of deficient production of $1,25(OH)_2D$. It is therefore likely that reduced synthesis of $1,25(OH)_2D$ in PHP type 1 arises as a consequence of two defects related to PTH resistance: (1) decreased renal phosphate clearance, which results in elevated plasma phosphate levels that inhibit activity of renal 1-α-hydroxylase; and (2) decreased PTH activation of the enzyme. The overall evidence suggests that the disturbances in calcium, phosphorous, and vitamin D metabolism in most patients with PHP type 1 result directly or indirectly from reduced responsiveness of both bone and kidney to PTH. Hypocalcemia results from impaired mobilization of calcium from bone, reduced intestinal absorption of calcium (via deficient generation of $1,25(OH)_2D$), and urinary calcium loss. Of these defects, the diminished movement of calcium out of bone stores into the extracellular fluid probably has the greatest role in producing hypocalcemia. Intensive treatment with calcitriol ($1,25(OH)_2D$) or other vitamin D analogs improves intestinal calcium absorption and bone calcium mobilization, restores plasma calcium to normal, and reduces circulating PTH levels. Thus, although PTH resistance appears to be the proximate biochemical defect, the major abnormalities in mineral metabolism found in patients with PHP type 1 can be largely explained on the basis of deficiency of circulating $1,25(OH)_2D$.

Molecular Classification of Pseudohypoparathyroidism

Hormone action may be divided conceptually into prereceptor, receptor, and postreceptor events; defects in each of these steps have been proposed as the basis of hormone resistance in PHP (Fig. 1). For example, a circulating inhibitor of PTH action has been proposed as a cause of PTH resistance on the basis of studies showing an apparent dissociation between plasma levels of endogenous immunoreactive and bioactive PTH in subjects with PHP type 1. Despite high circulating levels of immunoreactive PTH, the levels of bioactive PTH in many patients with PHP type 1 have been found to be within the normal range when measured with highly sensitive renal (de Deuxchaisnes *et al.,* 1981) and metatarsal (Bradbeer *et al.,* 1988) cytochemical bioassay systems. Furthermore, plasma from many of these patients has been shown to diminish the biological activity of exogenous PTH in these *in vitro* bioassays (Loveridge *et al.,* 1982). Currently, the nature of this putative inhibitor or antagonist remains unknown. The observation that prolonged hypercalcemia can remove or reduce significantly the level of inhibitory activity in the plasma of patients with PHP has suggested that the parathyroid gland may be the source of the inhibitor. In addition, analysis of circulating PTH immunoactivity after fractionation of patient plasma by reversed-phase high-performance liquid chromatography has disclosed the presence of aberrant forms of immunoreactive PTH in many of these patients (Mitchell and Goltzman, 1985). Although it is

conceivable that a PTH inhibitor may cause PTH resistance in some patients with PHP, it is more likely that circulating antagonists of PTH action arise as a consequence of the sustained secondary hyperparathyroidism that results from the primary biochemical defect.

By contrast, molecular studies have provided confirmation that defects in the PTH1R-G protein-coupled signaling pathway are responsible for PTH resistance in many patients with PHP type 1. Patients with PHP type 1a show decreased responsiveness to a variety of hormones that utilize cAMP as a second messenger, and cells from these patients have a 50% reduction in expression or activity of the α chain of Gs, the G protein that couples receptors to adenylyl cyclase (Fig. 1). Mutations in the *GNAS1* gene, which encodes $G\alpha_s$, have been identified in most cases of PHP type 1a. Patients with PHP type 1c also show resistance to multiple hormones, but have apparently normal levels of $G\alpha_s$. Multihormone resistance in these patients may be due to defects in other components of the signal transduction system that are not tissue specific, including some forms of adenylyl cyclase (Barrett *et al.,* 1989). Patients with PHP type 1a or 1c also have AHO. By contrast, hormone resistance is limited to PTH target tissues in patients with PHP type 1b. These patients have normal levels of $G\alpha_s$ in accessible cells and lack features of AHO. Defective imprinting of the *GNAS1* gene appears to be the basis for selective deficiency of $G\alpha_s$ in the proximal renal tubule.

Finally, patients with PHP type 2 have a normal urinary cAMP response to PTH but fail to generate a phosphaturic response. These subjects may have a defect in cAMP-dependent protein kinase A, one of its substrates or targets, or in another PTH signaling pathway (e.g., phospholipase C).

Pseudohypoparathyroidism Type 1a and Pseudopseudohypoparathyroidism

Cell membranes from patients with PHP type 1a show an approximately 50% reduction in expression or activity of $G\alpha_s$ protein (Levine *et al.,* 1986) (Fig. 5). This generalized deficiency of $G\alpha_s$ may impair the ability of PTH, as well as many other hormones and neurotransmitters, to activate adenylyl cyclase and thereby may account for multihormone resistance (Fig. 6). In addition to hormone resistance, patients with PHP type 1a also manifest the peculiar constellation of developmental and somatic defects that are collectively termed AHO (Fig. 7; see Clinical Features below) (Albright *et al.,* 1942).

Early studies of PHP type 1a led to the identification of families in which some individuals had signs of AHO but lacked apparent hormone resistance (i.e., pseudo-PHP). The observation that PHP type 1a and pseudo-PHP can occur in the same family first suggested that these two disorders might reflect variability in expression of a single genetic lesion. Support for this view comes from studies indicating that within a given kindred, subjects with either pseudo-PHP or PHP type 1a have equivalent functional $G\alpha_s$ deficiency (Fig. 5) (Levine *et al.,* 1986, 1988), and that a transition from hormone responsiveness to hormone resistance may occur (Barr *et al.,* 1994). It, therefore, seems appropriate to apply the term AHO to both of these variants in acknowledgment of the clinical and biochemical characteristics that patients with PHP type 1a and pseudo-PHP share (Mann *et al.,* 1962).

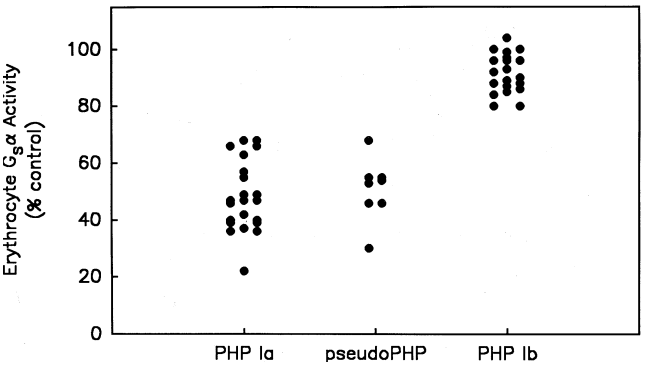

Figure 5 $G\alpha_s$ activity of erythrocyte membranes. $G\alpha_s$ is quantified in complementation assays with S49 cyc$^-$ membranes, which genetically lack $G\alpha_s$ but retain all other components necessary for hormone-response adenylyl cyclase activity. Activity is reduced approximately 50% in patients with AHO and subjects with either PHP type 1a or pseudo-PHP, but is normal in patients with PHP type 1b.

Molecular Defect in Albright Hereditary Osteodystrophy

The discovery that $G\alpha_s$ deficiency results from inactivating mutations in the *GNAS1* gene, located at 20*q*13.2–*q*13.3 (Levine *et al.,* 1991), first provided confirmation of autosomal dominant transmission of AHO and subsequently facilitated studies to address the unusual pattern of inheritance of PHP type 1a and pseudo-PHP. *GNAS1* is a complex gene (Kozasa *et al.,* 1988) composed of at least 16 exons, including 3 alternative first exons (Hayward *et al.,* 1998a,b; Swaroop *et al.,*

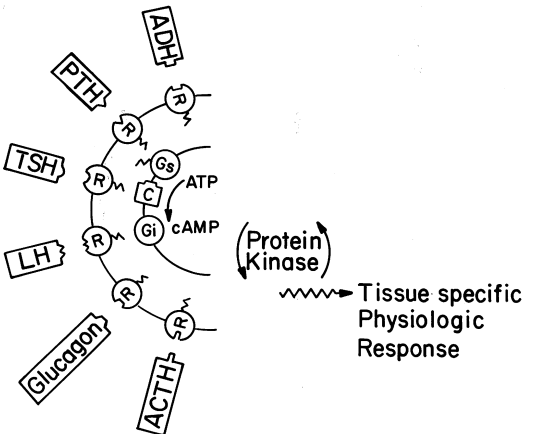

Figure 6 Model of multihormone resistance in subjects with $G\alpha_s$ deficiency.

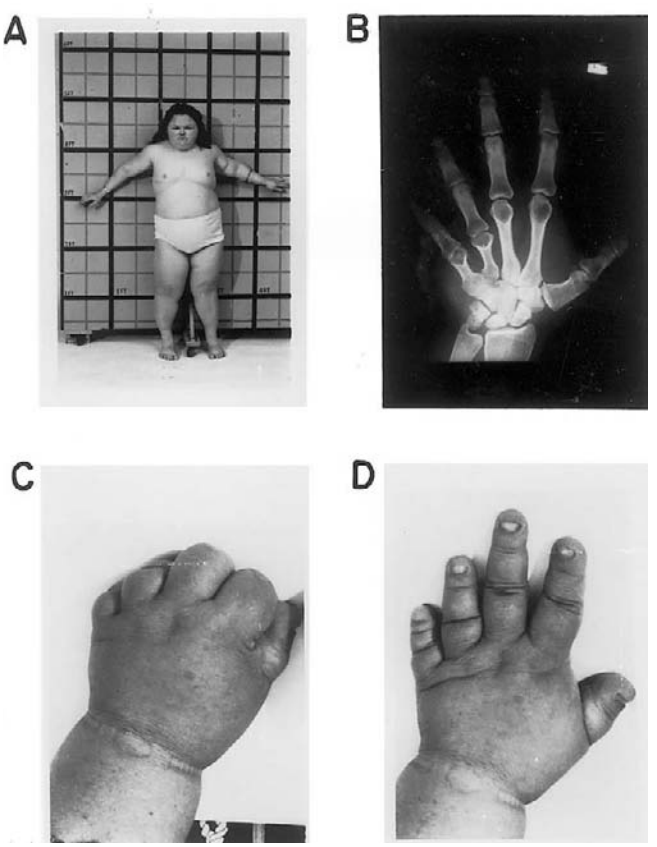

Figure 7 Typical features of Albright hereditary osteodystrophy. (A) A young woman with characteristic features of AHO; note the short stature, disproportionate shortening of the limbs, obesity, and round face. (B) Radiograph of patient's hand showing marked shortening of fourth and fifth metacarpals. (C) Archibald sign, with dimples in place of knuckles. (D) Brachydactyly of the hand, note thumb sign and shortening of the fourth and fifth digits.

1991) (Fig. 8). Alternative splicing of nascent transcripts derived from exons 1–13 generates four mRNAs that encode $G\alpha_s$ proteins. Deletion of exon 3 results in the loss of 15 codons from the mRNA, while use of an alternative splice site in exon 4 results in the insertion of a single additional codon into the mRNA. This produces two $G\alpha_s$ isoforms with apparent molecular weights of 45 kDa and two isoforms with apparent molecular weights of 52 kDa (Kozasa *et al.,* 1988). The long and short forms of $G\alpha_s$ exhibit tissue-specific patterns of expression (Bhatt *et al.,* 1988) and can both can stimulate adenylyl cyclase and open calcium channels (Mattera *et al.,* 1989). However, biochemical characterization of these isoforms has revealed subtle differences in the binding constant for GDP, the rate at which the forms are activated by agonist binding, efficiency of adenylyl cyclase stimulation, and the rate of GTP hydrolysis. The significance of these differences remains unknown (Jones *et al.,* 1990; Mattera *et al.,* 1989; Graziano *et al.,* 1989), but these distinctions imply the existence of as yet undefined roles for these G proteins (Novotny and Svoboda, 1998).

Additional complexity in the processing of the *GNAS1* gene arises from the use of first exons other than exon 1, which encodes sequences necessary for interaction with $G\beta\gamma$ and attachment to the plasma membrane (Evanko *et al.,* 2000). Accordingly, it is unlikely that these proteins can function fully as transmembrane signal transducers to couple receptors to intracellular signal generators. In one case, $G\alpha_s$ transcripts are produced with an alternative first exon (exon 1A) that lacks an initiator ATG codon. Smaller, $G\alpha_s$ proteins are predicted to be translated from an inframe ATG in exon 2, if translation occurs at all. Because these proteins lack amino acid sequences encoded by exon 1 (Ishikawa *et al.,* 1990) that are necessary for binding to $\beta\gamma$ subunits, it is unlikely that they function as conventional α chains. In two other instances unique transcripts are generated using additional coding exons that are present upstream of exon 1, the first exon present in transcripts used to generate functional $G\alpha_s$ protein. The more 5′ of these exons encodes the neuroendocrine secretory protein NESP55, a chromogranin-like protein, and is generated from a transcript that contains sequences derived from exon 2–13 of *GNAS1* in the 3′ nontranslated region (Leitner *et al.,* 1999; Ischia *et al.,* 1997). Accordingly, NESP55 shares no protein homology with $G\alpha_s$. Eleven kilobases further downstream is a second alternative exon, XLαs, that when spliced in-frame to exons 2–13 results in a transcript that encodes a larger, 51-kDa $G\alpha_s$ isoform. (Kehlenbach *et al.,* 1994). The XLαs protein is able to interact with $\beta\gamma$ chains through sequences in the carboxyl terminal region of the XL domain, which shows high homology to the exon 1-encoded portion of $G\alpha_s$ that promotes binding to $\beta\gamma$ dimers (Pasolli *et al.,* 2000; Klemke *et al.,* 2000). XLαs is targeted to the plasma membrane (Pasolli *et al.,* 2000), and can activate adenylyl cyclase (Klemke *et al.,* 2000). However, it is apparently unable to interact with heptahelical receptors (Klemke *et al.,* 2000) and thus its signal transduction properties are unique. Both NESP55 and XLαs are most highly expressed in neuroendocrine tissues.

Molecular studies of DNA from subjects with AHO have disclosed inactivating mutations in the *GNAS1* gene (Lin *et al.,* 1992; Iiri *et al.,* 1994; Luttikhuis *et al.,* 1994; Patten *et al.,* 1990; Weinstein *et al.,* 1990; Miric *et al.,* 1993; Schwindinger *et al.,* 1994; Farfel *et al.,* 1996; Shapira *et al.,* 1996; Fischer *et al.,* 1998; Jan de Beur *et al.,* 1998; Nakamoto *et al.,* 1998; Warner *et al.,* 1998; Ahmed *et al.,* 1998; Yu *et al.,* 1999) that account for a 50% reduction in expression or function of $G\alpha_s$ protein in accessible tissues (Table II). All patients are heterozygous, and have one normal *GNAS1* allele and one defective allele. Mutations in the *GNAS1* gene are heterogeneous, and include missense mutations (Patten *et al.,* 1990; Miric *et al.,* 1993; Schwindinger *et al.,* 1994; Farfel *et al.,* 1996; Warner *et al.,* 1998), point mutations that disrupt efficient splicing (Weinstein *et al.,* 1990) or terminate translation prematurely (Jan de Beur *et al.,* 1998), and small deletions (Weinstein *et al.,* 1990; Miric *et al.,* 1993; Weinstein *et al.,* 1992; Shapira *et al.,* 1996; Yu *et al.,* 1999; Fischer *et al.,* 1998) (Fig. 9). Although private mutations have been found in nearly all of the kindreds studied, a four-base deletion in exon 7 has been detected in multiple families (Weinstein *et al.,* 1992; Yu *et al.,* 1995; Ahmed

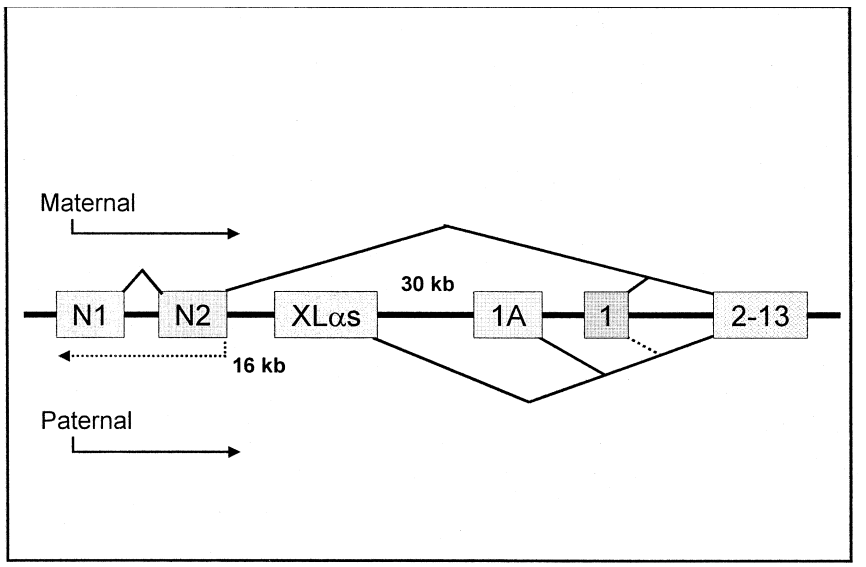

Figure 8 The *GNAS1* gene complex. The gene consists of 13 exons that encode the signaling protein $G\alpha_s$; upstream of exon 1 are three alternative exons, labeled exon 1A, XLαs, and NESP55. These three alternative first exons are spliced to exons 2–13 of the gene to produce unique transcripts and, at least in the case of NESP55 and XLas, distinct proteins (see text). Differentially methylated regions are located near the NESP55, XLas, and 1A exons and correspond to regulated expression via genomic imprinting. NESP55 is transcribed exclusively from the maternal allele, while XLas and exon 1A transcripts are derived exclusively from the paternal allele. Transcripts encoding Gas are generated from both alleles except in imprinted tissue (e.g., proximal renal tubule), where only the maternal allele is trancriptionally active.

et al., 1998; Yokoyama *et al.,* 1996; Walden *et al.,* 1999; Nakamoto *et al.,* 1998) and an unusual missense mutation in exon 13 (A366S; see below) has been identified in two unrelated young boys (Iiri *et al.,* 1994), suggesting that these two regions may be genetic "hot spots."

Most gene mutations lead to reduced expression of $G\alpha_s$ mRNA (Levine *et al.,* 1988; Carter *et al.,* 1987), but in some subjects the mutant allele produces normal levels of $G\alpha_s$ mRNA (Levine *et al.,* 1988; Carter *et al.,* 1987; Mallet *et al.,* 1982) that encode dysfunctional $G\alpha_s$ proteins (Schwindinger *et al.,* 1994; Farfel *et al.,* 1996; Iiri *et al.,* 1994; Warner *et al.,* 1998; Wu *et al.,* 2001). The replacement of arginine by histidine at codon 385 in the carboxyl terminal tail of $G\alpha_s$ selectively "uncouples" G_s from receptors and prevents receptor activation (Schwindinger *et al.,* 1994). Substitution of arginine by histidine at position 231 also prevents receptor activation of G_s, but by an entirely different mechanism (Farfel *et al.,* 1996). The replacement of arginine-231 hinders binding of GTP to the α chain, and thereby inhibits receptor-induced dissociation of $G\alpha_s$ from $G\beta\gamma$. Likewise, substitution of arginine at position 258 with tryptophan leads to increased GDP release and impaired receptor-mediated activation (Warner *et al.,* 1998).

Multiple Hormone Resistance in Pseudohypoparathyroidism Type 1a

Although biochemical hypoparathyroidism is the most commonly recognized endocrine deficiency in PHP type 1a, early clinical studies described additional hormonal abnormalities, such as hypothyroidism (Marx *et al.,* 1971; Werder *et al.,* 1975) and hypogonadism (Wolfsdorf *et al.,* 1978). Because available evidence suggests that $G\alpha_s$ is present in all tissues, generalized deficiency of this protein could be the basis for not only PTH resistance, the hallmark of PHP type 1a, but could also explain the decreased responsiveness of diverse tissues (e.g., kidney, thyroid gland, gonads, and liver) to hormones that act via activation of adenylyl cyclase (e.g., PTH, thyroid-stimulating hormone (TSH), gonadotropins, and glucagon) (Levine *et al.,* 1983a; Tsai *et al.,* 1989; Shima *et al.,* 1988). Primary hypothyroidism occurs in most patients with PHP type 1a (Levine *et al.,* 1983a). Typically, patients lack a goiter or anti-thyroid antibodies and have an elevated serum TSH with an exaggerated response to thyrotropin-releasing hormone (TRH). Serum levels of T_4 may be low or low normal. Hypothyroidism may occur early in life prior to the development of hypocalcemia, and elevated serum levels of TSH are not uncommonly detected during neonatal screening (Levine *et al.,* 1985; Weisman *et al.,* 1985; Yokoro *et al.,* 1990). Unfortunately, early institution of thyroid hormone replacement does not seem to prevent the development of mental retardation (Weisman *et al.,* 1985).

Reproductive dysfunction occurs commonly in subjects with PHP type 1a, particularly in women. Female patients with PHP type 1a may have delayed puberty, oligomenorrhea, and infertility (Levine *et al.,* 1983a). Plasma gonadotropins may be elevated, but are more commonly normal (Namnoum *et al.,* 1998). Some patients show an exaggerated serum gonadotropin response to GnRH (Wolfsdorf *et al.,* 1978; Downs *et al.,* 1983). Features of hypogonadism may be less obvious

Table II *In Vitro* Hormone Responsiveness of Tissues from Patients with PHP Type 1

Tissue	Form of PHP	Adenylyl cyclase response	Notes	Reference
Kidney	PHP type 1a (presumed) $n = 1$	Responsive to PTH. No normal controls.	[ATP] is high. Kidney obtained from autopsy.	Marcus *et al.,* 1971
Kidney	PHP type 1a $n = 1$	Abnormal response to PTH when [ATP] is low and no guanine nucleotides are present.	High [ATP] or addition of GTP normalizes response to PTH and replicates same activity as kidney from Marcus *et al.* (1971).	Downs *et al.,* 1983; Drezner and Burch, 1978
Cultured skin fibroblasts	PHP type 1a ($n = 5$) PHP type 1b ($n = 2$)	Normal responses to PGE_1, sodium fluoride, and cholera toxin in cells and membranes from both groups.		Bourne *et al.,* 1981
Cultured skin fibroblasts	PHP type 1a ($n = 5$)	Decreased response to PGE_1, sodium fluoride, guanine nucleotides.	Normal response to forskolin.	Levine *et al.,* 1983b
Cultured skin fibroblasts	PHP type 1b	Decreased cAMP response to PTH.	Decreased levels of PTH receptor mRNA; increases in some with dexamethasone.	Silve *et al.,* 1986; Silve *et al.,* 1990; Suarez *et al.,* 1995;
Cultured bone cells	PHP type 1b ($n = 1$)	Normal response to cholera toxin and PTH.		Murray *et al.,* 1993
Thyroid	PHP type 1a (presumed) ($n = 1$)	Basal and sodium fluoride-stimulated activities are normal; response to TSH is reduced.	Response is not affected by [ATP] or presence of GTP.	Mallet *et al.,* 1982
Platelets	PHP type 1a ($n = 3$) PHP type 1b ($n = 4$)	Maximal PGI_2-induced cAMP response is reduced in platelets from both groups.		Motulsky *et al.,* 1982
EBV-transformed lymphoblasts	PHP type 1a ($n = 3$) PHP type 1b ($n = 2$)	Adenylyl cyclase activity is normal in both groups.		Farfel *et al.,* 1982

in men with variable serum testosterone levels ranging from normal to frankly reduced. Testes may show evidence of a maturation arrest or may fail to descend normally. Fertility appears to be decreased in men with PHP type 1a. Deficiency of prolactin secretion (basal and in response to secretagogues such as TRH) had been reported in some patients with PHP type 1 (Carlson *et al.,* 1977), but later studies have not confirmed these early findings (Levine *et al.,* 1983a).

Obesity is common in subjects with PHP type 1a, as well as in patients with pseudoPHP. Obesity may reflect a defective lipolytic response to hormonal stimulation due to $G\alpha_s$ deficiency (Carel *et al.,* 1999; Kaartinen *et al.,* 1994). Others have hypothesized that $G\alpha_s$ deficiency results in reduced signaling of the melanocortin 4 receptor that is responsible for the disinhibition of satiety and hyperphagia observed in PHP 1a (Ong *et al.,* 2000).

Abnormal hormone responsiveness may occur in some tissues without obvious clinical sequelae. For example, the hepatic glucose response to glucagon is normal although plasma cAMP concentrations fail to increase normally (Levine *et al.,* 1983a; Brickman *et al.,* 1986). In other tissues significant hormone resistance does not occur despite the apparent reduction in $G\alpha_s$. Diabetes insipidus is not a feature of PHP type 1a, and urine is concentrated normally in response to vasopressin in patients with PHP type 1a (Moses *et al.,* 1986). Although there is a report of adrenal insufficiency in a single individual with PHP type 1a (Ridderskamp and Schlaghecke, 1990), hypoadrenalism is not a typical feature of PHP type 1a and adrenocortical responsiveness to corticotropin (ACTH) is normal (Levine *et al.,* 1983a).

PRIMARY SENSORY ABNORMALITIES

Patients with PHP type 1a frequently manifest distinctive olfactory (Weinstock *et al.,* 1986), gustatory (Henkin, 1968), and auditory (Koch *et al.,* 1990) abnormalities that are apparently unrelated to endocrine dysfunction. The molecular basis of these neurosensory deficits has become more obscure with the discovery of unique G proteins that regulate signal transduction pathways related to vision (Lerea *et al.,* 1986; Lochrie *et al.,* 1985), olfaction (Jones and Reed, 1989), and taste (McLaughlin *et al.,* 1992).

Henkin first reported gustatory disturbances in patients with PHP type 1 (Henkin, 1968), but biochemical methods that allow distinction between types 1a and 1b had not yet been developed. These patients had elevated thresholds for detection and recognition of sour and bitter taste. In addition, the olfactory threshold for detection and recognition of all vapors tested was similarly elevated. Treatment with calcium, parathyroid extract, or both did not restore taste or olfaction to normal. The subsequent identification of an odorant-sensitive adenylyl cyclase in frog and rat olfactory

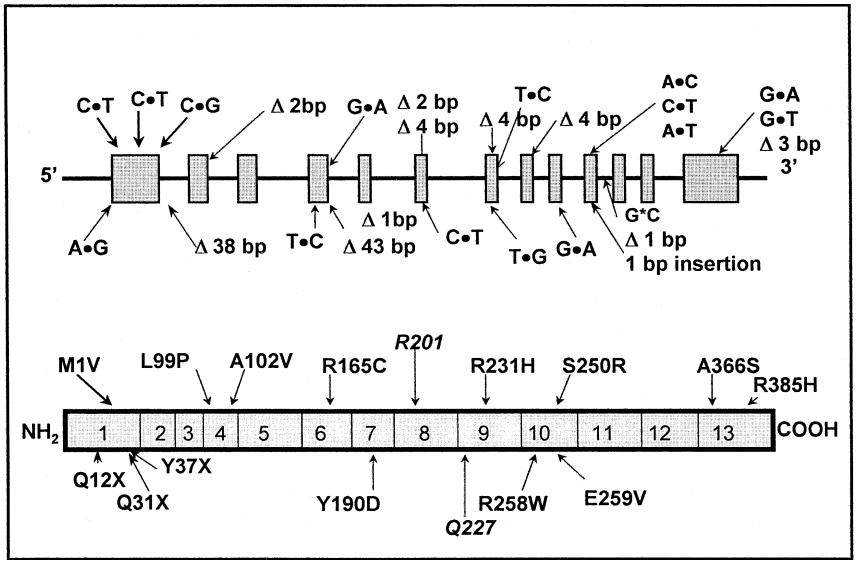

Figure 9 Mutations in the *GNAS1* gene. (Top) the human *GNAS1* gene, which spans over 20 k pairs and contains at least 13 exons and 12 introns. Unique mutations that result in *loss of* $G\alpha_s$ *function* have been identified in affected members of many unrelated AHO families; missense mutations are denoted by the symbol *. (Botton) The position of these missense mutations above the protein structure. Two polymorphisms are denoted by the symbol +, and the position of the unchanged amino acid is denoted beneath the predicted $G_{s\alpha}$ protein. The site of two missense mutations that result in gain of function (replacement of either Arg^{201} or Gln^{227}) in patients with MAS (Weinstein *et al.,* 1991; Schwindinger *et al.,* 1992; Shenker *et al.,* 1994, 1993; Levine *et al.,* 1994) or in sporadic tumors (Landis *et al.,* 1989; Lyons *et al.,* 1990) are depicted in italics. The mutation in exon 1 eliminates the initiator methionine codon and prevents synthesis of a normal $G_{s\alpha}$ protein (Patten *et al.,* 1990). The 4 base-pair deletions in exon 7 (Weinstein *et al.,* 1992) and exon 8 (Miric *et al.,* 1993), and the 1 base-pair deletion in exon 10 all shift the normal reading frame and prevent normal mRNA and/or protein synthesis. Mutations in intron 3 and at the donor splice junction between exon 10 and intron 10 cause splicing abnormalities that prevent normal mRNA synthesis (Weinstein *et al.,* 1990). The mutations indicated with an asterisk represent missense mutations (Miric *et al.,* 1993; Schwindinger *et al.,* 1994; Iiri *et al.,* 1994); the resultant amino acid substitutions are indicated in the schematic diagram of the $G\alpha_s$ protein at the bottom of the figure. Some of these mutations may prevent normal protein synthesis by altering protein secondary structure, but the arg → his substitution in exon 13 appears to encode an altered protein that cannot couple normally to receptors (Schwindinger *et al.,* 1994) and the ala → ser mutation encodes an activated $G\alpha_s$ protein that is unstable at 37°C (Iiri *et al.,* 1994).

neuroepithelium (Sklar *et al.,* 1986; Pace *et al.,* 1985) that was stimulated by a G_s-like protein (Pace and Lancet, 1986) prompted further investigation of olfactory function in patients with PHP type 1. Using quantitative tests to assess the ability of patients to identify common odorants, several early studies showed variably decreased olfactory ability in subjects with PHP type 1a by comparison to patients with PHP type 1b who have normal $G\alpha_s$ activity (Weinstock *et al.,* 1986; Ikeda *et al.,* 1988). Subsequent analyses, using comprehensive tests of odor detection, identification, and memory, demonstrated that patients with PHP type 1a as well as patients with PHP type 1b have olfactory dysfunction relative to matched controls and that patients with pseudo-PHP (PPHP) have relatively normal olfactory function (Doty *et al.,* 1997). These studies imply that other mechanisms (e.g., ones associated with PTH or PTHrP resistance) are responsible for the olfactory deficits of this disorder, and are consistent with the existence of a unique G protein in olfactory neuroepithelial cells, termed G_{olf} (Jones and Reed, 1989), that couples odorant receptors to a unique form of adenylyl cyclase (type III (Bakalyar and Reed, 1990)).

Koch *et al.* (1990) found that PHP type 1a patients with $G\alpha_s$ deficiency had symmetrical sensorineural hearing losses. The authors speculated that loss of the G protein–adenylyl cyclase complex causes progressive damage to cochlear hair cells induced by a disorder of the electrolyte composition of the inner ear fluids.

Alterations in visual function have also been described in patients with PHP type 1a. For example, decreased color discrimination and enhanced contrast sensitivity have been observed in patients with PHP type 1a (Jackowski *et al.,* 1992). The pathophysiological basis for these photoreceptor alterations is unknown, as $G\alpha_s$ seems to play no role in retinal phototransduction. Light activation of the specialized receptor proteins rhodopsin and opsin that are expressed in rods and cones, respectively, is coupled to stimulation of cyclic GMP phosphodiesterase by G proteins that were originally termed transducins (Lerea *et al.,* 1986; Lochrie *et al.,* 1985). Although these G proteins share substantial similarity

to G_s in subunit structure and mechanism of action, their expression is limited to retinal photoreceptor cells and they subserve unique signaling functions.

Neurological Abnormalities

Mild to moderate mental retardation is common in patients with PHP type 1a. Farfel and Friedman assessed intelligence in 25 patients with PHP type 1 whose $G\alpha_s$ activity had been determined (Farfel and Friedman, 1986). The authors found an association between mental deficiency and $G\alpha_s$ deficiency and speculated that reduced cyclic adenosine monophosphate (cAMP) levels in cortical tissue may lead to mental retardation. Other factors that might contribute to mental retardation in patients with PHP type 1a include hypothyroidism and hypocalcemia; however, efforts to control these have not prevented cognitive dysfunction in all patients, suggesting that $G\alpha_s$ deficiency may cause a primary abnormality of neurotransmitter signaling. Seizure disorders are also frequently reported. These may reflect atypical seizures due to hypocalcemia (cerebral tetany) or exacerbation of epileptic seizures. These seizures may occur prior to recognition of hypocalcemia (Faig *et al.,* 1992; Bonadio, 1989). There have been reports of psychosis in patients with AHO (Furukawa, 1991). As with patients with hormonopenic hypoparathyroidism, patients with PHP type 1 who have extensive calcifications of the basal ganglia may develop movement disorders (Klawans *et al.,* 1976; Blin *et al.,* 1991; Dure and Mussell, 1998; Vaamonde *et al.,* 1993; Uncini *et al.,* 1985; Tambyah *et al.,* 1993).

Phenotypic Variability in AHO

Molecular studies have provided a basis for $G\alpha_s$ deficiency, but they do not explain the striking variability in biochemical and clinical phenotype in subjects with AHO. Why do some $G\alpha_s$-coupled pathways show reduced hormone responsiveness (e.g., PTH, TSH, gonadotropins), whereas other pathways are clinically unaffected (ACTH in the adrenal gland and vasopressin in the renal medulla)? Perhaps even more intriguing is the paradox that $G\alpha_s$ deficiency can be associated with hormone resistance and AHO (PHP type 1a), AHO only (pseudo-PHP), progressive osseous heteroplasia (POH) (Shore *et al.,* 1999), or no apparent consequences at all (Miric *et al.,* 1993). These observations, when considered in the context of studies showing that the number of G_s molecules in cell membranes greatly exceeds the number of either receptor or adenylyl cyclase molecules (Levis and Bourne, 1992), raise issue with the hypothesis that a 50% deficiency of $G\alpha_s$ can impair hormone responsiveness. Indeed, *in vitro* studies of tissues and cells from subjects with PHP type 1a have often demonstrated normal hormonal responsiveness despite a 50% reduction in $G\alpha_s$ expression (Levine, 1996).

Several observations now suggest a molecular basis for the variable phenotypes that arise from identical *GNAS1* gene defects. First, clinical genetic studies have documented that PHP type 1a and pseudo-PHP frequently occur in the same family, but are not present in the same generation (Fig. 10). Second, analysis of published pedigrees has indicated that in nearly all cases maternal transmission of $G_s\alpha$ deficiency leads to PHP type 1a, whereas paternal transmission of the defect leads to pseudo-PHP (Fig. 10) (Wilson *et al.,* 1994; Levine *et al.,* 1986; Davies and Hughes, 1993; Nakamoto *et al.,* 1998). These findings are inconsistent with models in which chance determines phenotype or in which a second gene is interactive with the defective *GNAS1* gene, as both PHP type 1a and pseudo-PHP would be expected to occur with equal frequency and in the same sibship. An alternative explanation for the variable phenotypes that occur is that *GNAS1* expression

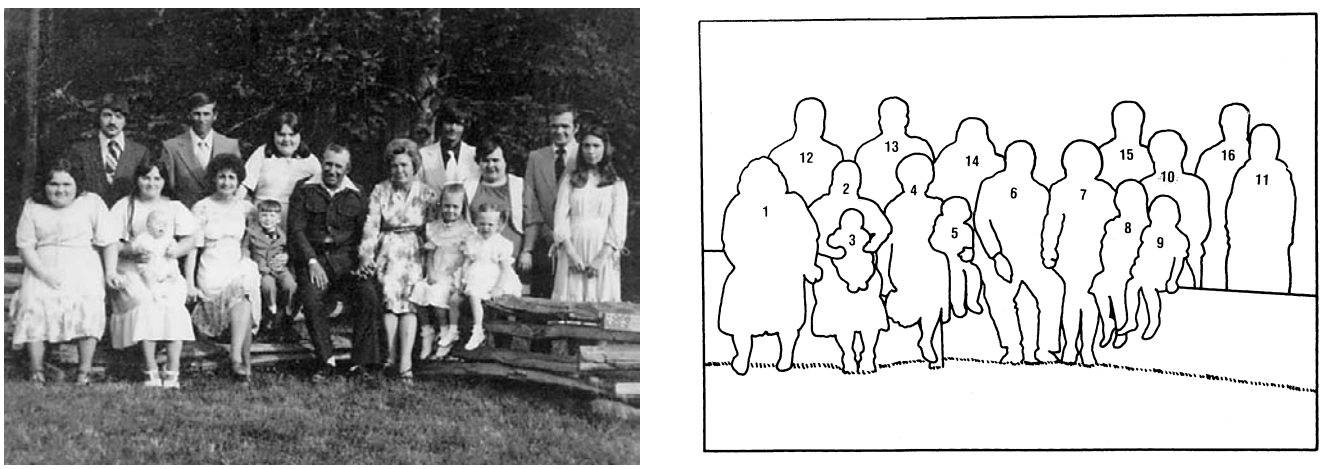

Figure 10 A large kindred containing members with AHO showing variable expression of the same mutation in the *GNAS1* gene (Weinstein *et al.,* 1990). There is increasing severity of $G\alpha_s$ deficiency with each generation. Subjects 1, 2, 14, and 10 have PHP type 1a with typical features of AHO and multihormone resistance. Their sisters, subjects 4 and 11, are unaffected. Subject 3, the son of patient 2, is the most severely affected member of the family and has multiple developmental defects. Subjects 5, 8, and 9, the children of unaffected sisters 4 and 11, respectively, are also normal. Patient 6, the father of the six women, is normal, whereas subject 7, the mother, carries the same gene defect yet her only manifestation of AHO is shortening of the distal phalanx of one thumb (i.e., pseudo-PHP). From Levine *et al.* (1982).

is modified by genomic imprinting (Davies and Hughes, 1993). Genomic imprinting is an unusual mode of regulation of gene expression that results in the expression of only one parental allele in somatic tissues. Thus, genomic imprinting can account for functional differences that arise as a consequence of the parental origin of a gene allele. Recent studies have indeed confirmed that the *GNAS1* gene is imprinted, but in a far more complex manner than had been anticipated. Three unique, alternative first exons, and their respective promoters, are located within the 35-kb upstream of the *GNAS1* exon 1 (Fig. 8). These promoters each have differentially methylated regions (DMR) that show allele-specific methylation that correspond to patterns of genomic imprinting and monoallelic transcription. The most 5′ of these exons encodes NESP55, which is expressed exclusively from the maternal allele and which is methylated at the DMR on the paternal allele. By contrast, the XLαs exon, which is only 11 kb downstream, is paternally expressed and shows methylation of the DMR on the maternal allele (Hayward *et al.,* 1998a,b). The exon 1A DMR is only 2 kb downstream from XLαs and is similarly methylated on the maternal allele and expressed from only the paternal allele (Liu *et al.,* 2000a). Despite the reciprocal imprinting in both the paternal and the maternal directions of the *GNAS1* gene, expression of Gα_s appears to be biallelic in all human tissues that have been examined thus far (Campbell *et al.,* 1994; Hayward *et al.,* 1998a,b), although monoallelic expression exclusively from the maternally derived allele has been documented in some tissues (e.g., renal proximal tubule) in murine models of PHP type 1a. These models have been developed through disruption of a single *Gnas* gene in murine embryonic stem cells (Yu *et al.,* 1998; Schwindinger *et al.,* 1998b), and although the heterozygous (*Gnas* +/−) knockout mice have reduced levels of Gα_s protein, they lack many of the features of the human disorder. Biochemical analyses of *Gnas* (+/−) mice demonstrate that Gα_s expression derives from only the maternal allele in some tissues (e.g., renal cortex) but from both alleles in most other tissues (e.g., renal medulla) (Weinstein *et al.,* 2000; Ecelbarger *et al.,* 1999; Yu *et al.,* 1998). Accordingly, mice that inherit the defective *Gnas* gene maternally express only that allele in imprinted tissues, such as the PTH-sensitive renal proximal tubule, with the result that there is no functional Gα_s protein. By contrast, the 50% reduction in Gα_s expression that occurs in nonimprinted tissues, which express both *Gnas* alleles, may account for more variable and moderate hormone resistance in these sites (e.g., the thyroid). Thus, variable hormonal responsiveness implies that haploinsufficiency of Gα_s is tissue-specific; that is, in some tissues a 50% reduction in Gα_s is still sufficient to facilitate normal signal transduction. Confirmation of this proposed mechanism in patients with AHO will require demonstration that the human Gα_s transcript is paternally imprinted in the renal cortex.

In AHO, inherited *GNAS1* gene mutations reduce expression or function of Gα_s protein. By contrast, in the McCune–Albright syndrome, post-zygotic somatic mutations in the *GNAS1* gene enhance activity of the protein (Schwindinger *et al.,* 1992; Weinstein *et al.,* 1991). These mutations lead to constitutive activation of adenylyl cyclase, and result in proliferation and autonomous hyperfunction of hormonally responsive cells. The clinical significance of Gα_s activity as a determinant of hormone action is emphasized by the description by Iiri *et al.* (1994) of two males with both precocious puberty and PHP type 1a. These two unrelated boys had identical *GNAS1* gene mutations in exon 13 (A366S) that resulted in a temperature-sensitive form of Gα_s (Fig. 9). This Gα_s is constitutively active in the cooler environment of the testis, while being rapidly degraded in other tissues at normal body temperature. Thus, different tissues in these two individuals could show hormone resistance (to PTH and TSH), hormone responsiveness (to ACTH), or hormone independent activation (to luteinizing hormone (LH)).

Pseudohypoparathyroidism Type 1b

Patients with PHP type 1b lack features of AHO, manifest hormone resistance that is limited to PTH target organs (Table I), and have normal Gα_s activity (Fig. 5) (Levine *et al.,* 1983a). Although patients with PHP type 1b fail to show a nephrogenous cAMP response to PTH (Fig. 3), they often manifest osteopenia or skeletal lesions similar to those that occur in patients with hyperparathyroidism, including osteitis fibrosa cystica (Fig. 4) (Kidd *et al.,* 1980). Cultured bone cells from one patient with PHP type 1b and osteitis fibrosa cystica were shown to have normal adenylyl cyclase responsiveness to PTH *in vitro* (Murray *et al.,* 1993). These observations have suggested that at least one intracellular signaling pathway coupled to the PTH receptor may be intact in patients with PHP type 1b.

Specific resistance of target tissues to PTH, and normal activity of Gα_s, had implicated decreased expression or function of the PTH1R as the cause for hormone resistance. In addition, cultured fibroblasts from some, but not all, PHP type 1b patients were shown to accumulate reduced levels of cAMP in response to PTH (Silve *et al.,* 1986, 1990) and contain decreased levels of mRNA encoding the PTH/PTHrP receptor (Suarez *et al.,* 1995). Several lines of evidence suggest that the primary defect in PHP type 1b is *not* in the gene encoding the PTH1R, however. First, pretreatment of cultured fibroblasts from subjects with PHP type 1b with dexamethasone was found to normalize the PTH-induced cAMP response and to increase expression of PTH1R mRNA (Suarez *et al.,* 1995). Second, molecular studies have failed to disclose mutations in the coding exons (Schipani *et al.,* 1995) and promoter regions (Bettoun *et al.,* 1997) of the PTH1R gene or its mRNA (Fukumoto *et al.,* 1996). Furthermore, linkage studies have demonstrated discordance between inheritance of PHP type 1b and PTH1R alleles, thus excluding defects even in regions of the gene that have not yet been examined by sequence analysis (Jan de Beur *et al.,* 2000). Third, mice (Lanske *et al.,* 1996) and humans (Jobert *et al.,* 1998) that are heterozygous for inactivation of the gene encoding the PTH1R do not manifest PTH resistance or hypocalcemia.

Finally, inheritance of two defective PTH1R genes results in Blomstrand chondrodysplasia, a lethal genetic disorder characterized by advanced endochondral bone maturation (Jobert *et al.,* 1998). Thus, it is likely that the molecular defect in PHP type 1b resides in other gene(s) that regulate expression or activity of the PTH1R.

Although most cases of PHP type 1b appear to be sporadic, familial cases have been described in which transmission of the defect is most consistent with an autosomal dominant pattern (Allen *et al.,* 1988; Winter and Hughes, 1980) In some of these kindreds, there appears to be a pattern of inheritance consistent with imprinting as observed in PHP type 1a (Juppner *et al.,* 1998; Wu *et al.,* 2001). Molecular genetic studies of multiplex PHP type 1b kindreds have used linkage analysis to map the location of the PHP type 1b gene to a small region of chromosome 20*q*13.3 near the *GNAS1* gene (Juppner *et al.,* 1998; Jan de Beur *et al.,* 2001a). Although it is possible that the PHP type 1b locus corresponds to a unique gene involved in PTH responsiveness that resides upstream of *GNAS1* (Juppner *et al.,* 1998), more recent observations imply that defective imprinting of *GNAS1* may account for selective deficiency of $G\alpha_s$ in the renal proximal tubule in PHP type 1b. Upstream of the first exon for $G\alpha_s$ is a DMR that is normally methylated on the maternal allele and unmethylated on the paternal allele, which is expressed (Fig. 8). Remarkably, subjects with PHP type 1b show a loss of methylation on the maternal allele, which permits biallelic expression of alternative transcripts that contain exon 1A as the first exon (Liu *et al.,* 2000b; Jan de Beur *et al.,* 2001b). It is conceivable that loss of maternal-specific imprinting of the exon 1A region, and consequent transcription of both alleles, may interfere with normal expression of $G\alpha_s$ transcripts from the maternal allele in imprinted tissues such as the renal proximal tubules, thereby leading to PTH resistance.

Pseudohypoparathyroidism Type 1c

Resistance to multiple hormones has been described in several patients with AHO who have do not have a demonstrable defect in G_s or G_i (Levine *et al.,* 1983a; Farfel *et al.,* 1981; Izraeli *et al.,* 1992). This disorder is termed PHP type 1c. The nature of the lesion in such patients is unclear, but it could be related to some other general component of the receptor–adenylyl cyclase system, such as the catalytic unit (Barrett *et al.,* 1989). Alternatively, these patients could have functional defects of G_s (or G_i) that do not become apparent in the assays presently available.

Pseudohypoparathyroidism Type 2

PHP type 2 is the least common form of PHP, and does not appear to have a clear genetic or familial basis. Patients do not have features of AHO. Renal resistance to PTH in PHP type 2 patients is manifested by a reduced phosphaturic response to administration of PTH, despite a normal increase in urinary cAMP excretion (Drezner *et al.,* 1973). These observations suggest that the PTH receptor–adenylyl cyclase complex functions normally to increase cyclic AMP in response to PTH, and are consistent with a model in which PTH resistance arises from an inability of intracellular cAMP to initiate the chain of metabolic events that result in the ultimate expression of PTH action.

Although supportive data are not yet available, a defect in cAMP-dependent protein kinase A has been proposed as the basis for this disorder (Drezner *et al.,* 1973). Alternatively, the defect in PHP type 2 may not reside in an inability to generate a physiological response to intracellular cAMP: a defect in another PTH-sensitive signal transduction pathway may explain the lack of a phosphaturic response. One candidate is the PTH-sensitive phospholipase C pathway that leads to increased concentrations of the intracellular second messengers inositol 1,4,5-trisphosphate and diacylglycerol (Civitelli *et al.,* 1988; Dunlay and Hruska, 1990) and cytosolic calcium (Gupta *et al.,* 1991; Civitelli *et al.,* 1989; Reid *et al.,* 1987; Yamaguchi *et al.,* 1987) (Fig. 1).

In some patients with PHP type 2 the phosphaturic response to PTH has been restored to normal after serum levels of calcium have been normalized by treatment with calcium infusion or vitamin D (Kruse *et al.,* 1989). These results point to the importance of Ca^{2+} as an intracellular second messenger. Finally, a similar dissociation between the effects of PTH on generation of cAMP and tubular reabsorption of phosphate has been observed in patients with profound hypocalcemia due to vitamin D deficiency (Rao *et al.,* 1985), suggesting that some cases of PHP type 2 may in fact represent vitamin D deficiency.

Clinical Features

Hypocalcemia

The *sine qua non* of PHP is target tissue resistance to the action of PTH, and the major clinical manifestations of PHP are the consequence of reduced concentrations of ionized calcium in blood and extracellular fluid. Hypocalcemia may produce neuromuscular, ectodermal, cardiac, and ocular changes. The primary hallmark of hypocalcemia is *tetany,* a condition characterized by increased neuromuscular excitability. Symptoms of tetany include carpopedal spasm, convulsions, circumoral and acral paresthesias, muscle cramps, and stridor. Laryngeal spasm occurs most commonly in young children and may be the presenting manifestation of severe hypocalcemia. Tetany may be potentiated by hypomagnesemia and can be mimicked by hypokalemia. Hypocalcemia may cause seizures with abnormal EEG patterns, or may also unmask a previously unsuspected seizure disorder or greatly aggravate known epilepsy. Latent tetany may be detected by tapping over the facial nerve to produce a facial twitch (Chvostek sign) or by inflating a blood pressure cuff above systolic pressure for 3 min to produce carpal spasm (Trousseau sign). A slightly positive Chvostek sign may occur in 15% of normal adults. Importantly, both of

these signs can be absent even in patients with severe hypocalcemia. The natural history of PHP is quite variable. Although PHP is congenital, hypocalcemia is not present from birth, and PTH resistance seems to arise gradually during childhood. The initial manifestations of tetany typically occur between 3 and 8 years of age, but the significance of these findings may not be appreciated and the diagnosis of hypocalcemia may be delayed for months or even years. Increasing levels of serum phosphate, PTH, and $1,25(OH)_2D_3$ were documented to precede the onset of hypocalcemia in one child as he advanced from 3 to $3\frac{1}{2}$ years of age (Tsang *et al.,* 1984). These findings seem to imply that the earliest manifestation of PTH resistance is the inability to induce a phosphaturic response. Indeed, we have found some very young infants that we identified through genetic screening to have mild hypercalcemia as an apparent response to increasing levels of PTH and $1,25(OH)_2D$, implying that skeletal responsiveness to PTH is maintained even as renal resistance evolves (unpublished observations). In a second report serial PTH infusions were used to evaluate hormone responsiveness. This child was shown to have a normal cAMP response at age 3 months when serum levels of calcium, phosphorous, and PTH were normal, but was found to have an abnormal cAMP response when retested at age 2.6 years after he had developed tetany and was found to be hypocalcemic (Barr *et al.,* 1994). At the time of his second PTH infusion, the child had markedly elevated serum concentrations of phosphorous and PTH and was receiving thyroxine for recently diagnosed hypothyroidism (Barr *et al.,* 1994). Some affected children show few symptoms of tetany and the diagnosis of PHP is recognized only later in life after hypocalcemia is inadvertently discovered or when features of AHO become obvious. Hypocalcemia may not always provide a clue to the clinical diagnosis of PHP, however, as some PHP patients are able to maintain a normal serum calcium level without treatment (i.e., normocalcemic PHP) (Drezner and Haussler, 1979).

As in other forms of hypoparathyroidism, patients may have coarse, brittle hair, dermatitis with eczema, and brittle nails. Chronic hypocalcemia and hyperphosphatemia may lead to soft tissue calcifications in patients with PHP. Posterior subcapsular cataracts develop frequently, and computerized tomography of the brain reveals calcification of the basal ganglion (Illum and Dupont, 1985) and frontal lobes in most patients. Occasional patients may also have calcifications in the heart (Schuster and Sandhage, 1992).

Skeletal Remodeling

PTH responsiveness of the skeleton appears to be variable in subjects with PHP. In contrast to the well-documented resistance of the kidney to PTH, there is less compelling evidence to support the notion that bone cells are resistant to PTH in PHP. There is a spectrum of bone disease in PHP: some subjects have apparently normal appearing bone while others have radiological or histological evidence of significant bone resorption (Burnstein *et al.,* 1985). Patients with PHP type 1a typically have little or no evidence of diffuse skeletal involvement, while patients with PHP type 1b often demonstrate evidence of osteopenia or hyperparathyroid bone disease, including osteitis fibrosa cystica (Fig. 4). Cultured bone cells from one patient with PHP type 1b and osteitis fibrosa cystica were shown to have normal adenylyl cyclase responsiveness to PTH *in vitro* (Murray *et al.,* 1993).

Patients with PHP may develop additional abnormalities in bone metabolism, including osteomalacia (Burnstein *et al.,* 1985), rickets (Dabbaugh *et al.,* 1984), renal osteodystrophy (Hall *et al.,* 1981), and osteopenia (Breslau *et al.,* 1983). These skeletal abnormalities result from excessive PTH or deficient $1,25(OH)_2D_3$.

Albright Hereditary Osteodystrophy

Subjects with PHP type 1a or pseudo-PHP typically manifest a characteristic constellation of developmental defects, termed Albright hereditary osteodystrophy, that includes short stature, obesity, a round face, shortening of the digits (brachydactyly), subcutaneous ossification, and dental hypoplasia (Fig. 7) (Albright *et al.,* 1942, 1952). Considerable variability occurs in the clinical expression of these features even among affected members of a single family, and all of these features may not be present in every case (Faull *et al.,* 1991). On rare occasion, it may be impossible to detect any features of AHO in an individual with $G\alpha_s$ deficiency (Fig. 10) (Weinstein *et al.,* 1990; Miric *et al.,* 1993).

Although patients with AHO may be of normal height and weight, approximately 66% of children and 80% of adults are below the 10th percentile for height. This reflects a disproportionate shortening of the limbs, as arm span is less than height in the majority of patients. Obesity is a common feature of AHO and about one-third of all patients with AHO are above the 90th percentile of weight for their age, despite their short stature (Fitch, 1982) (Fig. 7A). Patients with AHO typically have a round face, a short neck, and a flattened bridge of the nose. Numerous other abnormalities of the head and neck have also been noted. Ocular findings include hypertelorism, strabismus, nystagmus, unequal pupils, diplopia, microphthalmia, and a variety of abnormal findings on funduscopic exam that range from irregular pigmentation to optic atrophy and macular degeneration. Head circumference is above the 90th percentile in a significant minority of children (Fitch, 1982). Dental abnormalities are common in subjects with PHP type 1a and include dentin and enamel hypoplasia, short and blunted roots, and delayed or absent tooth eruption (Croft *et al.,* 1965).

Brachydactyly is the most reliable sign for the diagnosis of AHO, and may be symmetrical or asymmetrical and involve one or both hands or feet (Fig. 7). Shortening of the distal phalanx of the thumb is the most common abnormality; this is apparent on physical exam as a thumb in which the ratio of the width of the nail to its length is increased (so-called "Murder's thumb" or "potter's thumb," Fig. 7D). Shortening of the metacarpals causes shortening of the digits, particularly the fourth and fifth. Shortening of the metacarpals may also be recognized on physical exam as dimpling over the knuckles of a clenched fist (Archibald sign, Fig. 7C). Often a definitive diagnosis requires careful examination of radiographs of the

hands and feet (Fig. 7B). A specific pattern of shortening of the bones in the hand has been identified, in which the distal phalanx of the thumb and third through fifth metacarpals are the most severely shortened (Poznanski *et al.,* 1977; Graudal *et al.,* 1988). This may be useful in distinguishing AHO from other unrelated syndromes in which brachydactyly occurs, such as familial brachydactyly, Turner syndrome, and Klinefelter syndrome (Poznanski *et al.,* 1977).

In addition to brachydactyly, several other skeletal abnormalities are present in AHO. Numerous deformities of the long bones have been reported, including a short ulna, bowed radius, deformed elbow or cubitus valgus, coxa vara, coxa valga, genu varum, and genu valgus deformities (Fitch, 1982). The most common abnormalities of the skull are hyperostosis frontalis interna and a thickened calvarium. The skeletal abnormalities of AHO may not be apparent until a child is 5 years old (Steinbach *et al.,* 1965). Bone age is advanced 2–3 years in the majority of patients (Fitch, 1982). Spinal cord compression has been reported in several patients with AHO (Alam and Kelly, 1990).

Patients with AHO develop heterotopic ossifications of the soft tissues or skin (osteoma cutis) that are unrelated to abnormalities in serum calcium or phosphorous levels. Osteoma cutis is present in 25 to 50% of patients and is typically noted in infancy or early childhood. Occasionally, ossification of the skin is the presenting feature of AHO (Prendiville *et al.,* 1992; Izraeli *et al.,* 1992). Blue-tinged, stony hard papular or nodular lesions that range in size from pinpoint up to 5 cm in diameter often occur at sites of minor trauma and may appear to be migratory on serial examinations (Prendiville *et al.,* 1992). Biopsy of these lesions reveals heterotopic ossification with spicules of mineralizing osteoid and calcified cartilage. More extensive and progressive ossification that affects the deep connective tissues occurs in subjects with progressive osseous heteroplasia (POH), a rare genetic disorder with apparent autosomal dominant inheritance (Kaplan *et al.,* 1994). In its most specific characterization, POH does not share any other phenotypic features with AHO or display hormonal resistance. Recently, a mutation in *GNAS1* has been identified in a girl with POH-like heterotopic ossification, normal PTH responsiveness, and no features of AHO except mild brachydactyly of the fourth and fifth metacarpals (Eddy *et al.,* 2000). The discovery of *GNAS1* mutations in an overlap syndrome of AHO and POH subsequently led to the analysis of *GNAS1* in several POH kindreds. Heterozygous inactivating mutations in *GNAS1,* including the 4-bp deletion in exon 7 that is a mutational "hot spot" in AHO, were identified and paternal transmission of POH was confirmed by documenting inheritance of defective *GNAS1* alleles from fathers (Shore *et al.,* 1999). The mechanism by which heterozygous loss of function mutations in *GNAS1* leads to heterotopic bone formation is unclear. The association of POH with mutations in *GNAS1* further widens the phenotypic expression of $G\alpha_s$ deficiency, but the molecular modifiers that account for the variable phenotypes associated with identical gene defects in different families are unknown (see below).

The presence of these developmental and skeletal abnormalities does not necessarily indicate that a patient has AHO and $G\alpha_s$ deficiency. Features of AHO, particularly shortened metacarpals or metatarsals, may occur in normal subjects, as well as in patients with hormone deficient hypoparathyroidism (Moses *et al.,* 1974; Isozaki *et al.,* 1984; Le Roith *et al.,* 1979), renal hypercalciuria (Moses and Notman, 1979), and primary hyperparathyroidism (Sasaki *et al.,* 1985). Moreover, several features of AHO, for example obesity, round face, brachydactyly, and mental retardation, are common to other disorders (e.g., Prader–Willi syndrome, acrodysostosis, Ullrich–Turner syndrome, Gardener's syndrome), many of which are associated with unrelated chromosomal defects. In some instances overlapping clinical features between AHO and other syndromes may lead to confusion. For example, AHO in a mother and her daughter has been associated with a proximal 15*q* chromosomal deletion resembling that found in Prader-Willi syndrome (Hedeland *et al.,* 1992). A growing number of reports have described small terminal deletions of chromosome 2*q* in patients with variable AHO-like phenotypes. Terminal deletion of 2*q*37 [del(2)(*q*37.3)] is the first consistent karyotypic abnormality that has been documented in patients with an AHO-like syndrome (Wilson *et al.,* 1995; Phelan *et al.,* 1995). These patients have normal endocrine function and normal $G_{s\alpha}$ activity, however (Phelan *et al.,* 1995). Thus, high-resolution chromosome analysis, biochemical/molecular analysis, and careful physical and radiological examination are essential in discriminating between these phenocopies and AHO.

GENETICS

AHO, including PHP type 1a and pseudo-PHP, is inherited in an autosomal dominant manner via transmission of defects in the *GNAS1* gene (see above). The inheritance of other forms of PHP is less well characterized. Transmission of PHP type 1b is most consistent with an autosomal dominant pattern and appears to be linked to inheritance of a defective maternal *GNAS1* allele (Juppner *et al.,* 1998; Jan de Beur *et al.,* 1997, 2001b). Clinical and molecular evidence implicate genomic imprinting as the basis for development of hormone resistance in individuals with PHP types 1a and 1b who have mutations in *GNAS1*. PHP type 2 is typically a sporadic disorder, although one case of familial PHP type 2 has been reported (Van Dop, 1989).

Diagnosis of Pseudohypoparathyroidism

Clinical Evaluation

The diagnosis of PHP should be considered in any patient who has biochemical hypoparathyroidism. A low serum calcium level may be found during an evaluation of unexplained paresthesias or seizures, or may be discovered after multichannel analysis of a blood specimen obtained as part of a routine examination. PHP should be strongly suspected if the

serum concentration of PTH is elevated, although occasionally serum levels of PTH are "inappropriately" normal in subjects with PHP owing to confounding hypomagnesemia (Allen *et al.,* 1988) or other factors (Attanasio *et al.,* 1986). Hypocalcemia may be precipitated or worsened during times of "stress" on calcium homeostasis, such as during early pregnancy, lactation, or during an episode of acute pancreatitis. Although hypocalcemia is present in most patients with PHP by the end of the first decade of life, occasional patients do not develop hypocalcemia until they are in their 50s (Hamilton, 1980).

Cataracts and intracranial calcification, particularly of the basal ganglion, occur commonly in patients with all forms of chronic hypoparathyroidism, including PHP. The presence of these calcifications does not help to discriminate among the various causes of hypocalcemia and hyperphosphatemia (Litvin *et al.,* 1981), but does indicate that the mineral defects have been present for at least 5 to 10 years. Intracranial calcifications are readily detected when CT scanning is employed (Sachs *et al.,* 1982; Korn-Lubetzki *et al.,* 1980) and may occasionally be associated with symptoms such as Parkinson's disease (Pearson *et al.,* 1981). Other unusual presenting manifestations of PHP include neonatal hypothyroidism (Levine *et al.,* 1985; Weisman *et al.,* 1985), unexplained cardiac failure (Miano *et al.,* 1981), Parkinson's disease (Pearson *et al.,* 1981), and spinal cord compression (Cavallo *et al.,* 1980). Finally, relatives of patients with PHP should be evaluated for signs of PHP as expression of the disorder can be very subtle.

A diagnosis of PHP can be made with reasonable certainty when hypocalcemia is accompanied by hyperphosphatemia and elevated circulating levels of PTH. Further corroboration of the diagnosis requires demonstration of normal renal function and normal serum levels of magnesium and 25-(OH)-vitamin D. A positive family history lends further support to the diagnosis. The presence of AHO and manifestations of multihormone resistance, such as hypothyroidism or hypogonadism, favors a diagnosis of PHP type 1a (Levine *et al.,* 1983a). When most or all of these features are present, more sophisticated tests may not be necessary to confirm the clinical diagnosis. Serum calcium levels can fluctuate in patients with PHP, and may spontaneously change from low to normal and *vice versa,* thus contributing to the confusion regarding the distinction between PHP and pseudo-PHP (Breslau *et al.,* 1980; Drezner and Haussler, 1979). However, the abnormal cAMP response to administered PTH (below) does not become normal in PHP patients who become normocalcemic with or without treatment. Thus, the PTH infusion remains the most reliable test for distinguishing between these two variants (Fig. 3).

Biochemical Tests

The biochemical hallmark of PHP is the failure of the target organs, bone and kidney, to respond normally to PTH. Additional tests have been developed to identify subjects with PHP type 1a; these research tests, which are based on analysis of $G\alpha_s$ protein or the *GNAS1* gene, are only rarely indicated under typical clinical circumstances. The definitive diagnosis of PHP at the present time depends on the demonstration of a deficient cAMP, $1,25(OH)_2D_3$, or phosphate response to an active preparation of PTH. The classical tests of Ellsworth and Howard, and of Chase, Melson, and Aurbach involved the intravenous infusion of 200–300 USP units of bovine parathyroid extract (parathyroid injection, USP; Lilly) and subsequent measurement of urinary excretion of nephrogenous (or total) cAMP (Fig. 3) and phosphate. This relatively crude PTH preparation is no longer available, and has been replaced by synthetic peptides corresponding to the amino terminal region of human PTH (e.g., hPTH(1–34) peptide). Several diagnostic protocols have been described in which the response to an infusion of synthetic hPTH(1–34) peptide is used to differentiate among disorders of PTH responsiveness (Furlong *et al.,* 1986; McElduff *et al.,* 1987; Mallette, 1988). A standard protocol involves the infusion of this PTH fragment, 200 units in an adult and 3 units/kg body weight (200 units maximum) in children over the age of 3 years, intravenously over 10 min (Mallette, 1988; Mallette *et al.,* 1988) (Fig. 11). Test subjects should be in a fasting state and active urine output should be initiated and maintained by the ingestion of 200 ml of water per hour 2 hr prior to the infusion of PTH and continuing through the study. A baseline urine collection should be made in a 60-min period preceding the PTH infusion. Starting at time 0 urine should be collected in separate collections at the 0- to 30-, 30- to 60-, and 60- to 120–min time periods. Blood samples should be obtained at time 0 and at 2 hr after the start of PTH infusion for measurement of serum creatinine and phosphorus concentrations. Urine

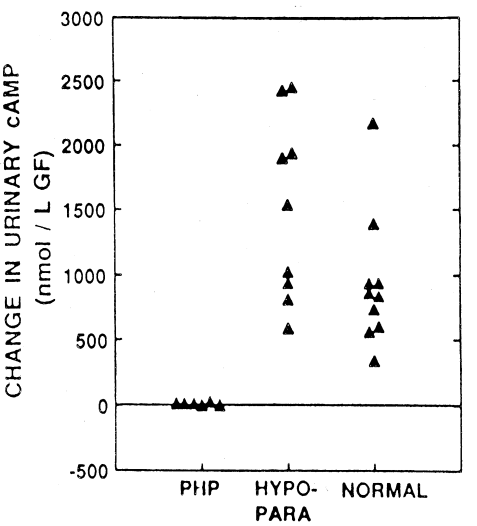

Figure 11 Change in urinary cAMP excretion in response to PTH infusion in patients with pseudohypoparathyroidism, and hypoparathyroidism and normal subjects. Reprinted with permission from Mallette *et al.* (1988)). The mean increase in cAMP excretion in the first 30-min collection period was 6.3 nmol/liter GF with individual values ranging from 0 to 20. In patients with hypoparathyroidism the increase was 1512 with the minimum being over 500 nmol/liter GF. In normal subjects the mean rise of cAMP was 937 nmol/L GF with a minimum increase of about 300.

samples should be analyzed for cAMP, phosphorus, and creatinine concentrations.

The preferred unit for expression of urinary cAMP is nmols/100 ml (or per liter) of glomerular filtrate (nmol/dl GF). The cAMP response during the first 30 min after the start of PTH infusion better differentiates patients with PHP type 1 from those with hypoparathyroidism and from normal subjects than other parameters of cAMP metabolism (Mallette *et al.,* 1988) (Fig. 11). The change in urinary cAMP/mg creatinine during the same 30-min period also discriminates well between patients with PHP and normal subjects or patients with hypoparathyroidism (Mallette *et al.,* 1988). Several metabolic abnormalities such as hypo- and hypermagnesemia and metabolic acidosis may interfere with the renal generation and excretion of cAMP in response to PTH (Rude *et al.,* 1976; Slatopolsky *et al.,* 1976; Beck and Davis, 1975; Beck *et al.,* 1975). The maximal urinary cAMP response to PTH increases after suppression of endogenous PTH in patients with PHP type 1, but nevertheless does not reach that of the normal range (Stone *et al.,* 1993) These abnormalities should be corrected if possible, but probably do not interfere with the interpretation of the test.

Calculation of the phosphaturic response to PTH as the percentage decrease in tubular maximum for phosphate reabsorption (percentage fall in TmP/GF) during the first hour after PTH infusion yields the best separation between normal subjects and patients with PHP or hypoparathyroidism (Mallette *et al.,* 1988) (Fig. 12). However, distinction between groups is also possible when the results are expressed as the fall in percentage tubular reabsorption of phosphorus (decrease in % TRP). A nomogram has been developed that facilitates calculation of $TmPO_4$/GFR (Walton and Bijvoet, 1975). TmP/GF is elevated in patients with PHP and hypoparathyroidism. Patients with hormone-deficient hypoparathyroidism have a steep fall in TmP/GF during the first hour after beginning the infusion of PTH. This fall does not occur in patients with PHP (for further details see references by Mallette *et al.* (1988; Mallette *et al.,* 1988)). Although a normal phosphate response may occur in PHP type 1 patients with serum calcium or PTH levels in the normal range (Stone *et al.,* 1993), in patients with PHP type 2 the phosphaturic response to PTH is not changed despite at least a 10-fold increase in cAMP excretion. Unfortunately, interpretation of the phosphaturic response to PTH is often complicated by random variations in phosphate clearance, and it is sometimes not possible to classify a phosphaturic response as normal or subnormal regardless of the criteria employed. More perplexing yet is the observation that biochemical findings that resemble PHP type 2 have been found in patients with various forms of vitamin D deficiency (Rao *et al.,* 1985). In these patients, marked hypocalcemia is accompanied by hyperphosphatemia due presumably to an acquired dissociation between the amount of cAMP generated in the renal tubule and its effect on phosphate clearance.

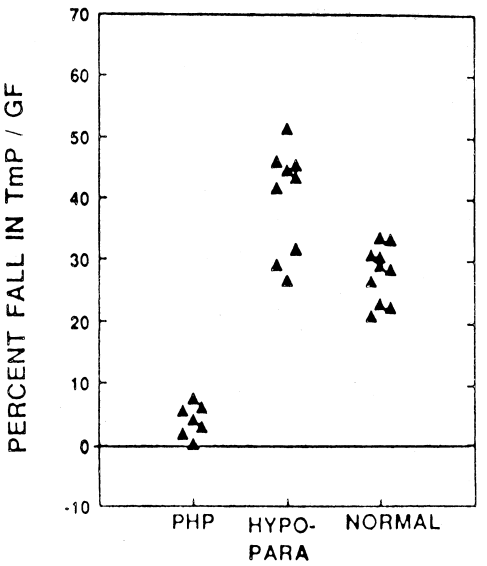

Figure 12 Percentage fall in TmP/GF from the baseline value in the first hour after infusion of PTH in patients with pseudohypoparathyroidism, and hypoparathyroidism and in normal subjects. Reprinted with permission from Mallette *et al.* (1988).

The plasma cAMP response to PTH can also be used to differentiate patients with PHP type 1 from normal subjects and from patients with hypoparathyroidism (Furlong *et al.,* 1986; Sohn *et al.,* 1984; Stirling *et al.,* 1991). Patients with PHP type 2 can be expected to have normal responsiveness, however. This test offers few advantages over protocols that assess the urinary excretion of cAMP, as changes in plasma cAMP in normal subjects and patients with hypoparathyroidism are much less dramatic than changes in urinary cAMP, and urine must still be collected if one wishes to assess the phosphaturic response to PTH. One reasonable indication for measuring the plasma cAMP response to PTH is the evaluation of patients in whom proper collection of urine is not possible, such as young children (Stirling *et al.,* 1991).

The plasma $1,25(OH)_2D_3$ response to PTH has been used to differentiate between hormone-deficient and hormone-resistant hypoparathyroidism (Miura *et al.,* 1990; McElduff *et al.,* 1987). In contrast to normal subjects and patients with hypoparathyroidism, patients with PHP had no significant increase in circulating levels of $1,25(OH)_2D_3$. This proposed test demonstrates nicely the difference in the pathophysiology between hypoparathyroidism and PHP. Its clinical relevance is probably limited to distinguishing type 1 from type 2 PHP where the expected increase in the latter form of PHP might be a more reliable parameter than the phosphaturic response to PTH.

Treatment

Urgent treatment of acute or severe symptomatic hypocalcemia in patients with PHP (or other forms of hypoparathyroidism) is best accomplished by the infusion of calcium intravenously. Vitamin D is not required. The

goal is alleviation of symptoms and prevention of laryngeal spasm and seizures. Hyperphosphatemia, alkalosis, and hypomagnesemia should be corrected. The serum calcium should be increased to the mid-normal range. The desired serum calcium levels can usually be obtained by injecting 1–3 g of calcium gluconate (93–279 mg of elemental calcium, 10–30 ml of 10% calcium gluconate) over a 10-min period followed by continuous infusion of calcium (up to 100 mg/hr) using a solution of 5% dextrose in water containing 100 ml of 10% calcium gluconate (930 mg of elemental calcium) per liter. A 10% solution of calcium chloride is available for intravenous use but it is very irritating to the veins. The serum calcium level should be measured at frequent intervals, and the amount of intravenous calcium should be adjusted accordingly. Electrocardiographic monitoring is advisable when patients are receiving digitalis-like drugs because increasing serum calcium levels can predispose to digitalis toxicity. Oral calcium and vitamin D therapy should be started as soon as possible and gradually adjusted to replace the need for intravenous calcium (Lebowitz and Moses, 1992).

The long-term treatment of hypocalcemia in patients with PHP involves the administration of oral calcium and vitamin D or analogs. Patients with PHP may require less intensive therapy than patients with PTH deficiency.

The goals of therapy are to maintain serum-ionized calcium levels in the normal range, to avoid hypercalciuria, and to suppress PTH levels. Patients with hypoparathyroidism have increased urinary calcium excretion in relation to serum calcium and are therefore prone to hypercalciuria (Litvak *et al.,* 1958). By contrast, patients with PHP have significantly lower urinary calcium in relation to serum calcium (Litvak *et al.,* 1958; Yamamoto *et al.,* 1988) and can tolerate serum calcium levels that are within the normal range without developing hypercalciuria (Mizunashi *et al.,* 1990).

Once normocalcemia has been attained attention should be directed toward suppression of PTH levels to normal. This is important because elevated PTH levels in patients with PHP are frequently associated with increased bone remodeling. Hyperparathyroid bone disease, including osteitis fibrosa cystica (Kidd *et al.,* 1980; Steinbach *et al.,* 1965; Kolb and Steinbach, 1962) and cortical osteopenia (Fig. 4) (Breslau *et al.,* 1983), can occur in patients with PHP type 1. These subjects may have elevated serum levels of alkaline phosphatase (Kolb and Steinbach, 1962) and urine hydroxyproline (Breslau *et al.,* 1983). In this regard calcitriol has an advantage over other vitamin D preparations since it may inhibit PTH release directly (Slatopolsky *et al.,* 1984) in addition to the indirect inhibition caused by elevating the serum calcium. Since thiazide diuretics effectively increase renal calcium reabsorption in patients with PHP (Breslau and Moses, 1978), these agents along with a low sodium diet can be utilized as an additional means of attaining higher serum calcium levels and better suppression of PTH without inducing hypercalciuria.

Oral calcium is usually administered in amounts from 1 to 3 g of elemental calcium per day in divided doses. To assure optimal absorption, oral calcium supplements should be taken with water or other fluids and with food in the stomach (Shangraw, 1989). Many considerations are involved in the selection of a calcium supplement, and none are unique to the treatment of PHP. Calcium carbonate is an inexpensive form of calcium that is very convenient owing to its high content of elemental calcium (40%). When taken with food, absorption of calcium from calcium carbonate is adequate even in achlorhydric patients. Due to the low content of elemental calcium in calcium lactate (13%) and calcium gluconate (9%), patients must take many tablets to obtain adequate amounts of calcium. Thus, these salts are inconvenient and are often not acceptable to the patients. Calcium citrate is 21% calcium and is well absorbed even in the absence of stomach acidity (Harvey *et al.,* 1988). Although more expensive than many other forms of calcium, calcium citrate has the advantage of causing fewer gastrointestinal side effects. For those who prefer a liquid calcium supplement, calcium glubionate is very palatable and contains 252 mg calcium/10 ml. Ten to 30 ml of a 10% calcium chloride solution (360 to 1080 mg calcium) every 8 hr may be very effective in patients with achlorhydria (Komindr *et al.,* 1989). Hyperchloremic acidosis may occur, which can be prevented by giving half of the calcium as chloride and half as carbonate simultaneously (Komindr *et al.,* 1989). Calcium phosphate salts should be avoided.

All patients with PHP who are hypocalcemic will require vitamin D or analogs in addition to calcium. Calcitriol, the active form of vitamin D, is the most popular physiological treatment choice in patients with PHP. Patients with PHP require about 75% as much calcitriol to maintain normocalcemia as do patients with hypoparathyroidism (Okano *et al.,* 1982). Almost all patients with PHP can be effectively treated with calcitriol in the amount of 0.25 μg twice a day to 0.5 μg four times a day. Because of the expense of calcitriol and the need to administer the drug several times per day, other vitamin D preparations may be preferred. PHP patients respond well to pharmacological doses of ergocalciferol and calcidiol. Ergocalciferol is the least expensive choice for vitamin D therapy, and provides a long duration of action (with corresponding prolonged potential toxicity). Patients with PHP require lower doses of vitamin D than patients with hypoparathyroidism (Okano *et al.,* 1982), an observation that reflects the response of bone and renal distal tubular cells to endogenous PTH (Breslau, 1989). Treatment with calcium and vitamin D usually decreases the elevated serum phosphate to a high normal level because of a favorable balance between increased urinary phosphate excretion and decreased intestinal phosphate absorption. In general, phosphate-binding gels such as aluminum hydroxide are not necessary.

Attention should be directed to a number of special situations. Because thiazide diuretics can increase renal calcium reabsorption in patients with PHP (Breslau and Moses, 1978), the inadvertent institution or discontinuation of these drugs may respectively increase or decrease plasma calcium levels. By contrast, furosamide and other

loop diuretics can increase renal clearance of calcium and depress serum calcium levels. The administration of glucocorticoids antagonizes the action of vitamin D (and analogs) and may also precipitate hypocalcemia. The development of hypomagnesemia may also interfere with the effectiveness of treatment with calcium and vitamin D (Rosler and Rabinowitz, 1973).

Estrogen therapy and pregnancy have particularly interesting effects on calcium homeostasis in patients with PHP. Estrogen therapy can reduce serum calcium concentrations in women with PHP type 1 (Breslau and Zerwekh, 1986) as well as women with hypoparathyroidism (Verbeelen and Fuss, 1979). In contrast, at the time of the menses, when estrogen levels are low, some well-treated hypoparathyroid women may develop symptomatic hypocalcemia with the cause remaining unknown (Mallette, 1992). The same phenomenon occurs occasionally in women with PHP. Hypocalcemic symptoms are relieved in 30–60 min by ingestion of 200–400 mg of elemental calcium.

Paradoxically, during the high-estrogen state of pregnancy, the patients of Breslau and Zerwekh remained normocalcemic without therapeutic amounts of calcium and vitamin D (Zerwekh and Breslau, 1986). During pregnancy serum $1,25(OH)_2D_3$ concentrations increased two- to three fold while the PTH levels were nearly half of what was present before pregnancy. After delivery serum calcium and $1,25(OH)_2D_3$ levels decreased and PTH rose (Breslau and Zerwekh, 1986). As placental synthesis of 1,25-dihydroxy vitamin D is not compromised in patients with PHP (Zerwekh and Breslau, 1986), the placenta may have contributed to the maintenance of normocalcemia during pregnancy in these patients. In contrast, patients with hypoparathyroidism may require treatment with larger amounts of vitamin D and calcium in the latter half of pregnancy (Caplan and Beguin, 1990).

Patients with AHO may require specific treatment for unusual problems related to their developmental and skeletal abnormalities. Patients with PHP type 1a should be treated for their associated hypogonadism and hypothyroidism. Ectopic calcification occurs in about 30% of patients with AHO (Fitch, 1982), but rarely causes a problem. However, at times large extraskeletal osteomas may occur (Prendiville *et al.,* 1992). These may require surgical removal to relieve pressure symptoms. Surgery may also be required to relieve neurological symptoms caused by ossification of ligaments (Firooznia *et al.,* 1985). Skeletal abnormalities such as deformed elbows, coxa valga and vara, and bowed tibia may require orthopedic evaluation and treatment. More commonly, treatment is directed at bone abnormalities of the feet. The symptoms are caused by deformities such as painful hyperkeratotic lesions beneath prominent metatarsal heads, bursitis of effected metatarsal phalangeal joints, dislocated toes that do not fit well into shoes, ulcerative lesions from malalignment, or pressure and exostoses (Kalajian, 1978). Measures which have been recommended to relieve pedal symptoms include reduction of hyperkeratotic lesions, padding of the plantar aspects of the feet to accommodate the lesions, soft orthotic devices that can be placed in shoes to cushion weight bearing, custom-made molded shoes, and various medical and physical therapies to relieve acute capsulitis and bursitis (Kalajian, 1978).

Conclusion

The discovery, and biochemical characterization, of the components of the PTH receptor signal transduction pathway have facilitated development of molecular approaches to the investigation of the pathophysiology of PTH resistance. Ultimately, the insights gained from studies of these unusual patients will provide new information concerning the physiological regulation of PTH responsiveness in classical target tissues, such as bone and kidney, as well as in nonclassical targets.

As with many other human disorders for which the disease gene has been identified, it is predicted that ability to diagnose these disorders on a molecular level will extend the clinical spectrum of disease. This prediction has already been fulfilled in AHO where defects in the *GNAS1* gene encoding $G\alpha_s$ have been found to produce highly variable phenotypes. More recent studies, exploring the basis for phenotypic variability in PHP type 1a and the molecular basis for PHP type 1b, have led to the unanticipated discovery of a remarkably complex mechanism of genomic imprinting that regulates *GNAS1* expression and which will provide the focus for much exciting future work.

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CHAPTER 65

Other Skeletal Diseases Resulting from G Protein Defects

Fibrous Dysplasia of Bone and McCune–Albright Syndrome

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Summary

Fibrous dysplasia (FD) is a benign focal bone lesion composed of fibrous connective tissue with interspersed spicules of immature woven bone and, occasionally, islands of cartilaginous tissue. The lesions usually expand concentrically from the medullary cavity toward the cortex, resulting in thinning of the surrounding cortical bone. These lesions may be asymptomatic but often present with local swelling, pain, deformity, or fracture. Involvement of the craniofacial bones may lead to symptoms of nerve entrapment. In the majority of cases patients present with a single focus of FD, referred to as monostotic fibrous dysplasia (MOFD). A minority of patients have multiple bone lesions, referred to as polyostotic fibrous dysplasia (POFD). Of the patients with POFD, a small number have either pigmented "café-au-lait" lesions on the skin and/or endocrinopathies resulting in autonomous hypersecretion of multiple hormones. This constellation is the McCune–Albright syndrome (MAS). With rare exceptions, all three syndromes occur sporadically and are not transmitted from generation to generation. It was previously proposed that MOFD is a disease entity distinct from POFD and MAS. Recent molecular biological examination of patient tissues or cells have determined that virtually all cases of MAS are due to widespread distribution of cells in which an activating mutation of the G_s α-subunit gene is present. This defect results in elevation of intracellular cyclic adenosine monophosphate (cAMP), resulting in a myriad of abnormalities in these patients. The same mutations have been identified in both MOFD and POFD lesions, suggesting that these really represent a more limited presentation of MAS. It is likely that elevated cAMP in mesenchymal precursor cells leads to overexpression of the Fos protein, a *trans*-activating protein that is the product of an immediate–early gene. Overexpression of Fos, as well as other abnormalities resulting from constitutive activation of G_s in these cells disrupts the normal program of proliferation and differentiation of these cells, resulting in FD. These changes also lead to increased expression of interleukin-6 (IL-6) from these cells, which stimulates osteoclast recruitment and activity. Although nonsurgical therapeutic options have been limited, recent early success with the bisphosphonate pamidronate raise hope that medical therapy may halt and even reverse FD.

Clinical Presentation

FD was first described as a distinct entity and named about 50 years ago (Lichtenstein and Jaffe, 1942). The majority of patients with FD have a single bone lesion (MOFD).

The remainder have multiple lesions (POFD) and, of these, a small fraction have MAS. It has been stated that the relative incidence of these three entities is 70% MOFD, 30% POFD, and less than 3% MAS (Nager *et al.,* 1982). However, if one considers the cooccurrence of FD and any other manifestation characteristic of MAS to be a form-fruste of MAS, then the relative incidence of MAS is significantly higher. The most commonly affected bones include the femur (most often the femoral neck and intertrochanteric region), tibia, humerus, ribs, and craniofacial bones (most often the maxilla) (Schlumberger, 1946; Harris *et al.,* 1962; Nager *et al.,* 1982). Other long bones such as the radius, ulna, and fibula are involved less frequently while the bones of the hands, feet, and spine are usually spared. FD is the most common benign bone lesion of the ribs (Schlumberger, 1946).

The most common symptoms associated with FD (more often associated with POFD) include deformities (often producing a limp), pathological fractures, pain, and nerve compression (Schlumberger, 1946; Harris *et al.,* 1962; Danon and Crawford, 1987). Deformities may include leg length discrepancy, outward bowing of the proximal femur (shepherd's-crook deformity), facial assymmetry, orbital displacement and visible or palpable bony enlargement. In some cases deformity of the chest wall may lead to restrictive pulmonary disease (Nager *et al.,* 1982; Schlumberger, 1946). Because of the lack of normal trabecular bone and the thinning of the surrounding cortical bone, pathologic fractures occur frequently and are often recurrent. Pain is caused by rapidly expanding lesions, pathological fractures, and secondary arthritic changes in nearby joints. The most common neurological complications include blindness, deafness and equilibrium disturbances due to compression of the occipital and auditory nerves. High output cardiac failure due to arteriovenous shunting through bone lesions has also been reported (Fischer *et al.,* 1970). Often, particularly in patients with MOFD, FD, may be asymptomatic and first diagnosed as an incidental radiologic finding (Schlumberger, 1946).

Usually FD is identified in the first decade of life either by the onset of symptoms or by screening patients who have other manifestations of MAS. It often progresses in early life but usually becomes quiescent after the third decade (Harris *et al.,* 1962; Danon and Crawford, 1987). It has been reported that advanced bone age in childhood may be associated with FD, even in the absence of endocrine abnormalities (Benedict, 1962; Mauras and Blizzard, 1986). In a small number of cases FD may significantly progress and be initially diagnosed during puberty, pregnancy, or the use of oral contraceptives (Stevens-Simon *et al.,* 1991; Schlumberger, 1946). Often active disease is associated with mildly to moderately increased serum alkaline phosphatase and other biochemical markers of bone turnover. In a small number of cases FD may undergo malignant degeneration, most often to osteosarcoma and occasionally to chondrosarcoma, fibrosarcomas, or other types of sarcoma (Tanner *et al.,* 1961; Yabut *et al.,* 1988; Ruggieri *et al.,* 1994). There is no concensus about whether prior radiotherapy increases the probability of malignant transformation. In contrast to POFD, MOFD usually has a benign clinical course (Schlumberger, 1946). The clinical manifestations of MAS will be summarized below (see Mc-Cune–Albright syndrome).

In some patients with FD, there is the coexistence of FD and intramuscular myxomas (Mazabraud's syndrome) (Mazabraud and Girard, 1957; Lever and Pettingale, 1983; Prayson and Leeson, 1993; Gober and Nicholas, 1993; Aoki *et al.,* 1995). Myxomas occur more frequently in patients with POFD and MAS. Generally these tumors are located in the vicinity of the FD, the thigh being the most common location. In contrast to sporadic myxomas, the myxomas in this syndrome tend to be large and often are multiple. They can easily be misdiagnosed as malignant sarcomas.

McCune–Albright Syndrome

MAS was first described as a specific syndrome distinct from neurofibromatosis and hyperparathyroidism in the 1930s (Albright *et al.,* 1937; McCune, 1936). The classical clinical triad which has generally defined the syndrome is the cooccurrence of sexual precocity, POFD, and areas of skin hyperpigmentation (café-au-lait spots). However, multiple other endocrine abnormalities, including thyroid nodules and hyperthyroidism, adrenal hyperplasia and hypercortisolism, pituitary tumors with acromegaly and hyperprolactinemia and hypophosphatemic rickets or osteomalacia, can also be present (Danon and Crawford, 1987; Mauras and Blizzard, 1986; Schwindinger and Levine, 1993). Other nonendocrine abnormalities are also occasionally present, affecting the liver, heart, thymus, spleen, bone marrow, gastrointestinal tract, and the brain (Weinstein *et al.,* 1991; Shenker *et al.,* 1993). However, many patients may present with only two of the features of the classic triad or one of these features and another endocrine or nonendocrine abnormality (Grant and Martinez, 1983; Rieth *et al.,* 1984; Danon and Crawford, 1987). These patients are also considered to have MAS. In any patient with characteristic café-au-lait spots, clinical evidence of FD, or any of the above endocrine abnormalities, particularly at a young age, the diagnosis of MAS should be considered.

The café-au-lait spots appear as single or multiple brown hyperpigmented flat macules that may be difficult to notice at birth, but become more obvious with age or with sun exposure (Danon and Crawford, 1987; Mauras and Blizzard, 1986). Albright originally stated that café-au-lait spots in MAS have irregular borders like the "coast of Maine," in contrast to those in neurofibromatosis in which the borders are smooth like the "coast of California" (Albright *et al.,* 1937). However, this distinction may be difficult to make and is not reliable in differentiating these two diseases. Often the café-au-lait spots are on one side of the body which usually corresponds to the side with bone involvement. The areas of pigmentation generally do not cross the midline. They are often arranged in a segmental pattern which follows the developmental lines of Blaschko (Happle, 1986). Melanocytes cultured from these lesions have increased numbers of

dendrites and melanosomes and increased levels of tyrosinase, the rate-limiting enzyme for the production of melanin (Kim *et al.,* 1999). These latter changes are likely due to increased intracellular cAMP concentrations. Rarely, MAS is also associated with alopecia (Schwartz *et al.,* 1996).

Sexual precocity may present as early as the first months of life. In girls premature menses is usually the first sign and occurs before breast development (Danon and Crawford, 1987; Mauras and Blizzard, 1986; Feuillan, 1993). Serum estrogen levels increase and decrease cyclically and correspond to the presence of large ovarian follicles which enlarge and regress in a cyclic manner (Comite *et al.,* 1984; Rieth *et al.,* 1984; Feuillan, 1993). There is no evidence of ovulation from the follicles and sometimes they remain as ovarian cysts. Measurements of serum gonadotropin pulsatility and the response to luteinizing-hormone-releasing hormone (LHRH) show suppression of gonadotropin secretion except in older girls who have developed secondary central puberty (Foster *et al.,* 1984). Usually females undergo normal development during adolescence and show normal reproductive function in adult life. Boys can also present with precocious puberty although less commonly than girls. Usual signs include testicular enlargement, secondary sex characteristics, and often evidence of spermatogenesis (Danon and Crawford, 1987; Mauras and Blizzard, 1986). In one case testicular enlargement was present without other evidence of puberty, which resulted from maturation of the tubules but not the Leydig cells (Giovanelli *et al.,* 1978). As in girls the sexual precocity is gonadotropin-independent. Sexual precocity in either sex is usually associated with advanced bone age. As expected, long-acting analogs of LHRH have been shown to be ineffective in the treatment of prepubertal patients (Comite *et al.,* 1984; Feuillan, 1993). Cyproheptadine has shown some success while the aromatase inhibitor testolactone has shown to have beneficial effects in girls (Feuillan *et al.,* 1993; Feuillan, 1993). Preliminary reports suggest that tamoxifen (Eugster *et al.,* 1999) and ketoconazole (Syed and Chalew, 1999) may also be useful for treatment of precocious puberty in MAS

Thyroid abnormalities can occur at any age, even soon after birth (Danon and Crawford, 1987; Mauras and Blizzard, 1986). The patient often presents with a nodular goiter and multinodular disease is often detected by ultrasound. The nodules are almost always benign and usually show increased radioiodine uptake (Feuillan *et al.,* 1990). In most cases with nodular disease hyperthyroidism is also present (Feuillan *et al.,* 1990; Mastorakos *et al.,* 1997). This may range from severe hyperthyroidism with many clinical manifestations to "biochemical" hyperthyroidism in which the thyroid hormone levels are in the upper normal range but the thyroid-stimulating hormone (TSH) is suppressed. Response of TSH to TSH-releasing hormone (TRH) has also demonstrated suppression of TSH. More minor sonographic abnormalities may also be present in patients who are euthyroid (Lair-Milan *et al.,* 1996). Hyperthyroidism may also contribute to accelerated bone growth. Thyroid pathology reveals no evidence of lymphocytic infiltration and thyroid antibodies are almost always undetectable. If hyperthyroidism is clinically significant patients can be treated with antithyroid drugs, radioiodine, or surgery.

Hypercortisolism in MAS is not as common as sexual precocity and hyperthyroidism (Danon and Crawford, 1987; Mauras and Blizzard, 1986). Often the major manifestation of hypercortisolism in children is decreased growth rate. Hypercortisolism can also result in clinically severe abnormalities and present very early in life. MAS patients with hypercortisolism may have an increased incidence of severe nonendocrine manifestations which include unexplained sudden death after adrenalectomy (Shenker *et al.,* 1993). Hormonal evaluation in these patients show that the adrenal glands are hypersecreting cortisol even in the absence of ACTH (Danon and Crawford, 1987; Mauras and Blizzard, 1986). Pathology usually is that of adrenal nodular hyperplasia although adrenal adenoma has also been reported (Benjamin and McRoberts, 1973; Kirk *et al.,* 1999). Most patients will require bilateral adrenalectomy.

Growth hormone excess (acromegaly) and hyperprolactinemia are also present in a small number of MAS patients (Danon and Crawford, 1987; Mauras and Blizzard, 1986). The clinical and biochemical features of acromegaly in MAS are similar to those of acromegaly in general, including elevated serum growth hormones which increase after administration of growth-hormone-releasing hormone (GHRH) or TRH and are not suppressed by glucose. In young children acromegaly will accelerate bone growth. Sometimes the deformities resulting from craniofacial FD may appear similar to those of acromegaly. Since the clinical manifestations are usually subtle and slowly progressive, all cases of MAS or FD should be periodically screened for the presence of acromegaly. Acromegaly in MAS may be associated with hyperprolactinemia. In some cases this may result in hypogonadism and galactorrhea although pubertal development is usually normal (Danon and Crawford, 1987; Mauras and Blizzard, 1986). Hyperprolactinemia in the absence of acromegaly has not been reported in MAS. Associated pathology is most commonly pituitary adenoma or nodular hyperplasia. In one case mammosomatotroph hyperplasia was associated with acromegaly and hyperprolactinemia (Kovacs *et al.,* 1984). Pituitary adenomas and hyperplasia have not been associated with excess secretion of any other pituitary hormones in MAS. The treatment options include surgical excision, radiotherapy, bromocryptine or pergolide, or long-acting somatostatin analogs. Surgery may be technically difficult or impossible if FD is present in the base of the skull. In these cases, radiotherapy may be contraindicated because of possible increase of malignancy in the surrounding bone.

Hyperphosphaturic hypophosphatemic rickets or osteomalacia has been associated with MAS and POFD (Ryan *et al.,* 1968; McArthur *et al.,* 1979; Cole *et al.,* 1983; Lever and Pettingale, 1983; Danon and Crawford, 1987; Mauras and Blizzard, 1986; Hahn *et al.,* 1991; Zung *et al.,* 1995). These patients show inappropriately decreased reabsorption of phosphate from the renal proximal tubule in the absence

of hyperparathyroidism. It has been postulated that a phosphaturic factor is secreted from areas of FD, similar to the mechanism of tumor-associated hyperphosphaturic hypophosphatemic rickets (Dent and Gertner, 1976; McArthur *et al.,* 1979). Others suggest that the hypophosphatemia is caused by a primary defect in the renal proximal tubule (Tanaka and Suwa, 1977; Lever and Pettingale, 1983). MAS patients with hypophosphatemia were demonstrated to have increased basal urinary cAMP, although some of this increase may possibly be related to increased glomerular filtration of cAMP (Zung *et al.,* 1995). The urinary cAMP response to exogenous PTH infusion was significantly blunted while the phosphaturic response was normal. The abnormal renal adenylyl cyclase activity is more consistent with a defect in the proximal renal tubule which leads to an abnormality in the production of cAMP, although the mechanism for hypophosphatemic rickets in MAS has not been clearly established. Osteomalacic changes have also been shown to be a common feature of fibrous dysplastic bone lesions in patients without the other clinical features of MAS (Bianco *et al.,* 2000). The hypophosphatemia is treated with vitamin D and oral phosphate therapy. Usually the osteomalacia or rickets is corrected but the hypophosphatemia is more resistant to therapy (Lever and Pettingale, 1983).

Hyperparathyroidism has been reported to occur with FD (primarily MOFD) in a few patients (Benedict, 1962; Firat and Stutzman, 1968; Ehrig and Wilson, 1972; Hammami *et al.,* 1997). Parathyroid adenoma was usually found surgically, and correction of hyperparathyroidism did not result in regression of the bone lesions. It is possible that most, if not all, of these patients have the autosomal dominant syndrome of hyperparathyroidism and fibroosseous tumors, a distinct clinical and genetic entity that has been subsequently recognized (Jackson *et al.,* 1990; Szabo *et al.,* 1995). Consistent with this, in the one case in which $G_s\alpha$ mutations were looked for, none were identified (Hammami *et al.,* 1997). Hyperparathyroidism has not been associated with MAS and it not clear if there is a true association between hyperparathyroidism and FD.

While the vast majority of MAS patients have abnormalities restricted to the bone, skin, and endocrine organs and have an excellent prognosis (Harris *et al.,* 1962; Lee *et al.,* 1986; Danon and Crawford, 1987), there are a small number of patients who present with one or more nonendocrine abnormalities which may lead to markedly increased morbidity and mortality (Shenker *et al.,* 1993). Patients with these features often present with the early development of extensive POFD or hypercortisolism (Harris *et al.,* 1962; Shenker *et al.,* 1993). Liver abnormalities include severe neonatal jaundice, elevated liver enzymes, and evidence of cholestatic and biliary abnormalities on liver biopsy (Shenker *et al.,* 1993; Silva *et al.,* 2000). Cardiac abnormalities may include cardiomegaly, persistent tachycardia, and unexplained sudden death in young patients. Atypical myocyte hypertrophy has also been noted on histological examination (Shenker *et al.,* 1993). The role that endocrine abnormalities, restrictive pulmonary disease, arteriovenous shunting, or a primary abnormality of myocardium and cardiac conduction plays in the pathogenesis of the cardiac manifestations is still unclear (Shenker *et al.,* 1993). Other abnormalities that are rarely associated with MAS include thymic hyperplasia, myelofibrosis with extramedullary hematopoiesis, gastrointestinal polyps, pancreatitis, breast and endometrial cancer, microcephaly, and other neurological abnormalities (Danon and Crawford, 1987; Shenker *et al.,* 1993).

Genetics

All forms of FD (MOFD, POFD, MAS), with very rare exceptions, occur sporadically without evidence of disease in more than a single family member. There is one report of POFD in multiple family members (Reitzik and Lownie, 1975). Two other reports suggest the inheritance of disease from mother to daughter. In the first family a mother was diagnosed as possible MAS while her daughter had POFD (Hibbs and Rush, 1952). In the second family a mother and daughter had both hyperparathyroidism and FD (Firat and Stutzman, 1968). It is possible that this latter family has the autosomal dominant syndrome of hyperparathyroidism and fibroosseous tumors (Jackson *et al.,* 1990; Szabo *et al.,* 1995). Recently two brothers with POFD born of nonconsanguinous unaffected parents were reported (Sarkar *et al.,* 1993). In one report, one of dizygotic twins had MAS (Caldwell and Broderick, 1947) while in another report one of monozygotic twins had MAS while the other had craniofacial FD (Lemli, 1977). These two instances are consistent with sporadic occurrence of the disease. There are no reported instances of MAS in more than one family member.

Radiological Findings

Nuclear scintigraphy with 99m-technetium methylene diphosphonate is the most sensitive method to survey the skeleton for FD (Pfeffer *et al.,* 1990). This should be performed in patients in whom MAS may be suspected due to an endocrine abnormality and/or pigmented skin lesion. It can also be used to survey the skeleton once FD has been diagnosed to determine extent of disease. On standard radiographs the lesions are typically cystic lesions which appear to expand from the medullary cavity to the cortex, usually expanding and thinning the surrounding cortical bone (Schlumberger, 1946; Mauras and Blizzard, 1986; Lucas *et al.,* 1995). In some cases the surrounding cortex becomes sclerotic, particularly if there has been a previous fracture. The lesions are usually radiolucent or exhibit a ground-glass appearance. Bone trabeculae are occasionally seen traversing the radiolucent lesions (Schlumberger, 1946). If a large amount of cartilage is present within the lesion calcifications may be visible (see Pathology) (Ishida and Dorfman, 1993). The lesions can also be sclerotic or mixed if there is a large amount of osseous tissue (Lucas *et al.,* 1995). FD is usually present in the metaphyses and diaphyses of long bones,

typically sparing the epiphyses (Albright *et al.,* 1937; Schlumberger, 1946; Mauras and Blizzard, 1986; Lucas *et al.,* 1995). Other lesions with a similar radiographic appearance include Paget's disease, osteitis fibrosa cystica, bone cysts, giant cell tumor, fibroma, chondroma, and other types of bone tumors (Schlumberger, 1946; Lucas *et al.,* 1995). Patients with POFD or MAS may show advanced bone age (Benedict, 1962; Mauras and Blizzard, 1986). Decreased bone density or radiographic features of rickets or osteomalacia is seen in patients with hypophosphatemia or hypercortisolism (Cole *et al.,* 1983; Danon and Crawford, 1987; Mauras and Blizzard, 1986)

Magnetic resonance imaging (MRI) has recently been shown to be useful in defining the extent of disease (Inamo *et al.,* 1993; Gober and Nicholas, 1993). MRI may be more sensitive and may delineate the borders of the lesions better than standard X-rays, particularly in areas in which standard X-rays are not very sensitive, such as the spine (Inamo *et al.,* 1993). Usually the lesions are hypodense on T1-weighted images but usually become brighter on T2- or STIR-weighted images (Inamo *et al.,* 1993; Gober and Nicholas, 1993; Jee *et al.,* 1996). It has been suggested that lesions that have a large amount of cartilage, decreased cellularity, or cystic changes have high signal intensity on T2-weighted images, while those that have a large amount of bone, increased cellularity, or sclerotic changes have low signal intensity on T2-weighted images. In one case MRI was also useful in identifying intramuscular myxomas, which are low signal intensity on T1-weighted images and high signal intensity on T2- or STIR-weighted images (Gober and Nicholas, 1993).

Pathology

The most prominent histological feature of FD is fibrous tissue composed of immature mesenchymal cells which appear to expand concentrically from the medullary cavity to the cortical bone (Fig. 1, see also color plate). Long, spindle-shaped fibroblasts are arranged in parallel arrays or in whirls and are embedded in a matrix with parallel collagen fibrils (Greco and Steiner, 1986). In some areas there appears to be a myxomatous matrix. The fibroblastic cells are of the osteoblastic lineage, as they express alkaline phosphate and other proteins associated with osteoblast differentiation (Riminucci *et al.,* 1997). Embedded in this fibrous tissue are spicules of immature woven bone mostly surrounded by flat lining cells similar to the fibroblasts in the fibrous areas (Greco and Steiner, 1986). These lining cells have retracted cell bodies, forming pseudolacunar spaces (Riminucci *et al.,* 1997, 1999b). This retraction is presumed to be secondary to increased intracellular cAMP, as cAMP produces similar cell shape changes in cultured osteoblasts (Riminucci *et al.,* 1997). In a few areas the woven bone trabeculae are lined by more typical osteoblasts. It appears that the osseous components of FD are formed through a process similar to membranous ossification. Unlike normal woven bone, the collagen fibrils at the surface are arranged perpendicular to the bone-forming surface (so-called Sharpey's fibers) (Riminucci *et al.,* 1997, 1999b; Bianco *et al.,* 2000). The woven bone in FD contains large osteocytic lacunae each containing multiple osteocytes (hyperosteocytic bone) (Riminucci *et al.,* 1997). Bone trabeculae in FD contain osteonectin, but not osteopontin or bone sialoprotein, proteins that are present in the matrix of normal woven bone (Riminucci *et al.,* 1997). Changes consistent with osteomalacia may be present within the bony component of FD lesions (Bianco *et al.,* 2000). Unlike normal bone, there is no evidence of replacement of the woven bone by mature lamellar bone. Usually the border between FD and normal bone is sharp and well demarcated.

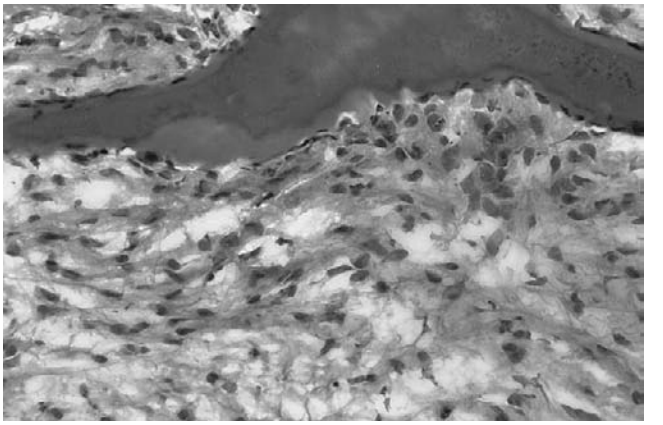

Figure 1 Histological section from a lesion of FD (H & E). Fibrous tissue containing elongated fibroblasts in a collagenous matrix dominate the section. One spicule of immature woven bone is present. Note that the bone spicule is surrounded in many areas by flattened cells which morphologically are different than cuboidal osteoblasts that normally are present in regions of bone formation. There are no cartilaginous elements in this section. (See also color plate.)

One recent study suggests that the histology of FD lesions is variable, with three major histological patterns depending on the location of the affected bone (Riminucci *et al.,* 1999b). The "Chinese writing" pattern, characterized by thin and disconnected bone trabeculae with interspersed fibrous tissue, is usually present in lesions located in the axial and appendicular skeleton. The "sclerotic/Pagetoid" pattern, characterized by dense, sclerotic trabecular bone forming an uninterrupted network and the presence of cement/arrest lines similar to the Schmorl's mosaic typically seen in Pagetic bone, is usually found in cranial bone lesions. The "sclerotic/hypercellular" pattern is present in FD lesions in gnathic (jaw) bones. This pattern is characterized by large trabeculae arranged in a parallel array with osteoblasts lining one side of the trabeculae. All three types of FD lesions have some common features, including the presence of retracted osteoblasts and Sharpey's fibers, that appear to be hallmarks for FD.

In some cases there are islands of hyaline cartilage which are surrounded by fibrous tissue, thus probably representing a metaplastic process (Greco and Steiner, 1986).

With few exceptions the cartilage appears normal and in some areas it undergoes endochondral ossification. In rare cases the cartilaginous component may be the dominant feature and this has been termed fibrocartilaginous dysplasia (Ishida and Dorfman, 1993). There may be some atypia suggesting chondrosarcoma but the presence of surrounding fibroosseous tissue is indicative of FD. Since endochondral ossification is often prominent, stippled or ring-like calcifications may be visible within these lesions on radiographs. Some lesions of FD contain calcified spherules, a typical feature of cemento-ossifying fibroma. These two benign fibrous lesions can sometimes be difficult to distinguish histologically and it has been suggested that they may represent a histological spectrum of a similar process (Voytek *et al.,* 1995). Other lesions which appear histologically similar to FD are osteitis fibrosa cystica, which is usually associated with hyperparathyroidism or chronic renal failure, and Paget's disease, which, unlike FD, usually develops in later life. In both of these diseases there is more active osteoclastic bone resorption within the lesions and no evidence of cartilaginous islands.

FD often invades the outer cortical bone through the action of increased numbers of surrounding osteoclasts (Riminucci *et al.,* 1997; Yamamoto *et al.,* 1996), resulting in cortical thinning and in some cases concentric bulging of the cortex. These osteoclasts have increased numbers of nuclei per cell (Yamamoto *et al.,* 1996). In rare cases these lesions may appear more aggressive, resulting in exophytic protuberances (Dorfman *et al.,* 1994) or even invasion into the surrounding soft tissue (Latham *et al.,* 1992). These lesions are benign and do not metastasize. Rarely, FD undergoes malignant degeneration, most often to osteosarcoma and less commonly to chondrosarcoma, fibrsarcoma, or malignant fibrohistiosarcoma (Yabut *et al.,* 1988; Ruggieri *et al.,* 1994)

Other tissues which may be abnormal pathologically in MAS include the skin, endocrine organs, liver, bone marrow, thymus, spleen and gastrointestinal tract. Histologically the pigmented macules appear similar to those seen in neurofibromatosis. However, in contrast to pigmented lesions from neurofibromatosis patients, those from MAS patients fail to develop giant pigmented granules within the melanocytes in response to dopa (Benedict, 1962). The affected endocrine tissues generally undergo a nodular hyperplasia or in some cases develop a hyperfunctioning adenoma (Danon and Crawford, 1987; Mauras and Blizzard, 1986; Weinstein *et al.,* 1991). The ovaries show the development of large follicular cysts with no evidence of ovulation. Endocrine organs may have pathological changes typically found in MAS in the absence of hormonal abnormalities (Danon and Crawford, 1987). In the small number of severely affected patients, numerous nonendocrine abnormalities have been described pathologically. These include cholestasis and biliary abnormalities in the liver, hypertrophy of the thymus and spleen, myeloid metaplasia with extramedullary hematopoiesis, subtle brain abnormalities, and gastrointestinal polyps (McCune and Bruch, 1937; Albright *et al.,* 1937; MacMahon, 1971; Danon and Crawford, 1987; Shenker *et al.,* 1993)

Pathogenesis

The first clues to the pathogenesis of the various syndromes in which FD is present originally came from observations of the skin and endocrine manifestations of MAS. It was first noted that in MAS the cutaneous hyperpigmentation often follows the developmental lines of Blaschko. Based upon this observation Happle proposed that the disease is likely to be caused by a dominant somatic mutation occurring early in development or a gametic half chromatid mutation (Happle, 1986). This early event would result in a mosaic with a widespead distribution of cells with a genetic abnormality. Since this disease is never inherited, it is likely that the mutation causing MAS is generally lethal if present in the germline. According to this model, the specific constellation of abnormalities within a given patient would be dependent on the distribution of mutant-bearing cells.

Evaluation of the endocrine abnormalities in MAS patients provided the second clue, which was important in hypothesizing where the specific genetic defect occurs. It was originally proposed that the hyperplasia and hyperfunction of diverse peripheral endocrine organs was the result of a hypothalamic defect (Hall and Warrick, 1972). Subsequent endocrine evaluations clearly demonstrated that in each case the abnormal endocrine glands were functioning autonomously (Danon *et al.,* 1975; Scully and McNeely, 1975; Mauras and Blizzard, 1986; D'Armiento *et al.,* 1983; Foster *et al.,* 1984; Feuillan *et al.,* 1990). An abnormality in these tissues resulting in excess intracellular cAMP could result in the diverse clinical manifestations of MAS since cAMP is known to stimulate the growth and/or function of endocrine organs which can be abnormal in MAS and the production of melanin pigment in melanocytes (Mauras and Blizzard, 1986; Lee *et al.,* 1986; Dumont *et al.,* 1989; Scully and McNeely, 1975). Mutations in the G_s α-subunit gene which lead to constitutive activation of G_s and cAMP production were identified in isolated human growth hormone-secreting pituitary tumors and thyroid tumors (Landis *et al.,* 1989; Lyons *et al.,* 1990; Suarez *et al.,* 1991), both of which are found in MAS. It therefore seemed likely that these same mutations may also be present in a more widespread distribution in MAS.

G proteins are a family of signal transduction proteins which transmit the signals between a large number of receptors for extracellular ligands and intracellular second messengers, such as enzymes and ion channels. Each G protein is a heterotrimer composed of a specific α, β, and γ subunit (Spiegel *et al.,* 1992). The α-subunit binds guanine nucleotide and interacts with specific receptors and effectors. In the inactive state, GDP is bound to the α-subunit and the GDP-bound α-subunit is associated with a tightly but noncovalently bound $\beta\gamma$ complex. When a ligand, such as a hormone, binds to its specific receptor, the occupied

receptor interacts with and activates the inactive heterotrimer, resulting in the exchange of GTP for GDP and dissociation of the α-subunit from the $\beta\gamma$ complex. Both GTP-bound α-subunit and $\beta\gamma$ subunits may regulate the activity of specific effectors. In the case of G_s, the G_s α-subunit ($G_s\alpha$) stimulates the activity of adenylyl cyclases, a family of enzymes which catalyze the formation of cAMP. The α-subunit has an intrinsic GTPase activity which hydrolyzes the bound GTP to GDP (Bourne *et al.,* 1990). This reaction functions as the "turn off" switch for the G protein. Therefore, modifications that inhibit the GTPase activity will result in constitutive activation and in the case of G_s will lead to overproduction of cAMP. ADP-ribosylation of amino acid residue Arg^{201} of $G_s\alpha$ by cholera toxin leads to constitutive activation of G_s by this mechanism (Spiegel *et al.,* 1992). Mutations which encode substitutions of amino acid residues Arg^{201} or Gln^{227} in $G_s\alpha$ have been found in human thyroid and growth hormone-secreting pituitary tumors and shown to result in constitutive activation (Landis *et al.,* 1989; Masters *et al.,* 1989; Graziano and Gilman, 1989). Targeted expression of the catalytic subunit of cholera toxin in somatotrophs in transgenic mice resulted in pituitary hyperplasia (Burton *et al.,* 1991).

Activating $G_s\alpha$ mutations coding for substitutions of residue Arg^{201} have been identified in tissues from many MAS patients (Weinstein *et al.,* 1991; Shenker *et al.,* 1993, 1994). In each case, one specific mutation (Arg^{201} to His or Cys) is detected in multiple tissues and in variable abundance, consistent with a widespread distribution of a population of cells harboring a somatic mutation. More recently an Arg^{201}-to-Ser mutation was identified in a POFD patient (Candeliere *et al.,* 1997) and an Arg^{201}-to-Gly mutation was identified in an MAS patient (Riminucci *et al.,* 1999a). A possible germline Arg^{201}-to-Leu mutation has also been reported in a severely affected patient with multiple skeletal, endocrine, and developmental abnormalities (Mockridge *et al.,* 1999). Substitutions of Gln^{227} have not been found in MAS. In each tissue the mutant allele was always present in equal or lower abundance than the wild-type allele, consistant with the mutation being dominant. These mutations are present in hyperplastic or adenomatous endocrine tissues, as well as in a café-au-lait lesions from MAS patients (Schwindinger *et al.,* 1992; Kim *et al.,* 1999). These mutations are also found in many nonendocrine tissues, some of which are abnormal (e.g., liver, heart, thymus) while others are normal (Weinstein *et al.,* 1991; Shenker *et al.,* 1993; Silva *et al.,* 2000). The role of these mutations in the pathogenesis of these nonendocrine manifestations needs to be defined. In Albright hereditary osteodystrophy (AHO), loss-of-function mutations of $G_s\alpha$ are associated with bone abnormalities and resistance to multiple hormones that stimulate cAMP production (Patten *et al.,* 1990; Weinstein *et al.,* 1990). After the discovery of these mutations in MAS tissues, elevated cAMP production has been demonstrated in MAS tissues *in vitro* and *in vivo* (Zung *et al.,* 1995; Yamamoto *et al.,* 1996; Marie *et al.,* 1997; Kim *et al.,* 1999).

Activating $G_s\alpha$ mutations have also been detected at high levels in bone lesions from MAS, POFD, and MOFD patients (Malchoff *et al.,* 1994; Shenker *et al.,* 1994, 1995; Alman *et al.,* 1996; Bianco *et al.,* 2000). Therefore all forms of FD can be considered to have the same underlying pathogenesis. Recent studies suggest that FD results from abnormal proliferation and differentiation of bone marrow stromal (CFU-F) cells (Bianco and Robey, 1999). Transplantation of skeletal progenitor cells derived from human FD lesions into immunocompromised mice leads to a lesion similar to that observed in human FD, with abnormal woven bone ossicles and lack of interspersed adipocytes or hematopoiesis (Bianco *et al.,* 1998, 2000). Interestingly, these lesions formed only when both normal and mutant cells were transplanted, suggesting that the formation of FD requires the presence of normal as well as mutant precursor cells (Bianco *et al.,* 1998).

The development of mature osteoblasts from pluripotential mesenchymal precursors in the marrow stroma involves a series of defined steps: commitment to the osteoblast lineage, a proliferative phase in which preosteoblasts divide and secrete collagen and other matrix proteins, and a postproliferative phase in which the cells express osteoblast-specific gene products (e.g., alkaline phosphatase, osteopontin, and osteocalcin) and form mineralized bone (Stein and Lian, 1993). In *in vitro* models of osteoblast differentiation, parathyroid hormone and forskolin, both stimulators of cAMP production, inhibit the differentiation of osteoprogenitor cells (Bellows *et al.,* 1990; Turksen *et al.,* 1990) while treatment with oligonucleotides antisense to $G_s\alpha$ promotes their differentiation (Nanes *et al.,* 1995). The cells present in lesions of FD appear poorly differentiated morphologically and have an increased proliferation rate (Marie *et al.,* 1997) and express markers of early, but not late, osteoblast differentiation (Marie *et al.,* 1997; Riminucci *et al.,* 1997). It therefore appears that in these cells the regulation of the normal differentiation program is disrupted.

Elevation of cAMP in osteoprogenitor cells may lead to FD by affecting a number of genes downstream in the signaling cascade (Fig. 2). Activated G_s has been shown to chronically increase the expression of c-*fos* and other oncogenes through the activation of cAMP-dependent protein kinase (protein kinase A (PKA)) (Gaiddon *et al.,* 1994). cAMP binds to the regulatory subunits of PKA and these subunits dissociate from the catalytic subunits. The catalytic subunits of PKA translocate to the nucleus and phosphorylate cAMP-responsive transcription factors such as cAMP response element binding protein (CREB) and the related cAMP response element modulator (CREM) proteins. Once phosphorylated, CREB and CREM can then bind to the promoters of cAMP-responsive genes, such as c-*fos,* resulting in increased transcription (Sassone-Corsi, 1995). Fos, the product of c-*fos,* binds with Jun, the product of the c-*jun* oncogene to form a heterodimer known as AP-1. AP-1 binds to the promoters of many genes and regulates their expression. AP-1-binding complexes are expressed at high levels in the proliferative phase of osteoblast develop-

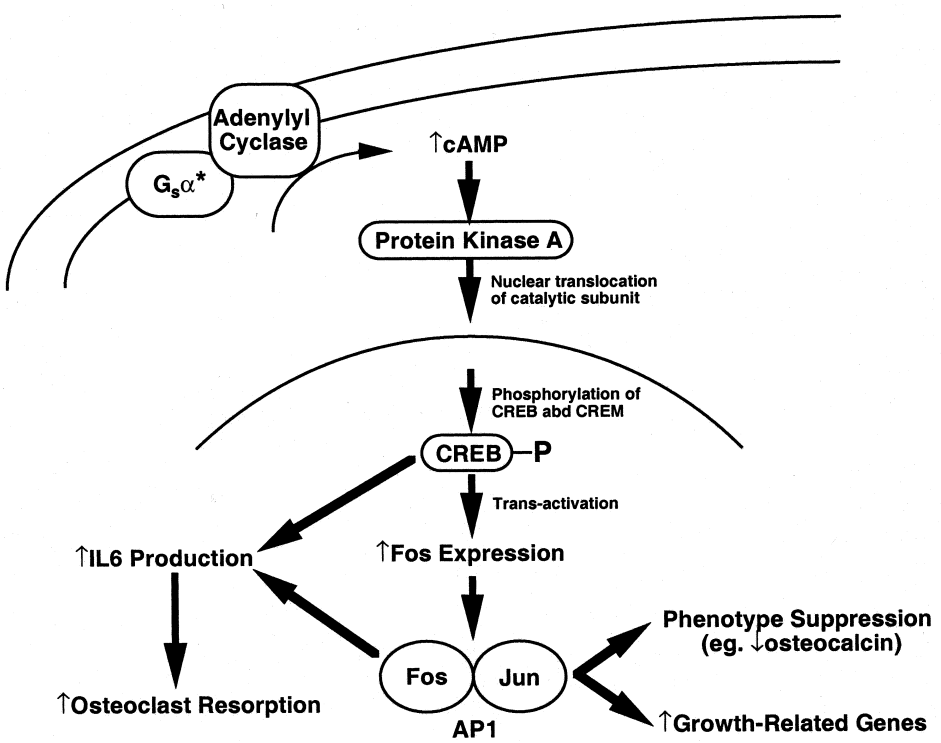

Figure 2 Possible mechanisms by which activated G_s in osteogenic cells may lead to FD. G_s activation increases the activity of the effector adenylyl cyclase, leading to increased intracellular cAMP levels. Binding of cAMP to the regulatory subunits of cAMP-dependent protein kinase (protein kinase A) allows release of its catalytic subunits, which translocate to the nucleus and phosphorylate proteins such as CREB and CREM. Phosphorylated CREB binds to promoters of cAMP-responsive immediate–early genes (e.g., c-*fos*) and increases their expression. Fos, the product of c-*fos,* binds with Jun to form AP-1. AP-1 is a transcription factor which increases the expression of growth-related genes and decreases the expression of osteoblast-specific genes, such as osteocalcin (phenotype suppression). CREB-P and AP-1 also stimulate the transcription of the IL-6 gene. IL-6 may be important in recruiting osteoclasts and stimulating osteoclastic bone resorption.

ment and markedly decrease upon the onset of differentiation (Stein and Lian, 1993). In the osteocalcin gene promoter AP-1 sites are found within two activating regions of the promoter, the vitamin D-responsive element (VDRE) and the osteocalcin (OC) box. It has been demonstated that the binding of AP-1 to these regions suppresses both the basal (OC box) and the vitamin D-induced expression (VDRE) of osteocalcin (Stein and Lian, 1993). This is one example of how high expression of AP-1 during the proliferative stage of osteoblast development can suppress the expression of a late marker of osteoblast differentiation.

Several lines of evidence suggest that overexpression of Fos is important in bone development *in vivo* and in the pathogenesis of FD. Fos has recently shown to be overexpressed in lesions of FD by *in situ* hybridization in specimens from eight patients, one of which was shown to have an activating $G_s\alpha$ mutation (Candeliere *et al.,* 1995). The abnormal expression of Fos was localized to the fibroblastic cells, which are presumed to be osteoblastic precursors. Overexpression of Fos in transgenic mice results in abnormal bone remodeling and bone lesions which are in many ways reminiscent of FD (Rüther *et al.,* 1987). Osteosarcomas which overexpress Fos develop in these animals after several months (Grigoriadis *et al.,* 1993). Fos is also overexpressed in many human osteosarcomas (Wu *et al.,* 1990). In contrast, mice with no expression of Fos develop osteopetrosis (Johnson *et al.,* 1992; Wang *et al.,* 1992).

Cells isolated from FD lesions also have markedly increased production if IL-6 (as well as IL-11). Increased local concentrations of IL-6 presumably recruit osteoclasts, allowing the FD lesion to expand by resorbing the surrounding normal cortical bone (Yamamoto *et al.,* 1996). Several lines of evidence show that the increased IL-6 production is the direct result of increased intracellular cAMP concentrations (Yamamoto *et al.,* 1996; Motomura *et al.,* 1998), and this is consistent with the presence of both cAMP-responsive elements and AP1 sites within the IL-6 promoter (Motomura *et al.,* 1998) (Fig. 2). The beta chain of platelet-derived growth factor (PDGF-B) has been shown to be abnormally expressed at high levels in FD and other aggressive fibrous bone lesions (Alman *et al.,* 1995). PDGF-B results in fibroblast proliferation and may be important in osteoclast activation. The increased levels of sex steroid receptors present in abnormal cells derived from areas of FD (Kaplan *et al.,* 1988; Pensler *et al.,* 1990) and the ability of sex steroids to increase PDGF-B expression (Alman *et al.,*

1995) may at least in part account for the progression of FD which is rarely observed during puberty, pregnancy, or the use of oral contraceptives (Stevens-Simon *et al.,* 1991).

Management

I will not discuss in detail the specific management of the various endocrine manifestations of MAS. They are generally managed with variable success by surgical removal of hyperplastic or adenomatous tissue or by specific medical therapy (e.g., somatostatin analog or bromocryptine for pituitary tumor, radioiodine or thionamides for hyperthyroidism, aromatase inhibitors for precocious puberty). One must be cautious that a small subset of patients, in which there are widespread manifestations including hypercortisolism, are at risk for perioperative sudden death. This complication may be secondary to either cardiomyopathy and arrythmia resulting from excess cAMP production in cardiac tissue or hormonal abnormalities (Shenker *et al.,* 1993). There is no specific therapy for the skin manifestations.

FD is often minimally symptomatic or asymptomatic and usually does not progress after the third decade. Generally fractures heal well with conservative management (Harris *et al.,* 1962). On occasion surgery is required for nonhealing fractures, severe pain or deformity, particularly on weight-bearing bones, or imminent signs of nerve compression (Harris *et al.,* 1962; Grabias and Campbell, 1977; Nager *et al.,* 1982; Edgerton *et al.,* 1985). Usually surgery involves excision or curretage and replacement with bone grafts. Until recently there were no other therapeutic options. Radiotherapy is contraindicated since it is generally ineffective and may increase the risk of sarcomatous degeneration, although this is controversial (Tanner *et al.,* 1961; Yabut *et al.,* 1988; Ruggieri *et al.,* 1994). Calcitonin, etidronate and clodronate have been administered to a few patients with no significant clinical improvement (Hjelmstedt and Ljunghall, 1979; Grant *et al.,* 1982; Cole *et al.,* 1983). Several uncontrolled trials have shown pamidronate to be effective in the treatment of FD, particularly with respect to improving bone pain and reversing some of the radiological abnormalites (Liens *et al.,* 1994; Chapurlat *et al.,* 1997; Lala *et al.,* 2000; Zacharin and O'Sullivan, 2000). Growth-plate widening was observed in one young patient, which reversed after the treatment was stopped (Liens *et al.,* 1994). More recent studies show pamidronate to be safe in children and adolescents.

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CHAPTER 66

Osteogenesis Imperfecta

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Introduction

Osteogenesis imperfecta (OI) is a highly variable heritable disease of bone characterized by recurring bone fractures. It is the most common single gene defect causing bone disease and its typical clinical presentation and natural history have been well chronicled in many review articles and book chapters. The disease has received attention from basic scientists because it has become the paradigm for mutations affecting a well-defined extracellular matrix protein. The results of these biochemical and molecular studies have provided the foundation for understanding the broad spectrum of heritable diseases of connective tissue, and OI will continue to be the disease that provides the breakthroughs for this class of heritable diseases. The purpose of this chapter is to provide an update to the well-described clinical classifications and point out recent advances in the diagnosis and therapy of the disease. Most of the chapter will review the consequences of mutations of type 1 collagen on the structure of the collagen fibers, the biology of the osteoblast and the pathophysiology of the resulting bone disease. It will emphasize how this information influences our understanding of osteoporosis and other heritable diseases of connective tissue. In addition outstanding problems requiring further research attention, particularly relating to cell and somatic gene therapy, will be presented.

Clinical Classifications and Variants of OI

The most widely used clinical classification for OI continues to be the one developed by Sillence that is based on disease severity (Sillence, 1988). For purposes of correlating clinical phenotype with the underlying genetic and pathophysiological basis of the disease, grouping the classes into deforming and nondeforming bone disease can be helpful even though this, too, is an arbitrary distinction. Three degrees of deforming OI, types II, III, and IV, are associated with decreasing severity of growth retardation and limb deformity and all result from a mutation affecting the structure of the type 1 collagen molecule (Table I). The consequences of this mutation on the structure of the extracellular matrix and the biology of bone cell are very similar, varying only in their degree of severity. In contrast most cases of nondeforming OI, type I, are the result of mutations affecting the production of an otherwise normal type I collagen molecule.

Deforming OI

The criteria for identifying individuals as OI type II, III, or IV are somewhat arbitrary. Although this classification helps to stratify patients according to their disease severity, the typing does not necessarily predict the natural history of the disease or the type of underlying genetic mutation. Furthermore, with the implementation of bisphosphonate in children, disease severity and natural history are being greatly improved and eventually may force a revaluation of this classification system.

Most infants that succumb to OI in the perinatal period are considered OI type II. The hallmarks of type II include shortened and deformed limbs in the presence of normal birth weight and length. The cranium is unusually soft and molded and may be fractured at birth. Intracranial bleeding may have occurred. The sclerae are deep blue. The limbs are deformed and short, raising the consideration of hypophosphatasia, achondrogenesis, and thanatophoric dwarfism in the diagnosis. Multiple fractures are seen on X-ray and the extremities appear broad and crumpled. The critical problem is neonatal pulmonary insufficiency that may lead to death in the first postnatal week. Faulty thoracic musculoskeletal development also limits respiratory function in the majority of cases.

Table I An All Inclusive Clinical and Molecular Classification of Osteogenesis Imperfecta

Molecular classification	Clinical classification	Clinical severity	Molecular mechanism
Dominant negative OI	Type II	Perinatal lethal	Glycine substitutions preferentially located in C terminal helical domain of either collagen chain.
	Type III	Progressive deforming	Glycine substitutions preferentially located in mid-helical domain of either collagen chain.
	Type IV	Moderately deforming	Glycine substitutions preferentially located in mid-helical domain of the a2 collagen chain.
	Type V	Moderately deforming	Non-type I collagen gene mutation.
	Type VI	Moderately deforming	Non-type I collagen gene mutation.
	Type I	Nondeforming	Glycine substitutions preferentially located in N terminal helical domain of either collagen chain.
	Sp1-associated osteoporosis	Osteoporosis associated risk factor	Excessive alpha-1 chain production may lead to accumulation of alpha-1(I) trimer molecules with the bone matrix.
	Noncollagen mutations	Mutation of proteins that interact with the collagen microfibril	Murine knock out studies suggest that noncollagenous proteins of bone may contribute to formation of a stable bone matrix. The osteoporosis of Marfan's may be an example.
Haploid insufficiency	Type I	Classical mild OI	Complete nonfunctional *Col1A1* allele usually due premature stop codon.
	Osteoporosis	Associated risk factor for osteoporosis	Partial inactivation of a *Col1A1* allele either due to a mutation affecting processing of the transcript or the activity of the promoter.

However, prolonged survival has been documented in occasional type II cases.

Infants born with fractures and deformity that survive the perinatal period are grouped as OI type III. The natural history of type III includes the presence of multiple fractures, significant molding of the calvarium, and deformity of the extremities at birth. Fractures may continue to occur in infancy and childhood precluding a normal pattern of ambulation. Deformity of the thoracic cage (pectus carinatum, pectus excavatum) may be present in early childhood and may advance as the scoliosis and vertebral compression increase. Type III OI subjects develop scoliosis at an early age. Vertebral compression, most commonly of the central or "codfish" type, begin shortly after birth and progress relentlessly prior to puberty (Ishikawa *et al.,* 1996). The volume of the thoracic cage decreases with age and eventually, cardiorespiratory insufficiency, including right-sided cardiac failure may occur. As adults these individuals are very short, frequently less than 4 ft in height. Skeletal involvement is associated with a severe defect in remodeling so that the appendicular bones are usually very narrow, although in some cases, a "broad bone" X-ray appearance is seen. In either event, the epiphyseal zone is markedly dysplastic which effectively limits endochondral and therefore, somatic bone growth. Because of severe limb deformity and a marked degree of osteopenia at an early age, type III subjects are wheelchair bound. Because of molding of the base of the skull, type III subjects are at risk for the development of basilar invagination that may cause brain-stem compression with both respiratory and neurologic complications and sudden death. Charnas reported communicating hydrocephalus in 17/76 subjects with OI (Charnas and Marini, 1993). Seizure disorders were observed in 5/76 cases. Brain-stem compression requiring surgical decompression or reinforcement of the craniocervical junction is extremely complex, requiring mechanical support, trans-oral clivectomy and decompression of the posterior fossa where respiratory center function is compromised (Bhangoo and Crockard, 1999; Sawin and Menezes, 1997).

Infants that appear normal at birth but develop deformity upon ambulation generally fall into the OI type IV group although this form can merge into features seen in OI type I. Moderate short stature is common, but ambulation is generally possible although with the aid of rods and braces. Radiologically, the extent of skeletal dysplasia in type IV disease is more severe than found in type I cases, but less than in type III OI. There is more severe osteoporosis with significant cortical and trabecular bone loss. Cystic changes may appear in the long bones. Vertebral compression fractures occur frequently. In addition to more severe scoliosis, there is moderate deformity of the pelvis and lower extremities that limits ambulation without support. Dentinogenesis imperfecta (DI) is most frequent in OI types III and IV and, overall, affects about 15% of OI patients among the different phenotypes (Lund *et al.,* 1998; Petersen and Wetzel, 1998). It appears as a bluish-brown translucent discoloration. Deciduous teeth tend to be more severely affected than permanent teeth. These teeth are soft because of a defect in underlying dentin (type I

collagen) so that the enamel layer is lost, which leads to extreme dental erosion even in children. Expert dental care is essential in affected children. Hearing loss, present in all OI phenotypes (Garretsen *et al.,* 1997), has its onset in childhood (Kuurila *et al.,* 2000) or during second or third decade. Both conductive, mixed and sensorineural hearing loss have been documented in OI (Shapiro *et al.,* 1982). The lesions are a combination of bony trauma (i.e., fractures of the stapes crura and footplate) and damage to neural structures in the inner ear. These lesions are not identical to those of otosclerosis.

Until recently there were no examples of OI resulting from mutations in genes other than type 1 collagen. As a result of forming an integrated research, diagnostic, and treatment team with the opportunity to examine the broad spectrum of patients with deforming OI, Gloreaux has identified at least two additional OI-like diseases (OI types V and VI) that are not a result of a mutation in the type I collagen genes. Type V OI has radiologic features of type IV OI with subtle differences including calcification of the interossieus membrane and a metaphyseal band adjacent to the growth plate. The disease is inherited as an autosomal dominant and no mutations with the type I collagen genes have been found (Glorieux *et al.,* 2000). Unlike OI type V which has been found in nonrelated families, OI type VI is restricted to an inbred Eskimo population. In this case linkage studies have been possible and have excluded type 1 collagen genes. The importance of these new clinical phenotypes is that it demonstrates that molecules other than type 1 collagen are essential for formation of a stable bone matrix and will eventually lead to a discovery of the role that these gene products play in bone biology. Murine knockout studies have also pointed to the importance of other matrix proteins to the formation of bone including osteonectin (Delany *et al.,* 2000) and biglycan (Xu *et al.,* 1998).

Another unifying feature of all the deforming forms of OI is the strong dominant negative properties of the mutation. In those cases where affected individuals choose to have children, the disease is passed on in an autosomal dominant manner. However, in most cases the disease results from a random and isolated mutation in the germ cell of a parent. Usually this event is a one-time occurrence and the likelihood of another affected individual is extremely small. However, in 5 to 10% of cases one of the parents sustained a somatic mutation during his or her embryonic development such that a proportion of their somatic and germ cells have an OI mutation (Cole and Dalgleish, 1995; Lund *et al.,* 1997). The mosaic parent does not have features of OI and most of their children have normal bones. However, if the child is conceived with the a germ cell carrying the OI mutation, then full-blown OI results. As a result of this possibility for recurrence, families with one child with OI who wish to have more children are advised to undergo prenatal testing (Raghunath *et al.,* 1994) or sensitive ultrasound analysis with the possibility that a subsequent child might be affected (Lachman, 1994; Tretter *et al.,* 1998).

Mild Nondeforming OI

Although this class of OI patients is relatively common, it is not unusual to be unappreciated by the family or their physician. In cases with blue sclera (Sillence *et al.,* 1993), mild short stature ligamentous laxity, mild scoliosis, and a strong family history of recurring fractures and osteoporosis, the diagnosis is not difficult. Radiographs are not diagnostic and fractures usually heal without deformity. In many cases there is not a high degree of morbidity and once puberty is attained, fractures are rare. Perhaps the most important reason for identifying these individuals is to initiate anti-osteoporosis therapy soon as menopausal symptoms have developed because these individuals are particularly susceptible to accelerated bone loss when sex steroids are lost (Bischoff *et al.,* 1999). Other patients who fit into this category can have symptoms that overlap with mild Ehlers Danlos syndrome. The overlap appears to be related to where the mutation is placed within the collagen molecule, making this type of mutation of interest to the structural biologist (Feshchenko *et al.,* 1998; Raff *et al.,* 2000). However, the importance to the clinician is to recognize that the two genetic phenotypes can coexist so that measures to strengthen the skeleton are initiated when appropriate.

Probably the most difficult situation that arises within this class of patients is child abuse. Although OI is frequently invoked as a potential explanation for the infants and young child with multiple fractures, it is rare that any of the clinical features that are characteristic of OI are present (Steiner *et al.,* 1996) and instead there usually is no family history of recurring fractures, short stature, or abnormal radiographs. Because OI can result from a new mutation within the type I collagen genes and potentially other extracellular matrix genes, lack of a family history does not exclude the diagnosis. Recently the term temporary brittle bone disease has been invoked to explain certain forms of multiple fractures in a setting without an obvious perpetrator (Paterson *et al.,* 1993) but the concept has not gained acceptance (Ablin and Sane, 1997; Chapman and Hall, 1997). A recent report finds that infants and children with this diagnosis have reduced bone mass by radiographic absorptiometry or computed tomography (Miller and Hangartner, 1999) but a credible mechanism for this clinical diagnosis is not yet available. Because analysis of bone density is so difficult in the infant, there are no accepted criteria for normal or diminished bone mass and instead diagnostic decisions are made by emotion and opinion and frequently in an antagonistic environment. This topic is probably best addressed in literature prepared by the osteogenesis imperfecta foundation (OIF) at (http://www.oif.org/tier2/childabuse.htm).

The relationship of mutations within the type I collagen genes and osteoporosis has been difficult to establish. In families where OI mutations and osteoporosis have been identified, careful clinical scrutiny usually identifies features of the disease (Shapiro *et al.,* 1992; Spotila *et al.,* 1991). Nonglycine substitutions in the helical domain have been found

in a few instances, but the significance of these findings is difficult to interpret (Spotila *et al.,* 1994). Quantitative trait linkage (QTL) studies of populations of individuals with osteoporosis have failed to demonstrate linkage to either type I collagen gene (Cardon *et al.,* 2000; Devoto *et al.,* 1998; Koller *et al.,* 1998; Niu *et al.,* 1999). However, a Sp1 polymorphism has shown a statistical association to individuals with osteoporosis (Grant *et al.,* 1996) in a number of convincing studies (Sainz *et al.,* 1999; Uitterlinden *et al.,* 1998; Weichetova *et al.,* 2000), although there appears to be certain population where this association cannot be demonstrated (Han *et al.,* 1999; Hustmyer *et al.,* 1999; Liden *et al.,* 1998; Nakajima *et al.,* 1999). Particularly intriguing is the observation that the Sp1 polymorphism, which is located at a transcriptional regulatory region in the first intron of the *Col1A1* gene, leads to a subtle increase in expression this genetic unit (Mann *et al.,* 2001). If this proves to be case at the protein level, the excess collagen α-1-(I) chains which are not incorporated into a heterotrimeric collagen molecule would form an α-1(I) trimer molecule. In mouse and man, severe OI results when both *COL1A2* genes failed to generate α-2(I) collagen chains because the bone matrix is composed of α-1(I) trimer molecules (see below). Osteopenia is observed when one of the two *COL1A2* genes does not function and a mixture of hetero and homotrimeric molecules accumulate. The Sp1 polymorphism suggests that an even more subtle imbalance between heterotrimer to homotrimer production can contribute to osteoporosis (Heegaard *et al.,* 2000; Keen *et al.,* 1999; McGuigan *et al.,* 2000) but that it is only one of a number of genetic and environmental factors contributing to diminished bone mass. This mild contribution will be missed by standard QTL studies and may be restricted to a specific population. If this explanation proves to be correct, a similar weak contribution from subtle mutations within the structural regions of type 1 collagen or mutations that affect regulation of either on the two collagen genes can be anticipated.

Needed Advances in Clinical Diagnosis and Management

In most cases the diagnosis of OI can be made on clinical grounds alone. Unfortunately radiographs are generally insensitive to the diminished bone mass associated with type 1 OI. DEXA has been shown to be effective in detecting the diminished bone mass associated with type 1 OI in adults although its application to children and particularly infants has not been adequately developed. Such standards would be particularly helpful in evaluating individuals with suspected abuse. pQCT might be more sensitive in young children to discriminate individuals with normal or diminished bone mass (Fredericks *et al.,* 1990; Keen *et al.,* 1999). Other clinical tests which may be helpful in the diagnosis OI are serum level of procollagen propeptide fragments reflecting the rate of type I collagen formation (Kauppila *et al.,* 1998; Lund *et al.,* 1998; Proszynska *et al.,* 1996) and the generation of collagen derived cross-links reflecting removal (Bank *et al.,* 2000). Here again standards need to be developed for age and sex, degree of sexual maturation, or menopause to make these tests of bone turnover useful in OI.

In many instances it is advantageous to know the underlying molecular abnormalities for purposes of genetic counseling and in some cases for predicting natural history. Frequently, molecular testing is also used to help in the evaluation of children with recurring fractures. Currently there are two sites in the United States that offer this service. One site http://www.pathology.washington.edu/clinical/byers.html) performs a screening test on fibroblasts derived from an affected individual for biochemical abnormalities of the collagen molecules followed by confirmation at the genetic level by DNA sequencing. The other site (http://www.som.tulane.edu/gene_therapy/matrix/) carries out a screening genomics analysis on DNA derived from peripheral blood or cultured fibroblasts and then identifies the mutation by DNA sequencing of abnormal migrating DNA fragments. Both sites have an excellent track record for finding mutations although not all the patients with OI yield an identified mutation. Tests that assess the intrinsic ability of bone cells to make a normal bone matrix are needed to assist in evaluation of individuals with otherwise unexplained bone fractures or osteoporosis.

Given the complexity in the clinical management of OI, a multidisciplinary clinical team approach to treatment is of greatest value for both the patient and the field. Not only are there significant orthopedic and medical issues, but problems of daily living are pervasive. Proper handling during infancy, mainstreaming within schools, driving an automobile, attending college, scoliosis and pulmonary insufficiency, neurological symptoms, pregnancy and genetic risk, and acceleration of bone disease after menopause are complex problems that are difficult for an individual clinician to manage and require an experienced and broad-based treatment team. Many of these issues are covered on the OIF web site (http://www.oif.org) and in their literature. The development of an OI registry that defines the underlying genetic mutation, characterizes the clinical phenotype, and tracks the response to intervention needs to be developed to broaden the experience of all clinicians who have to make diagnostic and treatment decisions. Such a resource might facilitate multicenter therapeutic trials as the most efficient manner of evaluating the efficacy of a treatment modality.

Animal Models of OI

Naturally arising forms of OI have been described in cows (Agerholm *et al.,* 1994) and dogs (Campbell *et al.,* 1997) and recently an experimentally produced form of OI has been introduced into sheep (Weiss, 2000). If these models are perpetuated into lines of affected animals, they will have value for evaluating orthopedic strategies. Because the maintenance of large animal models is extremely expensive, they will not be useful for the initial development of new medical or genetic strategies. However, once an intervention has the possibility of a clinical trial then a larger animal model will probably be a requirement for FDA approval.

To date, the mouse has contributed the most to our understanding of the disease and is the initial platform for evaluating new therapies. Lethal OI has been produced experimentally by insertion of a collagen transgene containing a glycine point mutation (Stacey *et al.,* 1988) or an internal deletion within the helical domain of the molecule (Khillan *et al.,* 1991). While transgene insertions are relatively easy to produce, there is significant variability in the clinical severity even within the same pedigree (Pereira *et al.,* 1994), reducing their value as an experimental model. OI mutations within the endogenous collagen gene yield a more reproducible phenotype. The oim/oim mouse arose from a spontaneous mutation within the *Col1A2* gene. Because the mutation produces a nonfunctional alpha-2(I) chain, the disease results from an accumulation of alpha-1(I) trimer molecules (Chipman *et al.,* 1993). It has been widely used even though this is a recessive form of OI that is quite unlike the more common dominantly inherited forms of OI. Recently a glycine substitution has been knocked into the *Col1A1* gene to produce a moderately severe form of OI (brit mouse) which has a stable phenotype and is very representative of the common forms of OI (Forlino *et al.,* 1999). The only murine model of type I OI is the heterozygous Mov 13 mouse in which one of the two *Col1A1* genes is nonfunctional due to a retroviral insertion within the first intron. The heterozygous mouse was initially thought to have normal bone and was used for generating a double knockout of the *Col1A1* gene, which has an embryonic lethal phenotype (Lohler *et al.,* 1984). However, more sensitive testing did demonstrate diminished bone mass and under production of type 1 collagen (Bonadio *et al.,* 1990). Unfortunately the mice succumb to leukemia in their early adulthood, limiting their value to basic bone research. A viable model for OI type 1 needs to be produced by genetic knock-in.

Murine models of mild OI (heritable osteoporosis) resulting from mutations other than type I collagen include the knockout mutations of biglycan (Xu *et al.,* 1998) and osteonectin (Delany *et al.,* 2000). Whether these cases represent a mutation that affects the structural stability of the extracellular matrix or indirectly affects the biology of the osteoblast is yet to be determined but these mice reveal the complexity of bone formation. The one naturally arising model that may be related to human forms of OI unrelated to the type 1 collagen gene is the fro/fro mouse (Muriel *et al.,* 1991; Sillence *et al.,* 1993). Direct analysis of collagen produced by these mice has not been informative and ongoing linkage studies should identify the abnormal genetic unit.

Molecular Basis of OI

A comprehensive listing of the mutations within type I collagen genes resulting in OI (Dalgleish, 1997) is now maintained in the OI mutation database (http://www.le.ac.uk/genetics/collagen). They can be broadly correlated with clinical severity with the deforming forms of OI being associated with mutations that interrupt the helical stability of the collagen molecule while most forms of type I OI being associated with underproduction of an otherwise normal type I collagen.

Deforming OI

Essentially all of these mutations act in a dominant negative manner; i.e., it is the presence of the abnormal gene product that causes the disease. Because of this mechanism the disease develops either as a sporadic new mutation or is inherited in an autosomal dominant manner. Those cases in which recurrence is observed represent germinal mosaicism in one parent and not recessive inheritance. The only known exception to this statement is a null mutation within the *Col1A2* gene (see below).

The three-dimensional structure of the collagen fibril can be altered by a substitution for glycine in the collagen (gly-x-y) triplet, inframe deletion, an inframe insertion, or exon skipping. Depending on the helical location of a mutation, these produce a variety of clinical pictures that range from lethal (OI type II) to severely deforming (OI type III) to mildly deforming (OI type IV). Glycine substitution in the helical domain of the collagen α-1(I) chain is the most common defect. Glycine, the smallest amino acid, is a critical component that must fit in a sterically restricted space where the three chains of the triple helix come together. Substitutions can occur in either base of the GGN codon for glycine and do not alter the length of the chain but disrupt helical stability. The eight potential amino acid substitutions are cysteine, alanine, arginine, aspartic acid, cysteine, glutamic acid, serine, valine, and tryptophan. How the substitution affects the conformation of the collagen helix is still not well understood and current biochemical analysis does not always predict clinical severity. Since the helix assembles from the C-terminal propeptide, a mutation in the C terminal helical and propeptide region results in greater instability and more severe disease while mutations located in the mid-helical domain tend to be less severe. However, mutations within the mid-helical domain can have a severe phenotype, suggesting that subdomains within the helix are critical for function beyond just contributing to an intact helical structure. Mutations located at the N-terminal domain of either chain can be extremely mild and fall into the category of type I OI.

Because the exons that encode the helical domain maintain the reading frame, mutations in the consensus donor or acceptor site can lead to exon skipping, producing a shortened helix that has the same effect on helical stability as a glycine substitution (Byers *et al.,* 1983). Much less common are mutations that delete a portion of the gene and along with it a number of inframe exons (Mundlos *et al.,* 1996) or mutations that insert a segment of intron that remains inframe with the entire transcript. In the latter case, a nonhelical segment is inserted within the helical domain disrupting the structure of the collagen helix (Wang *et al.,* 1996).

The one exception to the statement that severe disease results from a dominant negative mutation in either type I collagen gene is a null mutation of the *Col1A2* gene. Forma-

tion of the heterotrimeric collagen molecule requires that the α-2(I) chain account for 50% of the available chains at the time the procollagen molecule is assembled. When this requirement is not met, either because of underproduction of the α-2(I) chain or overproduction of the α-1(I) chain, then homotrimeric molecules are formed. Severity of disease depends on the balance between homotrimeric and heterotrimeric molecules within the bone matrix. This may explain why there is a spectrum of disease severity from OI type III when both *Col1A2* alleles are affected, to measurable osteopenia and fragility in the heterozygous state (McBride *et al.,* 1998; Saban *et al.,* 1996), to an association with osteoporosis due to the sp1 polymorphic alteration in the *Col1A1* gene. This variation in disease severity acts in a recessive manner or as quantitative trait in which gene dosage contributes to the severity of bone disease.

Nondeforming OI

The most common mutation causing type I OI reduces the output of otherwise normal type I collagen. Because of the 2:1 requirement for formation of heterotrimeric collagen, the level of *Col1A1* production directly influences the accumulation of normal type I collagen molecules. Reduced output from a single *Col1A1* allele reduces the production of heterotrimeric collagen and the unincorporated α-2(I) chains are degraded intracellularly. The genetic mechanism for a clinical phenotype resulting from complete inactivation of one allele is referred to as haploid insufficiency. In fact a spectrum of disease severity related to gene dosage is observed in the Mov 13 mouse. The homozygous Mov 13 is an embryonic lethal that does not reach the stage of skeletal development (Lohler *et al.,* 1984), while the heterozygous Mov 13 is a model for OI type I (Bonadio *et al.,* 1990). Similarly, the severity of osteopenia in type I OI is probably related to the degree that one of the *Col1A1* alleles underperforms. It would not be surprising to find that a more subtle underproduction from a *Col1A1* allele could be a contributing quantitative trait to the development of osteoporosis.

Mutations introducing a premature stop codon are the most frequent cause for a null *Col1A1* allele (Redford-Badwal *et al.,* 1996; Slayton *et al.,* 2000; Willing *et al.,* 1994). Premature stop codons arising in all but the terminal exon of a gene usually lead to rapid destruction of the transcript rather than producing a truncated protein. Termed nonsense mediated RNA decay, recently discovered nuclear proteins are able to recognize a premature stop codon and target it for rapid destruction (Serin *et al.,* 2001). This appears to be an important mechanism for preventing a truncated protein from expression, thus saving the cell from proteins with unintended function. Mutations of these surveillance genes are incompatible with development (Medghalchi *et al.,* 2001). Demonstrating that a truncated α-1(I) chain is produced from a *Col1A1* transcript *in vitro* serves as the basis for uncovering the presence and location of the stop codon (Bateman *et al.,* 1999). Otherwise finding the mutation by a molecular approach is laborious and can be missed.

A second mechanism for producing a null *Col1A1* allele is retention of an intron within the mature transcript. Intron retention instead of exon skipping can result when a mutation of a splice donor site is located in a small intron such that the combination of the intron and the flanking upstream and downstream exon is regarded as an acceptable exon (Stover *et al.,* 1993). However, the presence of the mutant donor site retains the transcript within the splicing apparatus of the nucleus (S35 domain) and it is eventually destroyed (Johnson *et al.,* 2000). While this is an uncommon cause of a null allele, it has provided insight into the normal pathway for splicing a complex transcript such as collagen and demonstrated that splicing of otherwise normal but small collagen introns is relatively slow. Because splicing of approximately 25% of the *Col1A1* introns are candidates to be affected this mechanism, it would not be surprising to find polymorphic changes within the gene that contribute to the rate of splicing and that are associated with mild osteoporosis.

Other causes for diminished transcriptional activity from a collagen gene are extremely rare. The Mov 13 mutation affects the transcriptional activity of the *Col1A1* gene by a mechanism that is unlikely to be seen in humans. Because there are elements within the collagen promoter necessary for activity in osteoblasts (Dodig *et al.,* 1996), mutation in these regions may exist but have not been identified to date. Mutation within the 3′ untranslated region affecting polyadenylation have been reported, and mutations in the 5′ untranslated region would be predicted have a phenotype but have not been observed.

Finally, a nonfunctional collagen gene can result from synthesis of a procollagen chain which is unable to incorporate within the triple helical molecule. Frameshift mutations within the terminal exon of either collagen gene have been identified that lead to synthesis of full sized procollagen chain which is rapidly degraded intracellularly when it fails to incorporate into the collagen molecule (Willing *et al.,* 1993). In the case of the *Col1A1* product, mild OI is observed, but when it occurs in the *Col1A2* product, homotrimeric molecules are formed, causing a more severe bone phenotype (see above).

Consequences of OI Producing Mutations

Formation on the Extracellular Matrix and Osteoid Mineralization

Many approaches have been employed to characterize the triple helical structure and intramolecular organization of collagen fibrils in normal and OI subjects. X-ray diffraction analysis of collagen-like peptides show the destabilizing effect of a glycine point mutation (Beck *et al.,* 2000) and studies using NMR and circular dichroism lead to a similar conclusion (Baum and Brodsky, 1999; Liu *et al.,* 1998;

Melacini *et al.,* 2000). At the intramolecular level, X-ray diffraction has shown small fibers with less well-defined lateral growth and more fiber disorganization in tissue obtained from OI subjects (McBride *et al.,* 1997). At the biochemical level, mutations that interrupt the helix decrease the thermal stability of procollagen molecules and render the molecule more susceptible to proteolytic attack by tissue proteases (Bachinger *et al.,* 1993). This may explain the observation that mutant collagen molecules are not uniformly distributed through matrix but are found on the surface of bone (Bank *et al.,* 2000). Because 25% of the collagen produced by cells containing a *Col1A1* mutation are normal and 50% of the collagen produced by cells containing a *Col1A2* mutation are normal, tissue proteases probably select against the mutant molecules (Bateman and Golub, 1994) allowing for a substrata of relatively normal collagen fibers to accumulate.

Transmission and scanning EM have shown that the periodicity of OI fibrils are normal but the fibrils are disorganized and have wide variation in fiber diameter (Eyden and Tzaphlidou, 2001). Fibril formation can be studied *in vitro* from components synthesized by cultured cells (Hashizume *et al.,* 1999; Holmes *et al.,* 1992, 2001; Hulmes *et al.,* 1995). The importance of other proteins that modify the size and organization of otherwise normal type I collagen fibrils has been revealed by EM studies. These changes can affect the mechanical properties of the collagen fibers formed *in vitro* (Christiansen *et al.,* 2000; Ottani *et al.,* 2001). For example the copolymerization of type V collagen within the type I collagen fibril influences the size and structure of the type I collagen fibril (Kypreos *et al.,* 2000; Mizuno *et al.,* 2001). Another modifier of the collagen fiber size is the incorporation of unprocessed type I procollagen producing another form of Ehlers-Danlos syndrome (EDS) that can overlap with features of type I OI. The EDS-OI-like symptoms appear to result from impairing cleavage of the procollagen propeptide secondary to glycine substitution disruption in the N-terminal helical domain. A similar problem might be expected with a mutation affecting cleavage of the C-terminal propeptide (Holmes *et al.,* 1996). Induced mutations in certain noncollagenous proteins such as decorin (Danielson *et al.,* 1997), fibromodulin (Ezura *et al.,* 2000; Svensson *et al.,* 1999), and microfibrillin (Kielty *et al.,* 1998) can affect the structure or organization of type I collagen fibers, indicating that physical interaction between the two components plays an important role in this process. It would not be surprising that OI mutations may affect some of these binding interactions, adding to the complexity of the bone phenotype.

The interaction of the mineralizing phase of new bone formation with the matrix has been studied by high-voltage electron microscopy (Landis *et al.,* 1996a,b), Fourier transform infrared microspectroscopic analysis (Cassella *et al.,* 1994, 2000), and small angle X-ray scattering (Fratzl *et al.,* 1996). These methods demonstrate that while the absolute amount of and composition of hydroxyapatite within a OI bone is probably not abnormal, the crystal structure is deformed and probably contributes to the overall weakened nature of the bone (Camacho *et al.,* 1999). Thus while the primary defect is in helix formation, the ultimate determination of bone strength reflects how the helix influences the interaction of noncollagenous proteins and mineral and at this point the critical features of the molecule that control these interactive events are not fully understood.

Intrinsic Properties of the OI Osteoblast

The presence of intracellular mutant procollagen molecules and the physiological response to the abnormal extracellular osteoid combine to have a profound impact on the biology of the osteoblast, which further influences the severity and natural history of disease. It has been appreciated for some time that the rough endoplasmic reticulum of OI fibroblasts are grossly dilated (Lamande *et al.,* 1995) and the secretion of fully formed procollagen is impaired (Fitzgerald *et al.,* 1999; Lamande and Bateman, 1999). The role that hsp47 chaperone protein has in determining the trafficking of normal and mutant molecules within these cells is likely to be an important determinant for the biology of the affected osteoblast (Kojima *et al.,* 1998). In fact, gene knockout of the hsp47 protein is an embryonic lethal in which type I procollagen is produced that is susceptible to protease digestion (Nagai *et al.,* 2000), suggesting that this chaperone protein plays an essential role in normal triple helix formation (Thomson and Ananthanarayanan, 2000). The retention of the mutant procollagen molecule also leads to posttranslational overmodification of the lysine residues in the helical domain that may further affect the quality of fibril formation.

The inherent ability of an OI fibroblast or bone cell to produce collagen and proliferate *in vitro* is impaired and probably is a consequence of the retained procollagen molecules with the distended rough ER. *In vitro* studies of osteoblasts derived from OI humans (Fedarko *et al.,* 1995, 1996) or OIM mice (Balk *et al.,* 1997) show diminished markers of osteoblastic differentiation as well as reduced rate of cell proliferation. However, the cells can be driven into osteoblastic differentiation when given exogenous BMPs. The explanation for this observation is not clear but may reflect the quality or quantity of the extracellular matrix that is made by the preosteoblastic cell that is necessary for osteoblast differentiation *in vitro.* Possibly the high rate of bone turnover that is characteristic of this disease (see below) may lead to exhaustion and/or premature senescence of stem cells capable of generating vigorous osteoblastic cells *in vitro.* If stem cell exhaustion is also present in intact bone, then an additional factor of a limited osteoblast number will contribute to the severity of the bone disease.

Physiological Demands on the OI Osteoblast

Intact bone, probably through the osteocyte, is able to sense its mechanical environment and initiate a new round of bone formation when the load on the region exceeds its

ability to carry it. This fundamental principle of bone biology is continuously called upon in OI because the matrix that is produced is never able to support the load placed on the skeleton. This situation is illustrated in the histology of OI bone that shows a state of high turnover characterized by increased numbers of osteoblasts and osteocytes (Jones *et al.,* 1999) and an increased number of osteoclasts. Dynamic labeling shows that increased number of double labeled surfaces of normal thickness (Rauch *et al.,* 2000). Biochemical markers for bone formation and resorption in growing children and adults do reflect the histological findings, although the measurements are variable because of differences in growth rate and the underlying mutation (Brenner *et al.,* 1994; Goans *et al.,* 1995; Lund *et al.,* 1998). Some of these variables can be overcome in murine models in which there is uniformity of the genetic defect and sufficient numbers of mice of similar age and sex.

Analysis of murine models is particularly instructive in appreciating the pathophysiology of the OI mutation. Because net total bone formation in OI bone is low and its intrinsic properties for matrix production in culture are impaired, the OI osteoblast or its lineage is viewed as underproductive. This view can lead to the conclusion that stimulation to increase its rate of matrix formation should be beneficial to the disease. However, the OI osteoblast lineage is under constant stimulation to proliferate to build up sufficient

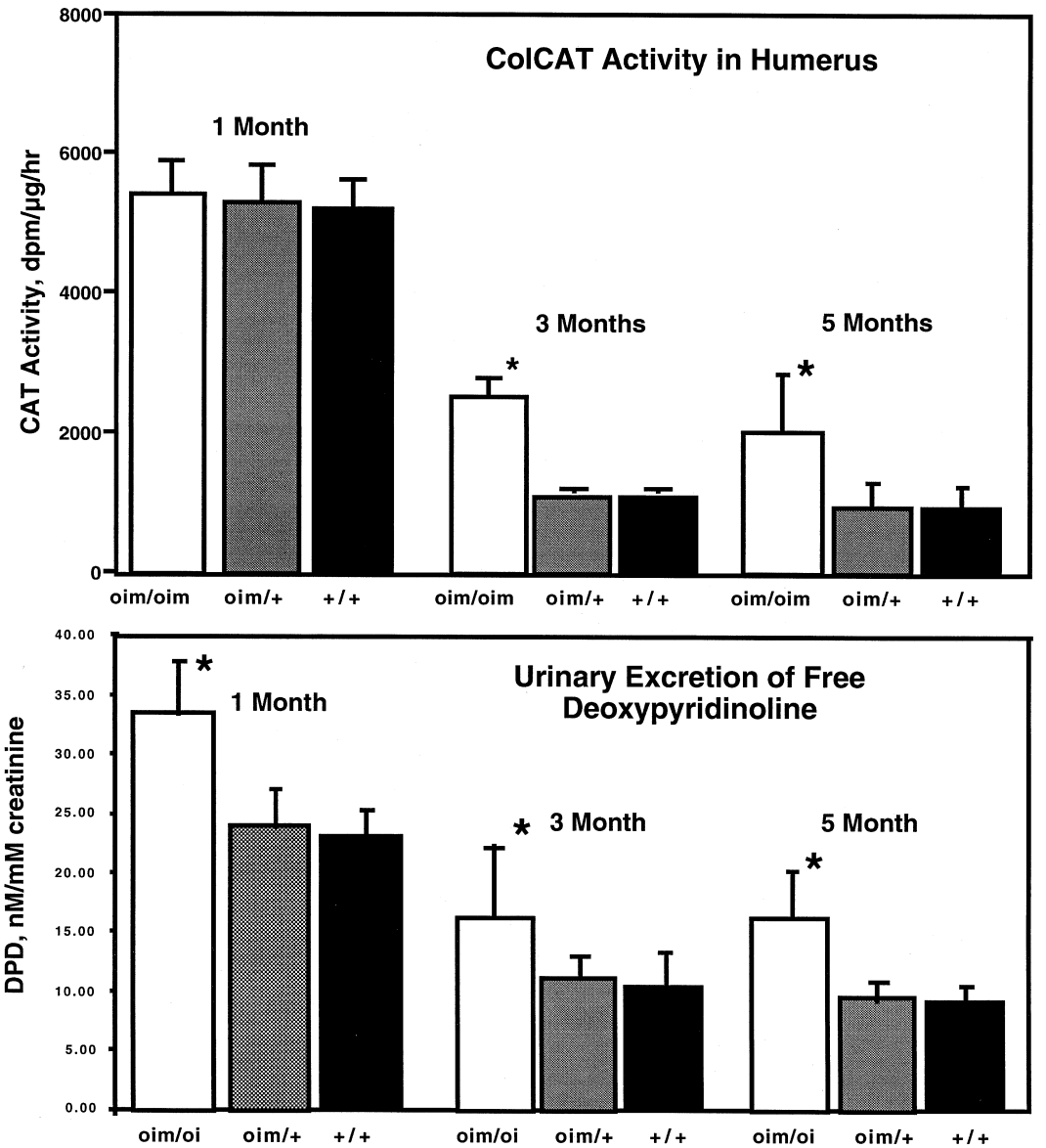

Figure 1 Relative balance of bone formation and resorption in OIM mice during the period of rapid somatic growth (1 month), adulthood (3 months), and older age (5 months). During somatic growth, the activity of the oim/oim osteoblast population does not exceed that of normal animals while bone resorption of the 1-month-old animal is greater than normal. Thus it is during the period of rapid bone growth that the deficit between bone formation and resorption is maximal. With stabilization of the skeleton, the continuous demand for new bone formation in the oim/oim mouse become evident and during this time the deficit between bone formation and resorption is less severe.

numbers of precursor cells to progress to full osteoblast differentiation and produce the matrix that was resorbed by the activated osteoclasts. The activated osteoblastic lineage can be demonstrated by measuring the content of *Col1A1* mRNA in OI bone or the activity of a type I collagen promoter transgene that is sensitive to osteoblastic activity. In both cases a high-level of transcriptional activity for type I collagen can be demonstrated relative to normal bone. The net effect is an uncoupling between the signals transmitted from the bone matrix to be bone lineage in which the bone cells do respond at the gene level but cannot deliver at the protein level. The lineage is already maximally stimulated in response to the activated osteoclastic pathways but the new matrix that is produced does not improve the mechanical properties of the bone.

Studies on the oim mouse model illustrates that it is the balance between matrix formation and resorption that determines bone strength in OI (Fig. 1). During periods of rapid linear growth, the deficit between formation and resorption is maximal because at this time bone turnover is enhanced even beyond the level that is responsive to mechanical forces. While normal bone has the reserve within the bone lineage to increase its rate of matrix formation, the OI bone lineage is already maximally stimulated so that it is during the period of linear growth that the deficit in net bone formation is most severe. This may explain why growth retardation and fractures are so severe in the rapidly growing child. With the completion of puberty and cessation of linear growth, bone remodeling slows and a balance between formation and resorption become more favorable. Thus puberty does not improve bone strength by stimulating the lineage but instead it stabilizes the skeleton and reduces the need for bone remodeling. When menopause reinstates a state of high bone resorption, the balance between formation and resorption again becomes unfavorable and fractures can return. In effect, the pathophysiology of OI can be viewed as a consequence of the activated osteoclast lineage and this probably explains the success of bisphosphonate treatment in OI.

Nondeforming OI secondary to an expressed collagen mutation probably represents a mild variant of deforming OI discussed above. However, OI type 1 resulting from haploid insufficiency of a *COL1A1* chain may have subtle differences. Analysis of the heterozygous Mov 13 mice show diminished bone volume and half-normal levels of col1a1 mRNA. This is consistent with human OI in which low levels of procollagen propeptide in blood reflect the null mutation in type I collagen-producing cells (Minisola *et al.,* 1994). Histomorphometry does show increase osteoblast cellularity and bone forming units and dynamic histomorphometry suggest a decrease in osteoid seams (McCarthy *et al.,* 1997). Excessive osteoclastic activity does not appear to be present. In both mouse and man with type I OI, significant skeletal remodeling becomes apparent upon sexual maturation so that the mechanical properties of the bone approach normal (Bonadio *et al.,* 1993; Jepsen *et al.,* 1997). While further analysis of a murine model that is healthy into adulthood is necessary, it would appear that the deficit between bone formation and resorption in type I OI is much less than in deforming forms of OI, particularly after the adult skeleton is established. Thus it is during adulthood that a relatively normal bone matrix is accumulated and fractures are uncommon. It is only during growth and in the menopause that this relationship is unfavorable, again underscoring the value of bisphosphonates for improving bone strength during these periods.

Therapy of OI

Antiresorptive Agents

Probably the most significant advance for OI since the previous edition of this text is the use of bisphosphonate in infants, growing children, and adults. Unlike previous agents that relied primarily on anecdotal evidence for success, a number of prospective studies have now shown that bisphosphonate can reduce fracture frequency, increase bone mineral content, and improve the radiographic assessment of the bone shape in growing children (Astrom and Soderhall, 1998; Glorieux *et al.,* 1998). Linear growth is not impaired and fractures heal at their expected rate. The findings are most dramatic in infants from one to three years of age in which the severity of the classification of the disease can be improved (Plotkin *et al.,* 2000). In both groups remarkable improvement is seen in the quality of life that includes motor function and muscle strength. Children begin to ambulate and adults find that their activities of daily living are easier to perform. Bone pain and diaphoresis, which is a major complaint often overlooked by physicians as well as families, is greatly diminished by the treatment. Equally positive results are being observed and adults although comprehensive studies have not yet been reported. Initial studies have used cyclical intravenous administration of pamidronate and currently prospective studies using oral forms of bisphosphonates are ongoing (Glorieux, 2000).

The success of bisphosphonate appears to be related to the unremitting osteoclastic activity. The effect of the drug can be monitored by measuring the level of collagen-derived cross-links in blood or urine. Clinical symptoms of bone pain and diaphoresis also correlate with the inhibitory effect of the drug on osteoclastic activity, suggesting that it is the process of high bone turnover and associated high blood flow, not unlike a pagetic lesion, that underlie these symptoms. Even though the matrix it now accumulates still contains the mutant collagen molecules, the balance between formation and degradation is improved and the accumulation of matrix improves bone strength relative a bone without matrix. The experience in children indicates that linear growth and repair of fractures is not compromised by the use of the bisphosphonate. Murine studies show less bowing and a diminished fractured frequency, even though bone strength by direct mechanical testing cannot be demonstrated. Further work is needed using animal models to confirm that the drug is acting primarily at the level of the osteoclast and

does not have other actions that contribute to clinical improvement. For example, if the bisphosphonate acts to reduce the level of bone turnover it might have the additional benefit of extending the longevity of the osteoblast lineage or allowing the resident osteoblast to achieve full differentiation and time to remodel the existing matrix from woven to lamellar bone.

Anabolic Agents

Growth hormone, IGF1 and PTH have the potential to increase bone mass. Except for a treatment protocol with growth hormone in children with deforming OI, most of the experience with these agents has been antidotal. Like all children who are initially started on growth hormone, OI children do experience an initial acceleration of growth rate. Because the treatment duration has been limited, a bone mass increase in excess to the increase in body size has not been reported, although stable ^{42}Ca isotopic studies demonstrate a improvement in mineral incorporation while on treatment (Vieira *et al.,* 1999, 2000). Given the underlying physiological basis of OI, it would be surprising that an agent which stimulates more bone turnover as part of its anabolic action would have a long-term beneficial effect. The osteoblast lineage is already maximally stimulated and the addition of agents which enhance osteoclastic activity will only contribute to the deficit between formation and degradation. The transient increasing growth rate that is seen in children with growth hormone occurs because the growth plate is stimulated to proliferate. If the bone that contains the growth plate cartilage (collar region and primary spongiosa) is no more structurally sound than before the stimulus, damage to the growth plate might be anticipated. Potentially the combination of growth hormone and bisphosphonate might provide a compromise that is acceptable and studies of with this combination are underway. However, this is another therapeutic setting requiring animal experimentation for concept validation. Furthermore, production of mice lines that are phenocopies of human OI with varying degrees of severity may point to the type of OI patient most appropriate for a particular combination of anabolic and/or antiresorptive drug (Antoniazzi *et al.,* 1996).

Cell and Gene Therapy

Because bisphosphonates do not correct the primary cause for OI and the effectiveness of their long-term use are still uncertain, steps to correct the underlying genetic mutation are being evaluated in both humans and mice. The possibility that gene therapy is a feasible strategy in OI came from the analysis of individuals who are somatic mosaic for an OI mutation but do not have evidence of bone disease. Those studies suggest that the deleterious effect of OI cells can be neutralized by the presence of normal cells so that if it were possible to introduce normal cells into an individual with OI, the severity of bone disease would be reduced. Furthermore, because bone turnover is high and the endogenous osteoblast lineage is activated in OI, introduction of normal cells into this environment would rapidly populate the bone with cells having a normal proliferative rate and making a normal matrix that would outproduce the effort from the resident OI cells. This treatment strategy requires the ability to introduce cells from the osteoblast lineage into OI subjects with the attendant problems of immune rejection unless a tissue-matched donor can be identified. Recent human transplant studies with bone marrow cells have been performed in a limited number of children with severe OI (Horwitz *et al.,* 1999, 2001). The success of these initial studies is difficult to assess at this time and again point to the importance of proof of principle experiment in animals before human experimentation is undertaken.

Assuming that the immune problems related to bone cell transplantation will be a major impediment for long-term engraftment of bone, a strategy needs to be developed in which the endogenous OI bone cells are engineered to correct the primary defect in type I collagen production *in vitro* followed by reintroduction into the affected host. This will require a two-step process in which the output from the mutant collagen allele is inhibited and a replacement collagen gene for the inactivated mutant gene is inserted. Once corrected, the engineered cells have to have the ability to engraft bone, proliferate, and participate in new bone formation. Each of these problems is a major research undertaking (Fig. 2).

Allele-specific suppression of a mutant collagen gene is potentially possible at either the genetic or the RNA level. Targeting the endogenous gene with a triplex-forming oligonucleotide (Faruqi *et al.,* 2000) or with a chimeric RNA–DNA oligonucleotide (Kren *et al.,* 1999) can correct a specified sequence. Although not all genes may be equally susceptible to this type of modification, if the change can be made it will be permanent and restore the output of collagen production to a normal level. This is a recently developed genetic approach that is likely to undergo further improvement which could make it the method of choice. More effort has been made at reducing the output at the level of RNA. While antisense constructs to a RNA transcript is unlikely to have allele specific discrimination, other strategies such as hammerhead (Klebba *et al.,* 2000), and hairpin (Lian *et al.,* 1999) ribozymes, U1snRNA (Beckley *et al.,* 2001), RNA transplicing (Caudevilla *et al.,* 1999; Mansfield *et al.,* 2000), and RNase P (Kawa *et al.,* 1998) do have such a potential. A detailed description on the background and mechanism of each antiRNA effector is beyond the scope of this chapter but is contained in the referenced articles. It is unlikely that any one approach will have sufficient strength and specificity to inhibit the mutation-containing transcript sufficiently to have a major impact on disease severity (Dawson and Marini, 2000). However, combining two or more of these antiRNA efforts that act at different compartments within the cell and by a different molecular mechanism may attain this goal.

Unlike correction at the level of the mutant gene, the antiRNA approach will require the introduction of a procollagen cDNA expression construct to replace the lost activity of the suppressed transcript. Expression of a collagen gene

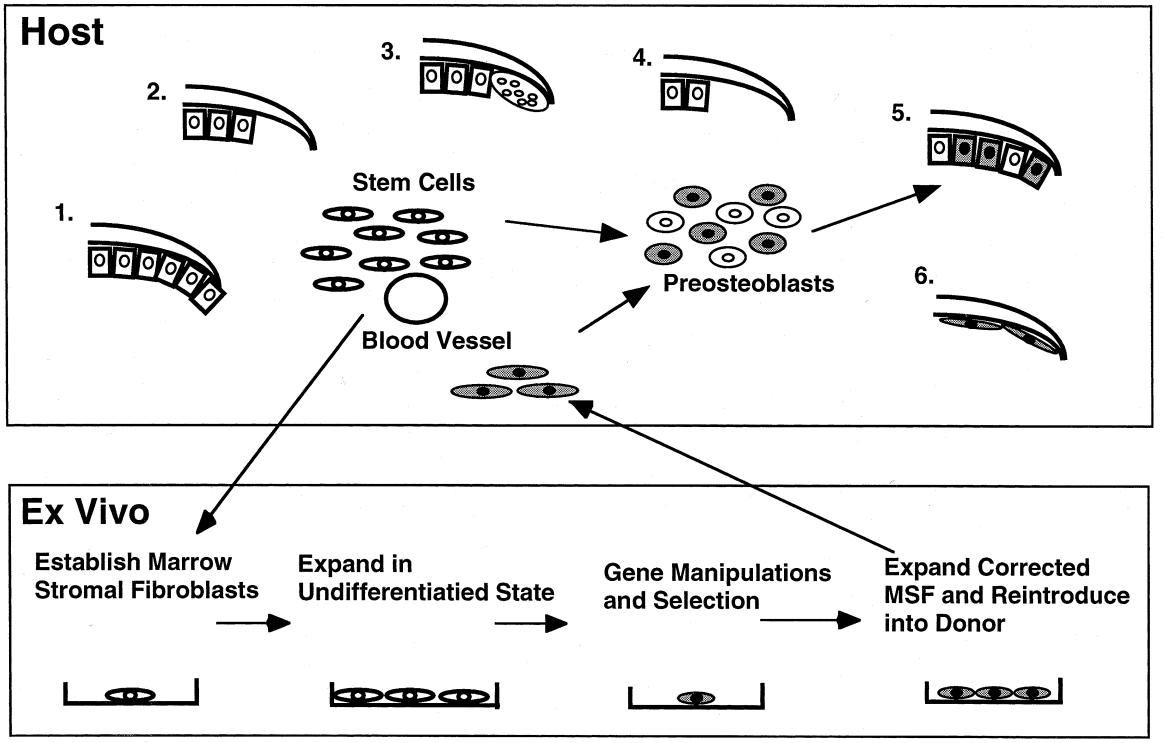

Figure 2 Strategy for somatic gene therapy for OI. The host bone is undergoing rapid and continuous bone turnover in which activated osteoblasts (1) start a cycle of bone remodeling (2). Osteoclasts remove damaged bone matrix (3) followed by a new wave of bone formation (4 and 5). Cells that contribute to the new round of bone formation are captured, probably by bone marrow aspiration, and grown in a culture dish under nondifferentiating conditions. Genetic manipulations include elimination and replacement of the mutant gene followed by expansion of the population of corrected cells. These cells are reintroduced into the host (darkened cells) and they begin to participate in the cycle of new bone formation. Over time the corrected cells outpopulate the endogenous cells and the quality of the bone matrix improves (5 and 6).

poses problems that differ from gene replacement for an enzyme or clotting factor. In bone, type I collagen production can account for 20% of total protein synthesis so that an extremely strong promoter is required to drive the replacement cDNA. In addition the cDNA has to be regulated so that α-1(I) chain production in excess of α-2(I) chains does not occur (see discussion of α-1(I) trimer above). Probably a type I collagen promoter fragment is the most appropriate promoter to achieve regulated expression. Another consideration is the vector to deliver the correcting construct. Although a collagen cDNA has been strongly expressed from an adenoviral construct at a high multiplicity of infection (Niyibizi *et al.,* 2000), this approach does not achieve permanent expression. Retrovectors have the size capacity and permanent integration needed to contain and express a collagen promoter–collagen cDNA construct. However, expression of the transgene can be suppressed after the transduced cells are reintroduced into the host (Cherry *et al.,* 2000). Modification of the retrovector that remove sequences responsible for suppression appear to overcome this problem, allowing for osteoblast-specific expression of the transgene throughout the life of the mouse (Stover *et al.,* 2001). It still remains to be demonstrated that this vector approach can achieve the level of collagen cDNA expression equivalent to one endogenous collagen allele.

Despite the genetic engineering problems, the most severe hurdle for somatic gene therapy for OI is the reintroduction of osteoprogenitor cells that are capable of homing to bone and participating in new bone formation. While there is no question regarding the ability of marrow stromal cells to differentiate into mature osteoblasts *in vitro* or in subcutaneous implant (Dennis *et al.,* 1999; Krebsbach *et al.,* 1997), demonstration that this is possible when administered systemically is still unconvincing. Most studies in man and mouse can demonstrate a low degree (1–5%) of engraftment of bone or bone marrow stroma as assessed by a transgenic or unique endogenous genetic marker (Ding *et al.,* 1999; Onyia *et al.,* 1998; Pereira *et al.,* 1998). In many cases, the marker gene does not discriminate whether this cell arises from a mesenchymal or macrophage lineage. Even when a relatively pure population of stromal cells are used for transplantation, small contamination from the myeloid lineage could belie stromal cell engraftment. Only one study has demonstrated expression of a transgene that is a marker of a differentiated osteoblast (Nilsson *et al.,* 1999) although the level of engraftment and its contribution to bone formation is hard to assess.

The underlying genetic abnormality also determines the success of stromal cell transplantation. When the disease is non-cell autonomous (i.e., the transplanted cells are

engineered to secrete a deficient soluble factor), an improvement in the disease phenotype occurs irrespective of where the cells established residence. This is most obvious when the transplanted cells express a cytokine (Brouard *et al.,* 1998) or clotting factor (Chuah *et al.,* 2000). This mechanism probably explains the recent success of bone marrow transplantation for vitamin D-resistant rickets (Miyamura *et al.,* 2000). Clinical improvement of a disease that is cell autonomous, such as OI, requires that the cells populate the affected bone, proliferate, differentiate, and participate in bone turnover. This is a standard that has not yet been met in any murine studies. The one exception is when the transplanted cells have undergone prolonged expansion *ex vivo* (Dahir *et al.,* 2000; Oyama *et al.,* 1999). For mouse this type of treatment rapidly leads to cell immortalization, making interpretation of a transplantation experiment difficult.

As new phamacologic or genetic therapies are developed, it will be increasingly necessary to establish a set of criteria that is used to judge its success relative to current therapies. While bisphosphonates still need further evaluation to fully appreciate their most effective use in different age and disease classifications, they do represent a significant advance in the treatment of OI and probably have to be regarded as the threshold that a new therapy has to exceed. Particularly as it relates to somatic gene therapy for OI, criteria need to be established that (a) demonstrate successful engraftment and (b) document improvements in bone health that are equivalent to or exceed medical therapy (Table II, column A). Whether the transplanted individual is mouse or man, it must be demonstrated that the transplanted cells populate the bone, expand in number over time, participate in the new bone formation, and provide a continuous source of new bone cells over the life of the transplanted subject. These studies are easier to perform in the mouse when the transplanted cells are engineered to contain visual transgenes driven by bone-specific promoters. Not only does the transgenic marker identify the source of the cell, it can reflect its level of participation in new bone formation. Moreover, this approach can be used to determine whether donor stem cells removed from the transplanted bone are still able to generate differentiated osteoblastic cells *in vitro.* If the transplanted cells are participating in new bone formation, the quality and quantity of the bone should improve over time. Radiographs should show remodeled bones with improved architecture and bone density measurements at well-defined sites will be increased in humans. μCT can be used to assess bone mass and architecture in mice. Bone histomorphometry and dynamic labeling studies should confirm these clinical measures of bone health. Particularly in the mouse, it should be possible to demonstrate improved mechanical properties of the bone after the intervention. Biochemical studies can also contribute to the impression of success by demonstrating that markers of new bone formation remain elevated while the level of markers reflecting bone degradation gradually subside.

Table II Criteria for Successful Therapy of OI

	A. Weight of criteria		B. Cell therapy	
Criteria	Man	Mouse	Man	Mouse
Cellular evaluation				
Cell engraftment	+	+	+	+
New bone formation	+++	+++	NR	NR
Increasing cell number; graft persistence	+++	+++	NR	NR
Bone evaluation				
BMD, QCT	+++	+++	+	NR
X-ray	++	+	NR	NR
Bone histology	+++	+++	+	+
Structural properties	NA	+++	NA	NR
Biochemical	+	++	NR	NR
Clinical criteria				
Quality of life	+++	+	NR	NR
Growth, wt gain	+++	+	++	+
Muscle strength	++	++	NR	NR
Fracture rate	+	+	+	NR

Note. NR, not reported; NA, not ascertainable; Assessment of current trials of bisphosphonate and cell transplantation in man and mouse.

The clinical evaluation of transplanted individuals is equally important to the assessment of success. Bone pain and diaphoresis are major symptoms that are daily facts of life for many patients with OI. Muscle weakness can be profound and in growing children greatly delay acquisition of motor milestones. Successful interruption of the pathophysiological cycle of OI appears to greatly reduce these symptoms. Bone pain diminishes, diaphoresis is greatly reduced and muscle tone improves. Children begin to ambulate and adults find that their activities of daily living are easier to perform. Because the symptoms are so profound for individuals with OI, they need to be recorded in a prospective manner as a clinical outcome measure. These types of measurements are difficult to record in the mouse, although cage activity can be quantitated and probably would be informative. Certainly fracture frequency, linear growth, and weight gain are important to record, although they can be influenced by many other factors unrelated to the intervention.

Even disregarding the molecular steps necessary to correct the genetic abnormalities in OI stromal cells, the success of transplantation of normal cells into a normal or OI host is relatively ineffective at this time (Table II, column B). Given the experimental difficulties of performing these studies in a controlled and quantitative manner in humans, greater emphasis must be placed on studies in experimental animals in which the problems inherent to the procedure can be identified and solved. Ultimately these problems will be solved and this will open a new era of therapeutic opportunities for OI and other osteopenic bone diseases.

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CHAPTER 67

Hereditary Deficiencies in Vitamin D Action

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Introduction

Hereditary deficiencies in vitamin D action can be due to disturbances in the synthesis of the hormonal form of the vitamin, 1,25-dihydroxyvitamin D1,($25(OH)_2$D-calcitriol), or defects in the interaction of calcitriol and its target tissues.

Vitamin D derived from endogenous production in the skin or absorbed from the gut is transformed into its active form by two successive steps: hydroxylation in the liver to 25-hydroxyvitamin D [25(OH)D], followed by 1α-hydroxylation in the renal proximal tubule to $1,25(OH)_2$D. Some other cells exhibit 1α-hydroxylase activity: placental decidual cells, keratinocytes, macrophages from various origins, and some tumor cells. The role of the extrarenal production of $1,25(OH)_2$D is unknown, and under normal conditions it does not significantly contribute to the circulating levels of the hormone. Hydroxylation at carbon 24, to produce 24,25-dihydroxyvitamin D [$24,25(OH)_2$D] or 1,24,25-trihydroxyvitamin D, is performed in a wide range of normal tissues and is believed to be important in the removal of vitamin D metabolites. All these enzymes are mitochondrial mixed-function oxidases containing cytochrome P450, and having ferredoxin and heme-binding domains. Cloning of cDNA for porcine 25-hydroxylase and the mouse, rat, and human 24- and 1-hydroxylase, as well as the genes for the latter two enzymes, has been recently reported (Akeno *et al.,* 1997; Fu *et al.,* 1997a; Jehan *et al.,* 1998; Jones *et al.,* 1999; Monkawa *et al.,* 1997; Murayama *et al.,* 1999; Ohyama *et al.,* 1993; Shinki *et al.,* 1997; Takeyama *et al.,* 1997). Hereditary defects along the cascade of $1,25(OH)_2$D synthesis lead to a deficiency in the vitamin D hormonal form.

Isolated deficiency of 25(OH)D is a very rare autosomal recessive disorder, attributed to, but not proven to be caused by deficiency in the hepatic vitamin D-25 hydroxylase activity (Gill et al. 2000).

This discussion will therefore concentrate on hereditary defects in the renal 25-hydroxyvitamin D-1 α-hydroxylase activity. Regarding interaction with the target tissue, as detailed elsewhere in this book, calcitriol effects are mediated via a high-affinity intracellular receptor—the vitamin D receptor (VDR). VDR acts as a ligand-modulated transcription factor that belongs to the steroid, thyroid, and retinoic acid receptors gene family (Baker *et al.,* 1988; Burmester *et al.,* 1988; Evans, 1988; Ozur *et al.,* 1991). Thus hereditary defects in the interaction of calcitriol and its target tissues could evolve from defects in hormone binding to VDR, defects in the interaction of the VDR or the hormone–VDR complex with DNA, or defects in the transcription modulation function.

Though VDRs were demonstrated in most, if not all, tissues, leading to the increased recognition of multiple target organs and actions of the hormone, it seems that most, if not all, clinical signs and symptoms associated with deficient vitamin D actions are due to perturbations in mineral metabolism.

$1,25(OH)_2$D is the most powerful physiological agent that stimulates active transport of calcium, and to a lesser degree phosphorus and magnesium, across the small intestine (DeLuca, 1984; Mayer *et al.,* 1984). Thus, deficiencies in vitamin D action will lead to a decrease in the net flux of mineral to the extracellular compartment, causing hypocalcemia and secondary hyperparathyroidism. Hypophosphatemia will

ensue, as a result of both reduced absorption of phosphorus due to deficient calcitriol action on the gut and increased renal phosphate clearance due to secondary hyperparathyroidism. Low concentrations of calcium and phosphorus in the extracellular fluid will lead to defective mineralization of organic bone matrix. Defective bone matrix mineralization of the newly formed bone and growth plate cartilage will produce the characteristic morphological and clinical signs of rickets, while at sites of bone remodeling, it will cause osteomalacia. Deficiencies in vitamin D action may impair the differentiation of osteoblasts and thus their functional capacity to mineralize bone matrix; this may be an additional mechanism leading to rickets and osteomalacia (Owen *et al.,* 1991; Reichel *et al.,* 1989).

As all hereditary deficiencies in vitamin D action will lead to the same clinical, radiological, histological, and most of the biochemical aberrations, those common features will be discussed first.

Clinical Features of Rickets and Osteomalacia

Children with hereditary deficiencies in vitamin D action will appear normal at birth and usually develop the characteristic features of rickets within the first 2 years of life. Defects in bone mineralization are particularly evident in regions of rapid bone growth, including, during the first year of life, the cranium, wrist, and ribs. Rickets at this time will lead to widened cranial sutures, frontal bossing, posterior flattening of the skull (craniotabes), bulging of costochondral junction (rachitic rosary), indentation of the ribs at the diaphragmatic insertion (Harrison's groove), and widening of the wrists. After the first year of life with the acquisition of erect posture and rapid linear growth, the deformities are most severe in the legs. Bow legs (genu varum) or knock-knee (genu valgum) deformities of various severity develop as well as widening of the end of long bones. If not treated, rickets may cause severe lasting deformities, compromise adult height, and increase susceptibility to pathological fractures.

The specific radiographic features of rickets reflect the failure of cartilage calcification and endochondral ossification and therefore are best seen in the metaphysis of rapidly growing bones. The metaphyses are widened, uneven, concave, or cupped and because of the delay in or absence of calcification, the metaphyses could become partially or totally invisible (Fig. 1). In more severe forms or in patients untreated for prolonged periods, rarefaction and thinning of the cortex of the entire shaft, as well as bone deformities and greenstick fractures, will become evident.

The clinical features of osteomalacia are subtle and could be manifested as bone pain or low back pain of varying severity in some cases. The first clinical presentation could be an acute fracture of the long bones, public ramii, ribs, or spine. The radiographic manifestations could be mild, e.g., generalized, nonspecific osteopenia or more specific, such as pseudofractures, commonly seen at the medial edges of long bones shaft.

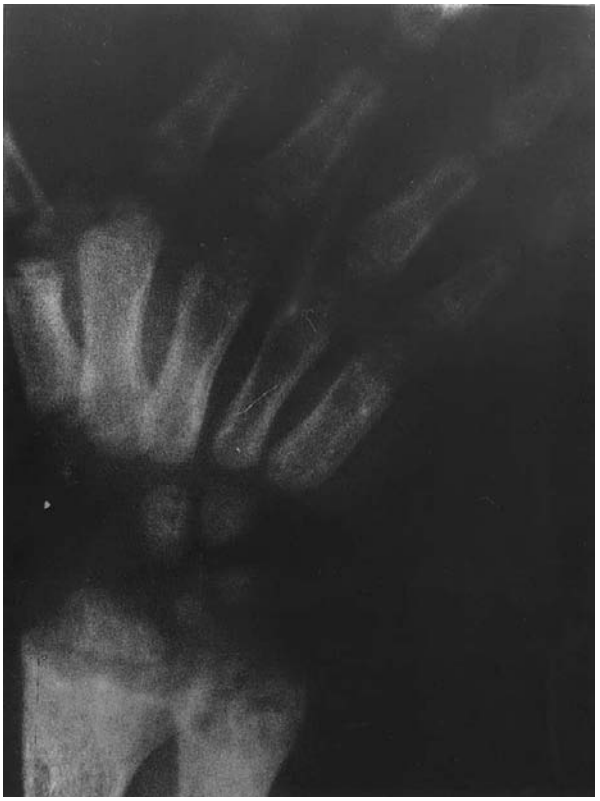


Figure 1 Radiographs of wrists and hands of a patient with VDDR. Note the signs of severe rickets and demineralization.

In hypocalcemic rickets and/or osteomalacia, as is the case in deficiencies in vitamin D action, there may exist radiographic features of secondary hyperparathyroidism such as subperiosteal resorption and cysts of the long bones.

The characteristic histological feature of rickets and osteomalacia is deficiency or lack of mineralization of the organic matrix of bone. Because in clinical practice a bone specimen can be obtained only from the iliac crest, the histological picture is osteomalacia. Osteomalacia is defined as excess osteoid (hyperosteoidosis) and a quantitative dynamic proof of defective bone matrix mineralization obtained by analysis of time-spaced tetracycline labeling (Parfitt, 1983; Teitelbaum and Bollough, 1979).

The biochemical parameters characterizing deficiencies in vitamin D action can be divided into those associated with vitamin D status, the primary disturbance in mineral homeostasis and the respective compensatory mechanisms, and changes in bone metabolism. Changes in mineral and bone metabolism will be similar in all states of deficient vitamin D action, while serum levels of vitamin D metabolites will characterize each of the two classes delineated in the introduction (Table I).

As previously discussed, deficiencies in vitamin D action will lead to hypocalcemia and secondary hyperparathyroidism. Thus, the characteristic biochemical features are low to low-normal concentrations of serum calcium (depending on compensatory parathyroid activity), low urinary calcium excretion, hypophosphatemia, increased serum immunoreac-

Table I Biochemical Features of Vitamin D-Dependent Rickets (VDDR) Types I and II

	Serum concentrations						
	Calcium	Phosphorus	Alkaline phosphatase	iPTH	25OHD	$1,25(OH)_2D$	$24,25(OH)_2D$
VDRR-I	↓	↓	↑	↑	N– ↑	↓	↑
VDRR-II	↓	↓	↑	↑	N– ↑	↑	↓

tive parathyroid hormone (iPTH) levels, increased urinary cyclic AMP excretion, and decreased tubular reabsorption of phosphate (the last two measures reflecting the biological activity of elevated iPTH). Biochemical markers associated with increased osteoid production such as bone-specific alkaline phosphatase and osteocalcin will be elevated in states of rickets and osteomalacia (Cole *et al.,* 1985). However, as $1,25(OH)_2D$ stimulates osteocalcin synthesis *in vivo* and *in vitro*, serum levels of this biochemical bone marker are unreliable measures in hereditary vitamin D-deficient states.

Hereditary Deficiency in $1,25(OH)_2D$ Production (Vitamin D-Dependent Rickets Type I)

Prader *et al.,* (1961) were the first to describe two young children who showed all the usual clinical features of vitamin D deficiency despite adequate intake of the vitamin, thus coining the name "pseudovitamin D deficiency." Complete remission was dependent on continuous therapy with high doses of vitamin D; thus the term "vitamin D-dependent rickets" (VDDR). However, remission of the disease could be achieved by continuous therapy with physiological (microgram) doses of 1α-hydroxylated vitamin D metabolites (Delvin *et al.,* 1981; Fraser *et al.,* 1973).

Family studies have revealed it to be an autosomal recessive disease. Linkage analysis in a subset of French-Canadian families assigned the gene responsible for the disease to chromosome 12q13 (De Brackeleer and Larochell, 1991; Labuda *et al.,* 1990). The gene encoding the 1α-hydroxylase of mouse kidney, human keratinocyte, and peripheral mononuclear cells was localized on chromosome 12q13.1–q13.3, which maps to the disease locus of VDDR-I (Fu *et al.,* 1997b; Kitanaka *et al.,* 1998; Smith *et al.,* 1999; St. Arnaud *et al.,* 1997, Wang *et al.,* 1998; Yoshida *et al.,* 1998). There are no direct measures of the renal enzyme proving defective 1α-hydroxylase activity. This is virtually impossible to obtain, because of both the difficulty in obtaining tissue and the low level of expression. There are, however, several indirect observations to support this etiology. First, circulating levels of 25-hydroxyvitamin D (25(OH)D) are normal or elevated, depending on previous vitamin D treatment. Second, serum concentrations of $1,25(OH)_2D$ are very low (Table I). Third, while massive doses (100–300 times the daily recommended dose) of vitamin D or 25(OH)D are required to maintain remission of rickets, physiological replacement doses of calcitriol are sufficient to achieve the same effect. Fourth, it was reported that cells isolated from the placenta of two women with this disease had deficient activity of the decidual enzyme 25(OH)D-1α-hydroxylase (Glorieux *et al.,* 1995). It is noteworthy that during the past 15 years or so, it was demonstrated that human decidual cells do produce $1,25(OH)_2D$ and that this enzyme was regulated by feedback mechanisms (Delvin and Arabian, 1987; Diaz *et al.,* 2000; Weisman *et al.,* 1979). Finally, the 1α-hydroxylase gene from more than 20 families with VDDR-I and some of their first-degree healthy relatives were analyzed by direct sequencing, site-directed mutagenesis, and cDNA expression in transfected cells. All patients had homozygous mutations while parents or other healthy siblings were heterozygous for the mutation. Most patients of French-Canadian origin had the same mutation causing a frameshift and a premature stop codon in the putative heme-binding domain. The same mutation was observed in additional families of diverse origins (Smith *et al.,* 1999). All other patients had either a base-pair deletion causing premature termination codon upstream from the putative ferredoxin and heme-binding domains, or missense mutations (Fu *et al.,* 1997b; Kitanaka *et al.,* 1999; Smith *et al.,* 1998; Wang *et al.,* 1998; Yoshida *et al.,* 1998). No 1α-hydroxylase activity was detected when the mutant enzyme was expressed in various cells. The sequence of the human 1α-hydroxylase gene from keratinocytes and peripheral blood mononuclear cells has recently been shown to be identical with the renal gene (Fu *et al.,* 1997b; Kitanaka *et al.,* 1999; Smith *et al.,* 1999; Wang *et al.,* 1998; Yoshida *et al.,* 1998), thus supporting the use of these accessible cells as a proxy to study the renal tubular enzymatic defect. Taken together, these observations support the notion that the etiology of this hereditary disease is a defect in the renal tubular 25(OH)D-1α-hydroxylase activity.

The beneficial therapeutic effect of high serum concentrations of 25(OH)D in patients treated with massive doses of vitamin D, while $1,25(OH)_2D$ levels remain low, may have several possible explanations. First, high levels of 25-(OH)D may activate the VDR whose affinity for this metabolite is approximately two orders of magnitude lower than for $1,25(OH)_2D$. Second, a metabolite of 25(OH)D may act directly on target tissues, and finally, high levels of 25(OH)D may drive the local production of $1,25(OH)_2D$ via a paracrine–autocrine pathway, assuming that the tissue enzyme is controlled differently than the renal and decidual enzyme.

The differential diagnosis of VDDR-I from other hereditary forms of hypocalcemic rickets and especially the one associated with defects in the vitamin D receptor–effector system is based on serum concentrations of calcitriol and

Table II Response to Treatment of Patients with Vitamin D-Dependent Rickets (VDDR) Types I and II

	Vitamin D or 25(OH)D		1α-Hydroxylated vitamin D metabolites	
	Physiological	Pharmacological	Physiological	Pharmacological
VDDR-I	−	+	+	Toxic
VDDR-II	−	− or +	−	− or +

Note. Physiological doses are those recommended or being used as replacement therapy. Pharmacological doses are 100 times and more of the physiological doses (see details in text).

the response to treatment with 1-α-hydroxylated vitamin D metabolites (Tables I and II).

A similar syndrome has been described and studied in a mutant strain of pigs where the mode of inheritance as well as the clinical and biochemical features are similar to the human disease (Fox *et al.,* 1985; Harmeyer *et al.,* 1982). Piglets affected by the disease have rickets, elevated 25(OH)D with low or undetectable $1,25(OH)_2D$ serum concentrations, normal specific tissue binding sites for tritiated $1,25(OH)_2D$, and no detectable activity of 25(OH)D-1α-hydroxylase in renal cortical homogenates. Thus, there is strong evidence that the disease state in the pig is caused solely by an inherited defect in the renal enzyme.

Hereditary Defects in the Vitamin D Receptor-Effector System (Vitamin D-Dependent Rickets Type II)

Introduction

This is a rare disorder and only ~60 patients have been reported (Balsan *et al.,* 1983; Bear *et al.,* 1981; Brooks *et al.,* 1978; Castells *et al.,* 1986; Chen *et al.,* 1984; Clemens *et al.,* 1983; Cockerill *et al.,* 1997; Eil *et al.,* 1981; Feldman *et al.,* 1982; Fraher *et al.,* 1986; Fujita *et al.,* 1980; Griffin and Zerwekh, 1983; Hawa *et al.,* 1996; Hewison *et al.,* 1993; Hirst *et al.,* 1985; Hochberg *et al.,* 1984; Liberman *et al.,* 1980, 1983b; Lin and Uttley, 1993; Lin *et al.,* 1996; Malloy *et al.,* 1997; Marx *et al.,* 1978, 1984; Mechica *et al.,* 1997; Rosen *et al.,* 1979; Saijo *et al.,* 1991; Simonin *et al.,* 1992; Sockalosky *et al.,* 1980; Takeda *et al.,* 1986, 1987, 1989; Tauchiya *et al.,* 1980; Yagi *et al.,* 1993; Whitfield *et al.,* 1996; personal communications).

Brooks *et al.* (1978) described an adult patient with hypocalcemic osteomalacia and elevated serum concentration of $1,25(OH)_2D$. Treatment with vitamin D, causing a further increase in serum calcitriol levels, cured the patient. The term vitamin D-dependent rickets type II (VDDR-II) was suggested to describe this disorder. However, reports on additional patients, about half of whom did not respond to vitamin D therapy, as well as *in vivo* and *in vitro* studies to be discussed below, seem to prove that vitamin D dependency is a misnomer. The term hereditary defects in the vitamin D receptor–effector system or end-organ resistance to $1,25(OH)_2D$ action is therefore more appropriate to describe the etiology and pathogenesis of this syndrome. However, due to convention and convenience, the term VDDR-II will be retained in this chapter.

Clinical and Biochemical Features

General Features

The clinical, radiological, histological, and biochemical features (except serum levels of vitamin D metabolites) are typical of hypocalcemic rickets and/or osteomalacia as previously discussed. Notable exceptions are two unrelated patients with hyperphosphatemia, despite elevated serum levels of iPTH (Liberman *et al.,* 1980; Yagi *et al.,* 1993), which can be the end result of long-standing and severe hypocalcemia, which paradoxically inhibits the phosphaturic response to PTH or represents an additional hereditary renal tubular defect.

In VDDR-II there is no history of vitamin D deficiency and no clinical or biochemical response to physiological doses of vitamin D or its 1α-hydroxylated active metabolites. Serum levels of 25(OH)D are normal or elevated (depending on preceding vitamin D therapy); $1,25(OH)_2D$ concentrations are markedly elevated before or during therapy with vitamin D preparations; and 24,25-dihydroxyvitamin D ($24,25(OH)_2D$)) circulating levels are inappropriately low (Table I).

The disease manifests itself as an active metabolic bone disease in early childhood. However, late onset at adolescence and adulthood was documented in several sporadic cases including the first report by Brooks *et al.* (1978) and Fujita *et al.* (1980). These patients represented the mildest form of the disease and had a complete remission when treated with vitamin D or its active metabolites. It is unclear if the adult onset patients belonged to the same hereditary entity, as no further studies on their VDR status have been published.

Ectodermal Anomalies

A peculiar clinical feature of VDDR-II patients, appearing in approximately two-thirds of the subjects, is total alopecia or sparse hair (Fig. 2). Alopecia usually appears during the first year of life and in one patient, at least, has been associated with additional ectodermal anomalies (Liberman *et al.,*

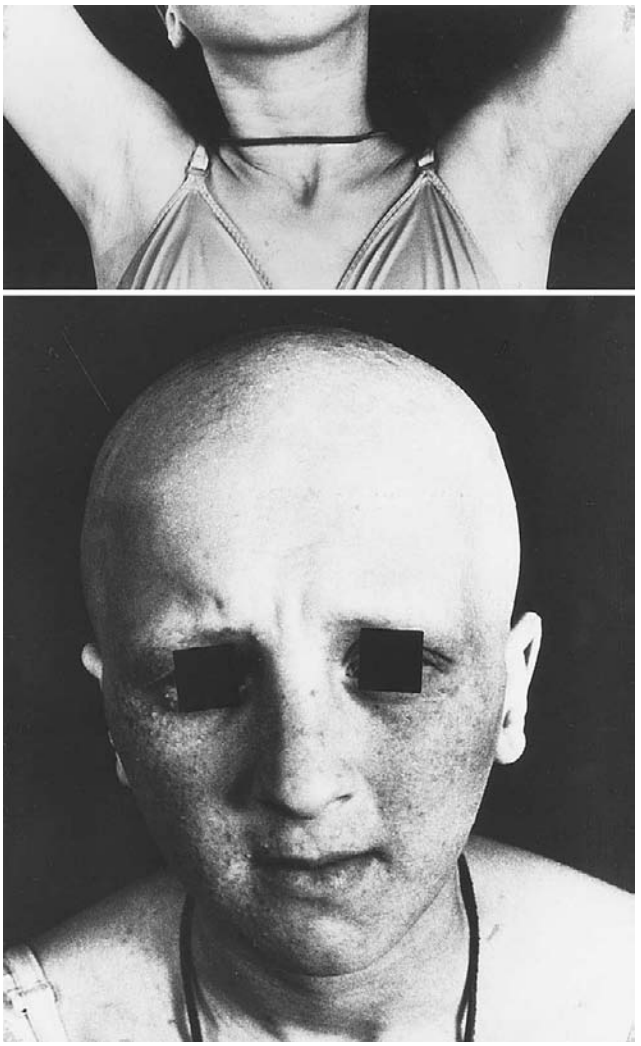


Figure 2 A patient with VDDR-II with total alopecia. Note, no scalp or axilla hair and no eyebrows or eyelashes.

1980). It seems that alopecia is a marker of a more severe form of the disease as judged by the earlier onset, the severity of the clinical features, the proportion of patients who do not respond to treatment with high doses of vitamin D or its active metabolites, and the extremely elevated levels of serum $1,25(OH)_2D$ recorded during therapy (Marx *et al.,* 1984, 1986). Though some patients with alopecia could achieve clinical and biochemical remission of their bone disease, none have shown hair growth. The notion that total alopecia is probably a consequence of a defective vitamin D receptor–effector system is supported by the following: alopecia has only been associated with hereditary defects in the VDR system, i.e., with end-organ resistance to the action of the hormone, and has not been recorded with hereditary deficiency in $1,25(OH)_2D$ synthesis, i.e., low circulating levels of the hormone; alopecia is present in kindreds with different defects in the VDRs; high-affinity uptake of tritiated $1,25(OH)_2D_3$ in the nucleus of the outer root sheath of the hair follicle of rodents has been demonstrated by autoradiography (Strumpf *et al.,* 1979); and the epidermis and hair follicle contain a calcium-binding protein that is partially vitamin D-dependent (Marx *et al.,* 1984). Finally, alopecia develops in homozygote VDR knockout mice (Li *et al.,* 1997; Yoshizawa *et al.,* 1997). Taken together, it could be hypothesized that an intact VDR–effector system is important for the differentiation of the hair follicle in the fetus, which is unrelated to mineral homeostasis.

VITAMIN D METABOLISM

Serum concentrations of $1,25(OH)_2D$ range from upper normal values to markedly elevated before therapy, but on vitamin D treatment may reach the highest levels found in any living system (100 times and more than the upper normal range) (Marx *et al.,* 1986). These values may represent the end results of four different mechanisms acting synergistically to stimulate strongly the renal 25(OH)D-1; α-hydroxylase. Three of the mechanisms are hypocalcemia, secondary hyperparathyroidism, and hypophosphatemia. The fourth mechanism may be a failure of the negative feedback loop by which the hormone inhibits the renal enzyme activity caused by the basic defect in the VDR–effector system. This was demonstrated in a patient with VDRR-II in remission (normal serum levels of calcium, phosphorus, and PTH) in whom a load of $25(OH)D_3$ had caused a marked increase in serum $1,25(OH)_2D_3$ concentration (Balsan *et al.* 1983; Marx *et al.,* 1984). It was reported recently that the 1α-hydroxylase gene expression was not suppressed by $1,25(OH)_2D_3$ in renal tubular cells from VDR knock out mice while it was suppressed in cells with normal VDR or heterozygote for the null mutation (Murayama *et al.,* 1999; Takeyama *et al.,* 1997).

$1,25(OH)_2D$ is a potent inducer of the enzyme 25(OH) D-24-hydroxylase *in vivo* and *in vitro*. Serum levels of $24,25(OH)_2D$ have been very low or inappropriately low in the face of the elevated concentrations of the hormone in patients with VDDR-II (Castells *et al.,* 1986; Fraher *et al.,* 1986; Liberman *et al.,* 1980; Marx *et al.,* 1984). This probably reflects defective stimulation of $24,25(OH)_2D$ production due to the basic deficiency in VDR activity. This assumption is supported by the observation that in mutant mice lacking the VDR, expression of 24(OH) ase was reduced to undetectable levels and the normal induction of the enzyme by $1,25(OH)_2D_3$ was not obtained (Takeyama *et al.,* 1997).

MODE OF INHERITANCE

In approximately half of the reported kindreds, parental consanguinity and multiple siblings with the same defect suggest autosomal recessive mode of inheritance (Marx *et al.,* 1984). Parents of patients who are expected to be obligate heterozygotes have been reported to be normal, i.e., no bone disease or alopecia and normal blood biochemistry. However, studies on cells (cultured dermal fibroblasts, Epstein–Barr transformed lymphoblasts, and mitogen-stimulated lymphocytes) obtained from parents of affected children revealed decreased bioresponses, decreased normal VDR protein and its mRNA, and a heterozygote genotype exhibiting both normal and mutant DNA alleles (Malloy *et al.,* 1989, 1990; Ritchie *et al.,* 1989; Takeda *et al.,* 1990). There is a striking

clustering of patients around the Mediterranean, including patients reported from Europe and America who originated from the same area. A notable exception is a cluster of some kindred from Japan (Fujita *et al.,* 1980; Tauchiya *et al.,* 1980, 1986, 1987, 1989; Yagi *et al.,* 1993).

Cellular and Molecular Defects

METHODS

The near ubiquity of a similar if not identical VDR–effector system among various cell types including cells originating from tissues easily accessible for sampling made feasible studies on the nature of the intracellular and molecular defects in patients with VDDR-II. The cells used were mainly fibroblasts derived from skin biopsies (Balsan *et al.,* 1983; Castells *et al.,* 1986; Chen *et al.,* 1984; Clemens *et al.,* 1983; Eil *et al.,* 1981; Feldman *et al.,* 1982; Fraher *et al.,* 1986; Griffin and Zerwekh, 1983; Hirst *et al.,* 1985; Hughes *et al.,* 1988; Liberman *et al.,* 1983b; Malloy *et al.,* 1989, 1990; Marx *et al.,* 1984; Ritchie *et al.,* 1989; Takeda *et al.,* 1989) and peripheral blood mononuclear (PBM) cells (Koren *et al.,* 1985; Ritchie *et al.,* 1989; Takeda *et al.,* 1986, 1990; Yagi *et al.,* 1993). PBM cells contain high-affinity receptors for $1,25(OH)_2D_3$ that are expressed constitutively in monocytes and are induced in mitogen-stimulated T-lymphocytes and Epstein–-Barr (EB) transformed lymphoblasts. All cells have been used to assess most of the steps in $1,25(OH)_2D_3$ action from cellular and subcellular uptake of the hormone to bioresponse as well as to elucidate the molecular aberrations in the VDR protein, RNA, and DNA levels.

The hormone–receptor interaction has been analyzed by several methods including binding characteristics of ^{3}H 1,25 $(OH)_2D_3$ to intact cells, nuclei or high salt cellular soluble extracts—so-called cytosol (Balsan *et al.,* 1983; Castells *et al.,* 1986; Chen *et al.,* 1984; Clemens *et al.,* 1983; Eil *et al.,* 1981; Feldman *et al.,* 1982; Fraher *et al.,* 1986; Hirst *et al.,* 1985; Hochberg *et al.,* 1984; Hughes *et al.,* 1988; Koren *et al.,* 1985; Liberman *et al.,* 1983a,b; Malloy *et al.,* 1989, 1990; Marx *et al.,* 1984; Ritchie *et al.,* 1989; Sone *et al.,* 1990; Takeda *et al.,* 1985; Yagi *et al.,* 1993), measurements of VDR protein content by monoclonal antibodies with radiological immunoassay or Western blot analysis (Malloy *et al.,* 1990; Ritchie *et al.,* 1989), immunocytochemical methods in whole cells (Barsony *et al.,* 1990), and characterization of the hormone–receptor complex on continuous sucrose gradient and nonspecific DNA–cellulose columns (Balsan *et al.,* 1983; Chen *et al.,* 1984; Clemens *et al.,* 1983; Eil *et al.,* 1981; Feldmam *et al.,* 1982; Hirst *et al.,* 1985; Hochberg *et al.,* 1984; Hughes *et al.,* 1988; Liberman *et al.,* 1983a,b; Malloy *et al.,* 1989; Marx *et al.,* 1984; Sone *et al.,* 1990).

The cloning and nucleotide sequencing of the human VDR gene made it feasible to study the molecular defects in patients with VDRR-II. The methods used included, among others, isolation of genomic DNA that encodes the structural portion of the human VDR, PCR amplification, screening, and sequencing of the amplified DNA fragments (Hughes *et al.,* 1988; Kristjansson *et al.,* 1993; Malloy *et al.,* 1990; Ritchie *et al.,* 1989; Sone *et al.,* 1990; Yagi *et al.,* 1993); cloning and sequencing of the VDR cDNA produced from isolated fibroblasts total RNA by reverse transcription and PCR amplification (Rut *et al.,* 1994; Saijo *et al.,* 1991; Weise *et al.,* 1993); recreation of the mutant receptor *in vitro* by introducing the appropriate base change in normal VDR cDNA by site-directed mutagenesis and measuring the biochemical and functional properties of the encoded VDR by transfecting an expression vector containing the mutant cDNA into mammalian cell lines (Hewison *et al.,* 1993; Hughes *et al.,* 1988; Malloy *et al.,* 1990; Ritchie *et al.,* 1989; Sone *et al.,* 1990; Yagi *et al.,* 1993); measurement of VDR mRNA by Northern blot analysis (Malloy *et al.,* 1990); and restriction enzyme mapping techniques (Yagi *et al.,* 1993).

TYPES OF DEFECTS

Studies with the above-mentioned methods in cells originating from a variety of patients with VDDR-II revealed heterogeneity of the cellular and molecular defects in the VDR–effector system. Based on the known functional properties of the VDR, three different classes of defects could be identified (Fig. 3).

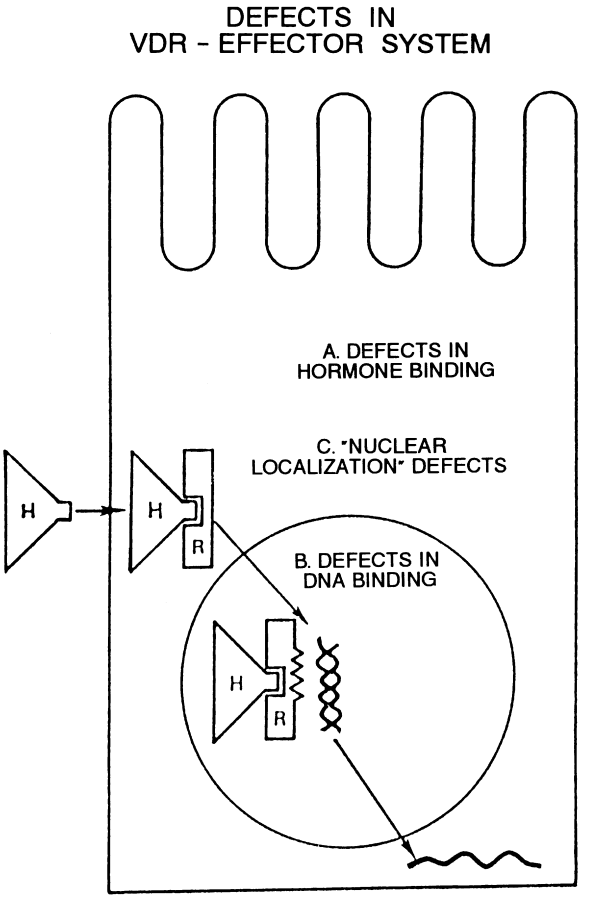


Figure 3 Three classes of defects in intracellular interaction of VDR, $1,25(OH)_2D$, nuclei, and/or DNA.

Deficient Hormone Binding There are three subgroups in this class: (i) No Hormone Binding. This is the most common abnormality observed and is characterized by unmeasurable specific binding of ^3H, $25(OH)_2D_3$ to either intact cells, nuclei, or cell extracts (Balsan *et al.,* 1983; Chen *et al.,* 1984; Cockeril *et al.,* 1997; Feldman *et al.,* 1982; Fraher *et al.,* 1986; Hawa *et al.,* 1996; Koren *et al.,* 1985; Kristjansson *et al.,* 1993; Liberman *et al.,* 1983a,b; Malloy *et al.,* 1990; Marx *et al.,* 1984; Mechica *et al.,* 1997; Ritchie *et al.,* 1989; Sone *et al.,* 1989). Studies in several kindreds with this defect (including an extended kindred with 8 patients studied) revealed undetectable levels of VDR by immunoblots on an immunoradiometric assay in most kindreds (Kristjansson *et al.,* 1993; Malloy *et al.,* 1990; Ritchie *et al.,* 1989; Weise *et al.,* 1993). DNA from these affected subjects exhibited a single base mutation in each kindred resulting in (a) conversion of a normal amino acid codon into a premature termination codon in the coding sequence of the VDR protein and (b) a frameshift in translation resulting in a premature stop codon. The truncated VDRs produced lacked hormone binding or both hormone and DNA binding domains (Fig. 4) (Cockeril *et al.,* 1997; Hawa *et al.,* 1996; Mechica *et al.,* 1997; Weise *et al.,* 1993). The recreated mutant VDR cDNA was expressed in mammalian cells, and the resulting mutant VDR was demonstrated to be the truncated protein that exhibited no specific hormone binding. Steady-state VDR mRNA levels were decreased or undetectable in patients' cells from one kindred (Malloy *et al.,* 1990; Ritchie *et al.,* 1989). An immunoradiometric assay for VDR did not detect the 148- and 291-amino-acid-long mutant VDR, though this portion of the receptor included both epitopes required for recognition (Weise *et al.,* 1993). These observations may indicate an unstable transcript and/or increased turnover. In cells cultured from parents of some patients, expected to be obligate heterozygotes, binding of ^3H, $25(OH)_2D_3$, VDR protein, and mRNA content of cells ranged from the lower limit of normal to about half the normal level.

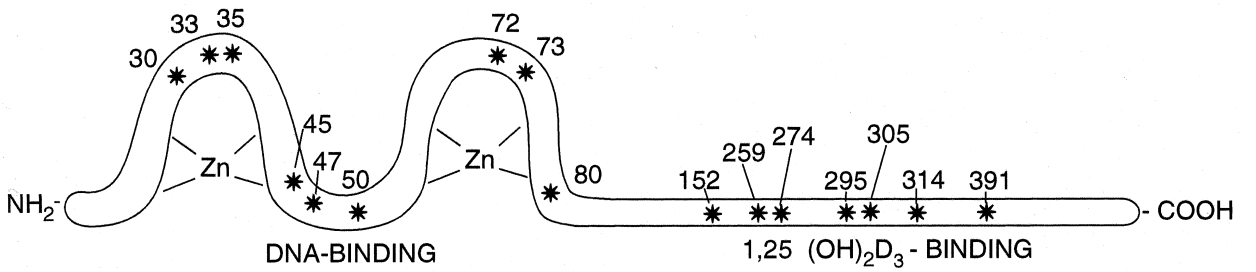
In one patient representing a kindred with no hormone binding, a missense mutation resulted in the substitution of the hydrophobic basic arginine-274 by the hydrophilic nonpolar leucine in the hormone binding region (Kristjansson *et al.,* 1993) (Fig. 4). In this patient, normal transcription in a transfection assay could be elicited by 1000-fold higher concentrations of calcitriol than needed for the wild-type receptor. However, no *in vivo* or *in vitro* stimulation of 25(OH)D-24-hydroxylase could be obtained by high concentrations of $1,25(OH)_2D_3$.

(ii) Defective Hormone Binding Capacity. In one patient representing one kindred, the number of binding sites in nuclei and high-salt soluble cell extracts was 10% of control, with an apparent normal affinity (Balsan *et al.,* 1983; Liberman *et al.,* 1983a,b).

(iii) Defective Hormone Binding Affinity. Binding affinity of tritiated calcitriol was reduced 20- to 30-fold, with normal capacity, in high-salt soluble dermal fibroblast extracts from one kindred (Castells *et al.,* 1986). An additional patient, representing a different kindred had a modest decrease of VDR affinity when measured at 0° C (Malloy *et al.,* 1997).

No studies on the molecular defect were performed in patients with the last two defects.

Deficient Binding to DNA Cell preparations derived from patients with this defect demonstrate normal or near normal binding capacity and affinity for ^3H $1,25(OH)_2D_3$ to nuclei of intact cells and to high-salt soluble cell extracts, as well as normal molecular size VDR of 48–50 kDa, as analyzed by immunoblot. Hormone receptor complexes, however, have



NORMAL	MUTANT
30 Arg (CGA)	Stop (TGA)
33 Gly (GGC)	Asp (GAC)
35 His (CAC)	Gln (CAG)
45 Lys (AAA)	Glu (GAA)
47 Phe (TTC)	Lle (ATC)
50 Arg (CGA)	Gln (CAA)
72 Arg (CGC)	Stop (TGA)
73 Arg (CGG)	Gln (CAA)
80 Arg (CGG)	Gln (CAG)

NORMAL	MUTANT
152 Gln (CAG)	Stop (TAG)
259 Gln (CAG)	Pro (CCG)
274 Arg (CGC)	Leu (CTC)
295 Tyr (TAC)	Stop (TAA)
305 His (CAC)	Gln (CAG)
314 Lle (ATC)	Ser (AGC)
391 Arg (CGC)	Cys (TGC)

Figure 4 Schematic presentation of the homozygous mutation in the VDR protein in VDDR-II. The asterisks depict sites of amino-acid substitutions due to point mutations and codon changes, using the numbering system of Baker *et al.* (1988).

decreased affinity to nonspecific DNA (peak elution from DNA–cellulose columns at 0.1 M KCl as compared with 0.2 M in normals) (Hirst *et al.,* 1985; Hughes *et al.,* 1988; Liberman *et al.,* 1986; Saijo *et al.,* 1991; Yagi *et al.,* 1993). A single nucleotide missense mutation within exon 2 or 3, encoding the DNA binding domain of the VDR, was demonstrated in genomic DNA isolated from dermal fibroblasts and/or EB-transformed lymphoblasts from 10 unrelated kindreds (Hughes *et al.,* 1988; Lin *et al.,* 1996; Malloy *et al.,* 1989; Mechica *et al.,* 1997; Rut *et al.,* 1994; Sone *et al.,* 1990; Saijo *et al.,* 1991; Yagi *et al.,* 1993). Eight different single nucleotide mutations were found in the ten kindreds (Fig. 4). Two apparently unrelated kindreds share the same mutation (Saijo *et al.,* 1991; Sone *et al.,* 1990).

All point mutations caused a single substitution of an amino acid that resides in the region of the two zinc fingers of the VDR protein that are essential for the functional interaction of the hormone–receptor complex with DNA. Interestingly, all these altered amino acids are highly conserved in the steroid receptor superfamily and seem to concentrate in three regions—the tip of the first zinc fingers (three mutations), the "knuckle" region, just C-terminal of the first zinc fingers (three mutations), and the C-terminal side of the second zinc fingers (two mutations). All of these single amino acid residue substitutions are associated with charge changes and thus affect hydrogen bonding. These aberrations, as well as changes in hydrophobicity, will have a deleterious effect on the interaction of the hormone–receptor complex with DNA.

Each of the mutants was recreated by introducing the appropriate base change into the normal VDR cDNA and then transfecting the mutated cDNA into mammalian cells. The functional properties of the resultant VDR expression product were indistinguishable from mutant DNA, i.e., normal hormone binding and deficient binding to nuclear extracts and nonspecific DNA (Hughes *et al.,* 1988; Saijo *et al.,* 1991; Sone *et al.,* 1990). Studies on cells obtained from parents of some of these patients revealed, as expected, a heterozygous state, i.e., expression of both normal and defective forms of VDR as well as normal and mutant gene sequences (Hughes *et al.,* 1988; Malloy *et al.,* 1989).

Deficient Nuclear Uptake The following features characterize the hormone–receptor–nuclear interaction in this defect—normal or near normal binding capacity and affinity of ^{3}H 1,25$(OH)_2D_3$ to high-salt soluble cell extracts with low to unmeasurable hormone uptake into nuclei of intact cells (Eil *et al.,* 1981; Hewison *et al.,* 1993; Liberman *et al.,* 1983b; Takeda *et al.,* 1989; Whitfield *et al.,* 1996). These features were demonstrated in skin-derived fibroblasts in all kindreds, in cells cultured from a bone biopsy of one patient (Liberman *et al.,* 1983b), and in EB-transformed lymphoblasts of one patient (Hewison *et al.,* 1993). Occupied VDR obtained from high-salt fibroblast extracts of two kindreds demonstrated normal binding to nonspecific DNA cellulose (Liberman *et al.,* 1986). Immunocytological studies in fibroblasts of a patient with this defect showed that immediately after 1,25$(OH)_2D_3$ treatment, VDR accumulated along the nuclear membrane with no nuclear translocation (Barsony *et al.,* 1990). Patients with this defect included a kindred with normal hair and several kindreds with total alopecia. Finally, almost all patients responded with a complete clinical remission to high doses of vitamin D and its active 1α-hydroxylated metabolites.

Attempts to characterize the molecular defect were carried in six kindreds. In three of them, no mutation in the coding region of the VDR gene was observed (Hewison *et al.,* 1993; pers. commun.). Studies in two kindreds revealed a normal molecular mass and quantitative expression of the VDR as judged by immunoblotting (Whitfield *et al.,* 1996). Complete sequencing of the VDR coding region revealed a different single nucleotide mutation in each kindred: ATC to AGC for isoleucine-314 to serine in one kindred, CGC to TCG altering arginine -391 to cysteine in the second kindred, and CAG to CCG altering glutamine-259 to proline in the third kindred (Fig. 4) (Cockeril *et al.,* 1997; Whitfield *et al.,* 1996), This region is considered to play a role in heterodimerization of VDR with RXR, and thus it has been suggested that these patients' receptors have defects that compromise RXR heterodimerization, which is essential for nuclear localization and probably for recognition of the vitamin D-responsive element as well. The fact that no mutation in the VDR coding region was observed in three additional kindreds with the same phenotypical defect may suggest that the genetic defect affects another component of the receptor effector system that is essential for the VDR function as a nuclear transcription factor. It has been recently shown that coactivation complexes are essential for the ligand-induced transactivation of VDR (Freedman, 1999). It is worthwhile to note that in one kindred originally described with this defect (Takeda *et al.,* 1989) a missense mutation at position 140 in exon 3, encoding the DNA binding domain was observed (Saijo *et al.,* 1991).

In Vitro Posttranscriptional and Transcriptional Effect of 1,25$(OH)_2D_3$

In vitro bioeffects of the hormone on various cells in patients with VDDR-II have been assayed mainly by two procedures, induction of 25(OH)D-24-hydroxylase and inhibition of mitogen stimulated PBM cells.

1,25$(OH)_2D_3$ induces 25(OH)D-24-hydroxylase activity in skin-derived fibroblasts (Balsan *et al.,* 1983; Castells *et al.,* 1986; Chen *et al.,* 1984; Clemens *et al.,* 1983; Feldman *et al.,* 1982; Fraher *et al.,* 1986; Gamblin *et al.,* 1985; Griffin *et al.,* 1983; Hewison *et al.,* 1993; Hirst *et al.,* 1985; Hughes *et al.,* 1988; Liberman *et al.,* 1983b; Malloy *et al.,* 1990; Ritchie *et al.,* 1989; Rut *et al.,* 1994; Sone *et al.,* 1990; Yagi *et al.,* 1993), mitogen-stimulated lymphocytes (Takeda *et al.,* 1990), and cells originating from bone (Balsan *et al.,* 1986) in a dose-dependent manner. In cells from normal subjects, maximal and half-maximal induction of the enzyme was achieved by 10^{-8} and 10^{-9} M concentrations of 1,25 $(OH)_2D_3$, respectively. Dermal fibroblast or PBM cells from VDDR-II patients with no calcemic response to maximal doses of

vitamin D or its metabolites in vivo did not show any 25(OH)D-24-hydroxylase response to very high concentration of $1,25(OH)_2D_3$ *in vitro,* while dermal fibroblasts from patients with a calcemic response to high doses of vitamin D or its metabolites *in vivo* showed inducible 24-hydroxylase with supraphysiological concentrations of 1,25 $(OH)_2D_3$, i.e., a shift to the right, *in vitro.* Physiological concentrations of $1,25(OH)_2D_3$ partially inhibit mitogen-induced DNA synthesis in peripheral lymphocytes with a half maximal inhibition achieved at 10^{-10} M of the hormone (Koren *et al.,* 1985; Takeda *et al.,* 1986). Mitogen stimulated lymphocytes from several kindreds with defects characterized as no hormone binding or deficient binding to DNA, with no calcemic response to high doses of vitamin D and its metabolites *in vivo*, showed no inhibition of lymphocyte proliferation *in vitro*, with concentrations of up to 10^{-6} M $1,25(OH)_2D_3$. (Fig. 5). Additional methods for measuring bioeffects of $1,25(OH)_2D_3$ on various cells *in vitro* were carried out only in few patients and included inhibition of dermal fibroblast proliferation (Clemens *et al.,* 1983), induction of osteocalcin synthesis in cells derived from bone (Balsan *et al.,* 1986), a mitogenic effect on dermal fibroblasts (Barsony *et al.,* 1989), and stimulation of cGMP production in cultured skin fibroblasts (Barsony and Marx, 1988). It is noteworthy that in all assays mentioned and without exception, each patient's cells showed severely deficient responses.

With the elucidation of the molecular defects in VDDR-II, the transactivation abilities of naturally occurring mutant or recreated mutant VDRs were evaluated in a transcriptional activation assay. The human osteocalcin gene promoter fused to chloramphenicol acetyltransferase (CAT) gene reporter plasmid was transfected into the patients or into normal fibroblasts (Hewison *et al.,* 1993; Hughes *et al.,* 1988; Malloy *et al.,* 1990; Ritchie *et al.,* 1989; Saijo *et al.,* 1991; Sone *et al.,* 1989; Yagi *et al.,* 1993). Treating normal transfected cells with $1,25(OH)_2D_3$, caused a concentration-dependent induction of transcription, measured by increased CAT activity. No induction of transcription was observed in cells originating from patients with defects characterized as no hormone binding (Kristjansson *et al.,* 1993; Malloy *et al.,* 1990; Ritchie *et al.,* 1989) or deficient binding to DNA (Hughes *et al.,* 1988; Saijo *et al.,* 1991; Sone *et al.,* 1990; Yagi *et al.,* 1993). Moreover, in a cotransfection assay, the addition of a normal human VDR cDNA expression vector to the transfected plasmid that directed synthesis of a normal VDR restored hormone responsiveness of resistant cells. Finally, in a patient characterized as deficient nuclear uptake defect no mutation was identified within the coding region of the VDR gene; no induction of 25(OH)D-24-hydroxylase activity by up to 10^{-6} M $1,25(OH)_2D_3$ was observed in cultured skin fibroblasts, but there was normal transactivation by $1,25(OH)_2D_3$ in the transcriptional activation assay (Hewison *et al.,* 1993).

CELLULAR DEFECTS AND CLINICAL FEATURES

Normal hair was described with most phenotypes of the cellular defects, the exception being patients with deficient hormone binding capacity and affinity, but this could be due to the fact that only one or two kindreds were described per subgroup. Normal hair is usually associated with a milder form of the disease, as judged by the age of onset, severity of the clinical features, and usually the complete clinical and biochemical remission on high doses of vitamin D or its metabolites.

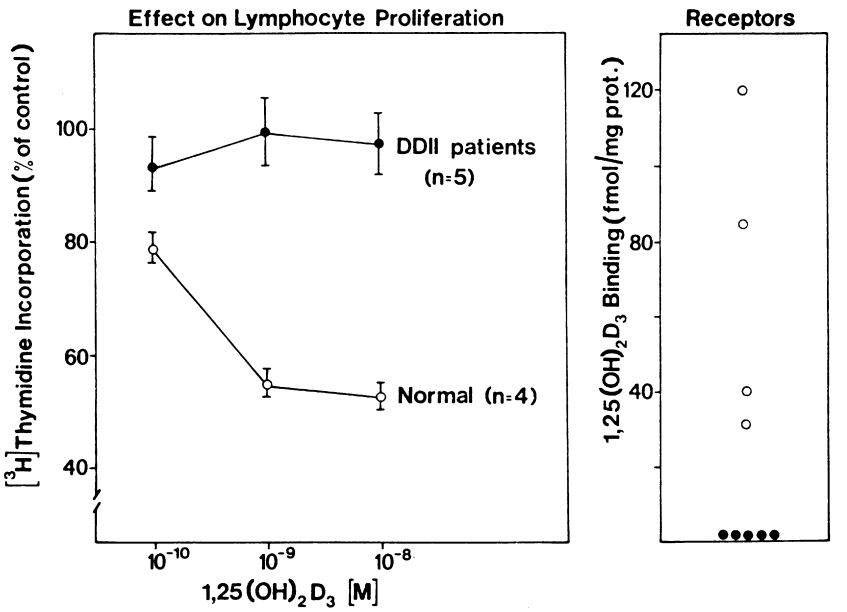


Figure 5 Effect of increasing concentrations of $1,25(OH)_2D_3$ on mitogen-induced lymphocyte proliferation from patients with VDDR-II (DD-II) and normal controls. The numbers of specific $[^3H]1,25(OH)_2D_3$ binding sites are depicted (right) in the same cell populations. VDRR-II patients tested here are with the defect characterized as no hormone binding.

One patient with normal hair seemed to display resistance both *in vivo* (no clinical remission on circulating calcitriol level up to 100 times the mean normal adult values) and *in vitro* (no induction of 25(OH)D-24-hydroxylase activity in dermal fibroblasts by up to 10^{-8} M $1,25(OH)_2D_3$ (Fraher *et al.,* 1986). However, it may be argued that the patient did not receive full treatment for a long enough period of time (only 10 weeks of total treatment with the various vitamin D metabolites). Only approximately half of the patients with alopecia have shown satisfactory clinical and biochemical remission to high doses of vitamin D or its active 1α-hydroxylated metabolites, but the dose requirement is ~10-fold higher than in patients with normal hair (Marx *et al.,* 1986).

It seems that patient defects characterized as deficient hormone binding affinity and deficient nuclear uptake achieve complete clinical and biochemical remission on high doses of vitamin D or its active 1α-hydroxylated metabolites. Most of the patients with other types of defects could not be cured with high doses of vitamin D or its metabolites. However, it should be emphasized that not all of the patients received treatment for a long enough period of time and with sufficiently high doses (see Treatment, below).

Diagnosis

Clinical features of early-onset rickets with no history of vitamin D deficiency, total alopecia, parental consanguinity, additional siblings with the same disease, serum biochemistry of hypocalcemic rickets, elevated circulating levels of $1,25(OH)_2D$, and normal to high levels of 25(OH)D (Table I) support the diagnosis of VDDR-II. The issue becomes more complicated when the clinical features are atypical, i.e., late onset of the disease, sporadic cases, and normal hair. Failure of a therapeutic trial with calcium and/or physiological replacement doses of vitamin D or its metabolites may support the diagnosis but the final direct proof requires the demonstration of a cellular, molecular, and functional defect in the VDR–effector system.

Based on the clinical and biochemical features, the following additional disease states should be considered: (1) extreme calcium deficiency—a seemingly rare situation, described in a group of children from a rural community in South Africa, who consumed an exceptionally low-calcium diet of 125 mg/day (Pettifor *et al.,* 1981). All had severe bone disease with histologically proven osteomalacia, biochemical features of hypocalcemic rickets with elevated serum levels of $1,25(OH)_2D$, and sufficient vitamin D. Calcium repletion caused complete clinical and biochemical remission. Nutritional history and the response to calcium supplementation support this diagnosis. (2) Severe vitamin D deficiency—during initial stages of vitamin D therapy in children with severe vitamin D-deficient rickets, the biochemical picture may resemble VDDR-II, i.e., hypocalcemic rickets with elevated serum calcitriol levels. This may represent a "hungry bone syndrome," i.e., high calcium demands of the abundant osteoid tissue becoming mineralized. This is a transient condition that may be differentiated from VDDR-II by a history of vitamin D deficiency and the final therapeutic response to replacement doses of vitamin D.

Treatment

In about half of the kindreds with VDDR-II, the bioeffects of $1,25(OH)_2D_3$ were measured *in vitro* (see above). An invariable correlation (with one exception) was documented between the *in vitro* effect and the therapeutic response *in vivo;* i.e., patients with no calcemic response to high levels of serum calcitriol showed no effects of $1,25(OH)_2D_3$ on their cells *in vitro* (either induction of 25(OH))D-24-hydroxylase or inhibition of lymphocyte proliferation) and vice versa. If the predictive therapeutic value of the *in vitro* cellular response to $1,25(OH)_2D_3$ could be substantiated convincingly, it may eliminate the need for time-consuming and expensive therapeutic trials with massive doses of vitamin D or its active metabolites. In the meantime, it is mandatory to treat every patient with VDDR-II irrespective of the type of receptor defect.

An adequate therapeutic trial must include vitamin D at a dose that is sufficient to maintain high serum concentrations of $1,25(OH)_2D_3$, as the patients can produce high hormone levels if supplied with enough substrate (Brooks *et al.,* 1978; Marx *et al.,* 1978). If high serum calcitriol levels are not achieved, it is advisable to treat with 1α-hydroxylated vitamin D metabolites in daily doses of up to 6 μg/kg or a total of 30–60 μg and calcium supplementation of up to 3 g of elemental calcium daily; therapy must be maintained for a period sufficient to mineralize the abundant osteoid (usually 3–5 months). Therapy may be considered a failure if no change in the clinical, radiological, or biochemical parameters occurs during continuous and frequent follow-up while serum $1,25(OH)_2D$ concentrations are maintained at ~100 times the mean normal range.

In some patients with no response to adequate therapeutic trials with vitamin D or its metabolites, a remarkable clinical and biochemical remission of their bone disease, including catch-up growth, was obtained by treatment with large amounts of calcium. This was achieved by long-term (months) intracaval infusions of up to 1000 mg of calcium daily (Balsan *et al.,* 1986; Bliziotes *et al.,* 1988; Weisman *et al.,* 1987). Another way to increase calcium input into the extra cellular compartment is to increase net gut absorption, independent of vitamin D, by increasing calcium intake (Sakati *et al.,* 1986). This approach is limited by dose and patient tolerability and was actually used successfully in only one patient.

Several patients have shown unexplained fluctuations in response to therapy or in presentation of the disease. One patient after a prolonged remission became completely unresponsive to much higher doses of active 1α-hydroxylated vitamin D metabolites (Balsan *et al.,* 1983), and another patient seemed to show amelioration of resistance to $1,25(OH)_2D_3$ after a brief therapeutic trial with $24,25(OH)_2D_3$ (Liberman *et al.,* 1980). In several patients, spontaneous healing occurred

in their teens (Hochberg *et al.,* 1984) or rickets did not recur for 14 years after cessation of therapy (Takeda *et al.,* 1989).

Animals Models

Some New World primates (marmoset and tamarins) that develop osteomalacia in captivity are known to have high nutritional requirements for vitamin D and maintain high serum levels of $1,25(OH)_2D$, thus exhibiting a form of end-organ resistance to $1,25(OH)_2D$ (Adams and Gacad, 1988; Liberman *et al.,* 1985; Shinki *et al.,* 1983; Takahashi *et al.,* 1985). Cultured dermal fibroblasts and EB-virus transformed lymphoblast have shown deficient hormone binding capacity and affinity (Adams and Gacad, 1988; Liberman *et al.,* 1985). It has been observed that marmoset lymphoblasts contain a soluble protein of 50–60 kDa that binds $1,25(OH)_2D_3$ with a low affinity but high capacity and thus may serve as a sink that interferes with the hormone binding and its cognate receptor (Gacad and Adams 1993). The same group described another protein present in the nuclear extract of these cells capable of inhibiting normal VDR–RXR binding to the vitamin D response element (Arbelle *et al.,* 1996).

It is of interest that these New World primates also exhibit a compensated hereditary end-organ resistance to the true steroid hormones including glucocorticoids, estrogens, and progestins (Lipsett *et al.,* 1985). This of course raises the interesting possibility that the defect in the hormone–receptor–effector system involves an element shared by all the members of this superfamily of ligand-modulated transcription factors.

Recently, VDR knockout mice have been created by targeted ablation of the first or second zinc finger (Li *et al.,* 1997; Yoshizawa *et al.,* 1997). Only the homozygotic mice were affected. Though phenotypically normal at birth, after weaning, however, they become hypocalcemic, develop secondary hyperparathyroidism, rickets, osteomalacia, and progressive alopecia. The female mice with ablation of the first zinc finger are infertile and show uterine hypoplasia and impaired folliculogenesis. Otherwise, both VDR cell mutant mice show clinical, radiological, histological, and biochemical features that are identical to the human disease VDRR-II. Supplementation with a calcium-enriched diet can prevent or treat most of the disturbances in mineral and bone metabolism in these animal models.

Concluding Remarks

Hereditary deficiencies in vitamin D action are rare disorders. The importance of studying these diseases stems from the fact that they represent a naturally occurring experimental model that helps to elucidate the function and importance of vitamin D and the VDR–effector system in humans *in vivo.*

VDRs are abundant and widely distributed among most tissues studied and multiple effects of calcitriol are observed on various cell functions *in vitro.* Yet, the clinical and biochemical features in patients with VDDR-I and -II seem to demonstrate that the only disturbances of clinical relevance are perturbations in mineral and bone metabolism. This demonstrates the pivotal role of $1,25(OH)_2D$ in transepithelial net calcium fluxes. Moreover, the fact that in patients with extreme end-organ resistance to calcitriol, calcium infusions correct the disturbances in mineral homeostasis and cure the bone disease may support the notion that defective bone matrix mineralization in VDRR-I and -II is secondary to disturbances in mineral homeostasis. Characterization of the molecular, cellular, and functional defects of the different natural mutants of the human VDR in VDDR-II demonstrates the essentiality of the VDR as the mediator of calcitriol action and the importance and function of its different domains. Furthermore, to function biologically, the VDR must associate with additional partners, i. e., $1,25(OH)_2D$, an RXR isoform, a specifically defined DNA region, and activator complexes. This notion has been based primarily on *in vitro*-created point mutations and *in vitro* functional assays. However, the acid test for the relevance of the structure–function relationship is the demonstration of *in vivo* effects in general and deficient function under pathological conditions in particular. Thus, studies in patients with hereditary deficiencies in vitamin D action are the essential link between molecular defects and physiological relevant effects.

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CHAPTER 68

Oncogenic Osteomalacia

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Introduction

Osteomalacia is a metabolic bone disease with two major histomorphometric abnormalities: increased amounts of osteoid tissue and a reduced bone formation rate. Most cases of disease are caused by a defect in phosphate and/or vitamin D metabolism (Parfitt, 1990). In oncogenic osteomalacia (tumor-induced osteomalcia) renal phosphate wasting, low serum 1,25 dihydroxyvitamin D levels and osteomalacia occur in association with a variety of benign or malignant neoplasms, disappearing upon removal or irradiation of the neoplasm (Lyles *et al.,* 1980; Drezner, 1993). Patients with oncogenic osteomalacia have diffuse bone pain and complain of fatigue. The bone pain can be localized to the lower back, the pelvis, and/or the femurs. Occasionally the patients develop fractures of the ribs, pelvis, femoral neck, or tibia. The fractures may occur with minimal trauma or be pseudofractures which occur more commonly in patients with osteomalacia. In addition to skeletal pain and fatigue, patients may have a proximal myopathy, making it difficult for them to climb steps or rise from a squatting position.

The syndrome was first described by McCance in 1947, when a patient with vitamin D-resistant osteomalacia of 7 years duration was cured by the resection of a tumor of degenerated osteoid from the right femur. Oncogenic osteomalacia has similar clinical features with x-linked hypophosphatemic rickets (XLH) and autosomal dominant hypophosphatemic rickets (ADHR): hypophosphatemia, renal phosphate wasting, inappropriately low serum $1,25(OH)_2$ vitamin D levels, and osteomalacia. New knowledge about the genetic and molecular defects in these three renal phosphate wasting syndromes have shed light on our understanding of normal phosphate homeostasis. Recently a factor(s) is postulated to cause renal phosphate wasting in the genetic disorder x-linked hypophosphatemic rickets, possibly making its pathophysiology similar to that of oncogenic osteomalacia (Meyer *et al.,* 1989; Nesbitt *et al.,* 1992).

Hypophosphatemia, the *sine qua non* for the diagnosis of oncogenic osteomalacia, can have been present for months to years before the diagnosis of osteomalacia is entertained. The hypophosphatemia (a fasting serum phosphorus level of < 2.5 mg/dl) is caused by a reduced renal phosphate reabsorption threshold. The renal phosphate wasting is quantitated by measuring a renal tubular maximum for the reabsorption of phosphorus per liter of glomerular filtrate (TmP/GFR) (Walton and Bijvoet, 1975). Serum $1,25(OH)_2D_3$ levels are reduced in these patients. Since hypophosphatemia is a primary stimulus for increasing renal production of $1,25(OH)_2D_3$, a low $1,25(OH)_2D_3$ level in a patient with oncogenic osteomalacia indicates an impairment of vitamin D metabolism in the proximal renal tubules (Lyles and Drezner, 1995). Gastrointestinal absorption of both calcium and phosphorus is reduced, which, coupled with renal phosphate wasting, leads to a negative phosphate balance. Parathyroid hormone (a hormone that lowers the TmP/GFR) levels have been measured in many patients with oncogenic osteomalacia, and, with few exceptions (Shenker and Grenkin, 1984; Cheng *et al.,* 1989), the levels are normal. Some patients with oncogenic osteomalacia have aminoaciduria and/or glycosuria, indicating additional abnormalities in the proximal renal tubule (Leehey *et al.,* 1985). Radiographic abnormalities include osteopenia, pseudofractures, fractures, and coarsened trabeculae from the osteomalacia. In growing children, widened epiphyseal plates occur an indicator of rachitic disease (Drezner, 1993).

Tumors

Most tumors that cause oncogenic osteomalacia are of mesenchymal origin and are found in either bone or soft tissue. Patients with these tumors range in age from 17 to 88

years. Tumor locations include the lower limbs, nasopharynx, mandible, ethmoid sinus, skull, cervical spine, upper extremities, and the abdominal wall (Ryan and Reiss, 1984; Renton and Shaw, 1976). Most tumors are skeletal with involvement of long bones; however, occasionally widespread osteolytic lesions are found (Daniels and Weisenfeld, 1979; Moncrieff *et al.,* 1978). Many tumors are small, being only a few centimeters in size which can explain the difficulty in detection and the delay in diagnosis. Neoplasms causing osteomalacia generally are benign and tumor types can include: hemangiomas (Daniels and Weisenfeld, 1979; Yoshikawa *et al.,* 1964; Salassa *et al.,* 1970; Renton and Shaw 1975; Turner and Dalinka, 1979; Camus *et al.,* 1980; Schapira *et al.,* 1995), dermatofibromas (Cai *et al.,* 1994), hemangioperioytoma (Miyauchi *et al.,* 1988; Yoshikawa *et al.,* 1964; Renton and Shaw, 1975; Linovitz *et al.,* 1976; Morita, 1976; Crouzet *et al.,* 1980; Sweet *et al.,* 1980; Chacko and Joseph, 1981; Seshadri *et al.,* 1985; Gitelis *et al.,* 1986; McClure and Smith, 1987; Schultze *et al.,* 1989; Papotti *et al.,* 1988), bone and soft tissue giant cell tumors (Seshadri *et al.,* 1985; Papotti *et al.,* 1988; Krane, 1965; Drezner and Femgios, 1977; Prowse and Brooks, 1987), mesenchymal lung tumor (Schapira *et al.,* 1995), osteoblastoma (Fukumoto *et al.,* 1979), non-ossifying and ossifying fibromas (Linovitz *et al.,* 1976; Asnes *et al.,* 1981, Parker *et al.,* 1990; Leehey *et al.,* 1985), fibroangioma (Aschinberg *et al.,* 1977), and mixed connective tissue variants (Lejeune *et al.,* 1979; Reid *et al.,* 1987; McGuire *et al.,* 1989). Malignant neoplasms have also been associated with oncogenic osteomalacia and these types include: carcinoma of the prostate, (Lyles *et al.,* 1980; Hosking *et al.,* 1975; Murphy *et al.,* 1985; McMurtry *et al.,* 1993; Nakahama *et al.,* 1995), carcinoma of the breast, (Dent and Stamp, 1978), oat cell carcinoma, (Taylor *et al.,* 1984; VanHeyningen *et al.,* 1994), multiple myeloma, (Maldonado *et al.,* 1975; Rao *et al.,* 1987; Pope and Belchetz, 1993), osteosarcoma, (Cheng *et al.,* 1989; Nomura *et al.,* 1982; Wyman *et al.,* 1977), sarcoma (Stanbury, 1972), angiosarcoma (Linovitz *et al.,* 1976), malignant fibrous histiocytoma (Willhoite, 1975), chondrosarcoma (Firth *et al.,* 1985), and malignant neurinoma (Hauge, 1956). Most of the early reports of oncogenic osteomalacia in association with prostatic carcinoma were in men who were also receiving diethylstilbestrol therapy for their disease (Lyles *et al.,* 1980; McGuire *et al.,* 1989; Hosking *et al.,* 1975; Murphy *et al.,* 1985). This suggested that estrogen therapy, with its recognized ability to lower the renal phosphate reabsorption threshold (Nassim *et al.,* 1955; Citrin *et al.,* 1984), was necessary to make oncogenic osteomalacia manifest in patients with this neoplasm. More recently two additional reports have described oncogenic osteomalacia in patients with carcinoma of the prostate whose only endocrine therapy for their cancer was orchiectomy (McMurtry *et al.,* 1993; Nakahama *et al.,* 1995). Thus, prostatic carcinoma appears capable of causing this syndrome *de novo.* Other diseases not traditionally considered to be neoplastic in nature can cause a form of oncogenic osteomalacia, and these disorders include epidermal nevi (Aschinberg *et al.,* 1977; Carey *et al.,* 1986; Goldblum and Headington, 1993), neurofibromatosis (Saville *et al.,* 1955; Weinstein and Harris, 1990; Konishi *et al.,* 1991), Albright's syndrome (Lever and Pettingale, 1983), Paget's disease of bone (D'Amore *et al.,* 1990), and fibrous dysplasia (Dent and Gertner, 1976).

A wide variety of meschemychymal tumors cause oncogenic osteomalacia and their diversity underscores the histologic complexity of these tumors. Weidner and Cruz (1987) showed that the histologically polymorphous mesenchymal neoplasms can be classified into four distinct morphological patterns: (1) primitive—appearing, mixed connective tissue tumors, (2) osteoblastoma-like tumors, (3) nonossifying fibroma-like tumors, and (4) ossifying fibroma-like tumors. The most frequently occurring type of these tumors, the mixed connective tissue variant, is characterized by variable numbers of primitive stromal cells growing in poorly defined sheets with occasional clusters on osteoclastic-like giant cells and vascularity is often prominent. The primitive stromal cells are probably the source of the hormonal factor(s) that cause the syndrome. Whether similar mesenchymal elements are present in tumors of epidermal and endodermal origin that are associated with oncogenic osteomalacia are unknown. However, fibrous mesenchymal elements are present in many neural tumors and metastatic prostatic carcinoma frequently have osteoblastic lesions with different amounts of fibrous tissue proliferation. Thus it is possible that oncogenic osteomalacia occurs in neoplasms of epidermal and endodermal origin when there are some of the mesenchymal elements present.

Pathophysiology

The mechanism(s) by which neoplasms cause the oncogenic osteomalacia syndrome is unknown. The general consensus is that offending tumors cause the syndrome by secreting a factor(s) which causes the renal phosphate wasting, resulting in hypophosphatemia and impair conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ resulting in low serum $1,25(OH)_2$ vitamin D_3 levels. Serum calcium levels are normal, as are levels of parathyroid hormone and parathyroid hormone-related peptide. In affected subjects resection of the tumor results in normalization of serum phosphorus levels because the TmP/GFR increases into the normal range. Also serum $1,25(OH)_2D_3$ levels normalize rapidly when the tumor is removed.

Several studies support the hypothesis that tumors cause oncogenic osteomalacia by secreting a phosphaturic factor(s). Aschinberg *et al.* (1997) induced phosphaturia in a puppy by injecting a homogenate of an epidermal nevus from a child with an oncogenic osteomalacia syndrome. However a homogenate of skin and tissue culture medium for the epidermal nevus did not cause phosphaturia in the puppy. Miyauchi *et al.* (1988) hetertotransplanted a hemiangiopericytoma from a patient with oncogenic osteomalacia into athymic nude mice. The heterotransplanted tumor increased urinary phosphate excretion in mice bearing the hemangigpericy-

toma. Nitzan *et al.* (1989) showed that a fibrosarcoma from a man with long standing oncogenic osteomalacia lowered serum phosphorus levels in athymic nude mice heterotransplanted with the tumor. The investigators grew the fibrosarcoma in tissue culture, and tested whether culture medium from the fibrosarcoma affect renal phosphate transport in a monkey proximal tubule cell culture line. In this study the culture media had no effect on phosphate transport in the cultured proximal tubule cells. Cai *et al.* (1994) cultured a sclerosing hemangioma from a patient with oncogenic osteomalacia. They showed culture medium from these tumor cells inhibited phosphate transport in cultured oppossum-kidney cells. The heat-labile factor with a mass between 8000 and 25,000 Da had some immunologic similarity to parathyroid hormone but it was neither parathyroid hormone nor parathyroid hormone-related peptide. The factor has been named "phosphatonin" and is very likely to be different from parathyroid hormone. Furthermore, phosphatonin did not influence phosphorus transport through a cyclic-AMP-dependent mechanism, did not increase calcium and phosphate mobilization from bone and did not influence bone remodeling.

Abnormal vitamin D metabolism has been recognized as a part of oncogenic osteomalacia since Drezner and Feingios (1977) reported low serum $1,25(OH)_2D_3$ levels in a patient with oncogenic osteomalacia caused by a giant cell tumor of bone. Now low $1,25(OH)_2D_3$ levels are considered an essential part of this syndrome. When serum $1,25(OH)_2D_3$ levels have been followed in patients undergo tumor resection, the low levels of the active vitamin D metabolite return to normal within 1 day. Two studies have shown that neoplasms causing oncogenic osteomalacia inhibit renal 25-hydroxyvitamin D-1α-hydroxylase activity, one in heterotransplanted athymic nude mice with prostatic carcinoma and the other in kidney cell cultures exposed to tumor extracts from an hemiangiopericytoma (Miyauchi et al., 1988; Lobaugh et al., 1984). It is unknown whether neoplasms eleaborate a single or multiple factors to inhibit phosphate reabsorption and impair 25 hydroxyvitamin D-1α-hydroxylase activity. To date, no experiments have been published to show whether these factors are caused by a single or several molecules. The observation that all patients with oncogenic osteomalacia and hypophosphatemia have inappropriately low serum $1,25(OH)_2$ vitamin D levels favors the concept of a single factor causing both defects (Kumar, 2000).

Patients with the inherited renal phosphate wasting disorder XLH have biochemical abnormalities similar to patients with oncogenic osteomalacia. Patients with XLH have hypophosphatemia, a low TmP/GFR, normal serum calcium levels, normal or elevated parathyroid hormone levels, inappropriately low serum $1,25(OH)_2$ vitamin D levels and osteomalacia. Although it would appear that the disorders may have a similar etiology, this is only partially correct.

The genetic mutation associated with XLH has been identified and the mutation involves the inactivation of an endopeptidase PHEX (phosphate regulating gene with homologies to endopeptidases located on the X chromosome) (Francis *et al.,* 1995). Although the mechanism(s) by which this mutation influences phosphate transport is unknown, several investigators have postulated that PHEX metabolizes a putative phosphate-regulating hormone called phosphatonin (Quarles and Drezner, 2001). According to this hypothesis, secondary to the inability of mutated PHEX in XLH subjects, phosphatonin, the putative phosphate regulating hormone, accumulates, interacts with the kidney and inhibits phosphate transport. At present, this hormone has not been isolated or cloned.

The inherited renal phosphate wasting disorder, autosomal dominant hypophosphate rickets has many similar biochemical characteristics to oncogenic osteomalacia and XLH including hypophosphatemia, renal phosphate wasting, inappropriately low serum $1,25(OH)_2$ vitamin D levels and osteomalacia (Econs and McEnery, 1997). The genetic mutation in this disorder is in fiborblast growth factor 23 (FGF 23) (White *et al.,* 2000). The biological actions of FGF 23 upon renal phosphate handling are unknown at present. More recently, White *et al.* (2001) have shown that FGF 23 was produced by four tumors causing oncogenic osteomalacia.

The hypothesis is that FGF 23 is phosphatontin, produced by tumors causing oncogenic osteomalcia in large amounts and unable to be metabolized by the normal endopeptidase in subjects and results in the oncogenic ostomalacia syndrome. In such subjects, tumor removal (the source of increased amounts of "phosphatontin," possibly FGS 23) causes resolution of the syndrome. In XLH, the phosphate wasting syndrome occurs because the abnormal endopeptidase PHEX cannot metabolize phosphatonin made by osteoblasts (Guo and Quarles, 1997) which results in the renal phosphate wasting disorder. Clearly, much more work will be required to provide adequate data to prove this hypothesis.

Differential Diagnosis

For many patients with oncogenic osteomalacia, making the initial diagnosis can take months to years. In the author's experience patients with bone pain, muscle weakness and hypophosphatemia have been seen for up to 3 years before the hypophosphatemia is evaluated; however, some cases have been followed up to 17 years before the diagnosis is made. When the hypophosphatemia is recognized, first it is important to establish that the low serum phosphate levels are due to a low renal phosphate reabsorption threshold. This is done by measuring the TmP/GFR (Waltan and Bijroet, 1975). If the offending neoplasm is not clinically apparent, next it is necessary to establish that the hypophosphatemia is acquired rather than having been present throughout life. Two other inherited disorders with renal phosphate wasting that can be confused with oncogenic osteomalacia include x-linked hypophosphatemic rickets/osteomalacia and autosomal dominant hypophosphatemic rickets/osteomalacia (Lyles and Drezner, 1995; Seriver *et al.,* 1977). If hypophosphatemia has been present throughout life, family members should have fasting serum phosphorus levels measured to help establish the diagnosis of XLH or ADHR. Finally, persistant hypophos-

phatemia is seen with increased secretion of parathyroid hormone and parathyroid hormone-related peptide. In patients with hyperparathyroidism, serum clacium levels are usually elevated unless there is vitamin D deficiency. Patients with elevated PTHnp levels almost always have a malignancy as well as elevated serum calcium levels.

The Fanooni syndrome represents a group of diseases, both genetic and acquired, in which hypophosphatemia occurs from a reduced TmP/GFR. This syndrome also has additional renal tubular defects causing loss of glucose, bicarbonate and amino acids. The disease, depending upon severity, may have associated osteomalacia, rickets, or short stature. Genetic diseases causing the Fanconi syndrome include: cystinosis, tyrosinemia galactosemia, Wilson's disease, and fructose intolerance (Lyles and Drezner, 1995). Acquired forms of this disease include heavy metal poisoning, hematologic malignancies, connective tissue disease and use of outdated tetracycline (Lyles and Drezner, 1995).

Another hypophosphatemic disorder is hereditary hypophosphatemic rickets with hypercalcuria (Tieder *et al.,* 1985). This rare genetic disease has hypophosphatemia due to a low TmP/GFR and hypercalcuria. In contrast to other diseases in which renal phosphate wasting occurs, serum $1,25(OH)D_3$ levels are elevated, which causes enhanced gastrointestinal absorption of calcium and hypercalcuria.

Since tumors may be small, a thorough physical examination may locate the neoplasm. Routine radiographs of the skeleton including the skull with special sinus views are helpful. Magnetic resonance imaging (MRI) is more efficient than CT scanning for identifying bone or soft-tissue mensenchymal tumors (Sundaram *et al.,* 1986, 1988). MRI scanning is also useful in planning for surgical resection. Proof of a tumor causing oncogenic osteomalacia requires resolution of biochemical abnormalities and the osteomalacia upon removal of the tumor. Since some neoplasms such as prostate carcinoma, oat cell carcinoma of the lung, and multiple myleloma may not be completely removed or eradicated, the diagnosis may be inferential. The biochemical profile and bone biopsy may suggest this diagnosis.

Therapy

The most important therapy for oncogenic osteomalacia is removal of the offending neoplasm. When this can be accomplished the syndrome remits and the patient does well. Unfortunately certain mesenchymal tumors, e.g. giant cell tumors of bone may recur or prostatic carcinoma cannot be eradicated (Miyauchi *et al.,* 1988; Harvey *et al.,* 1992; Leight *et al.,* 1993; Evens and Azzopard, 1972; McClure and Smith, 1989; Weidner *et al.,* 1985). In cases where the neoplasm can not be resected therapy with oral phosphate supplementation (1000–2000 mg daily in divided doses) and $1,25(OH)_2D_3$ (calcitriol 1.2–2.0 μg daily in divided doses) can improve the serum phosphate and $1,25(OH)_2D_3$ levels. Studies have shown that such therapy can improve bone pain and result in healing the osteomalacia on bone biopsy (Drezner and Feingios, 1977; Lobaugh *et al.,* 1984). Some investigators have shown that higher doses of oral phosphate supplements and calcitriol are necessary to raise serum phosphorus levels and heal the osteomalacia (Cheng *et al.,* 1989; Nomura *et al.,* 1982; Harvey *et al.,* 1992; Evans and Azzopard, 1972; Leicht *et al.,* 1990). Others have shown incomplete or unsatisfactory responses to this therapy (Shenker and Grenkin, 1984; Nitzan *et al.,* 1989; Scully *et al.,* 1989). Nevertheless, in a patient who cannot have their tumor completely removed, a trial of therapy with oral phosphate supplements and calcitriol should be considered since there is no other effective therapy. Such doses of medication can cause hypercalcuria, hypercalcemia, and reductions in glomerular filtration rates (Lyles and Drezner, 1995). It is necessary to follow both serum calcium and phosphorus levels as well as urinary calcium excretion and creatinine clearance. Some patients can develop secondary hyperparathyroidism, so PTH levels should also be followed (Firth *et al.,* 1985; Olefsky *et al.,* 1972).

Oral phosphate therapy is usually well tolerated, although 5–10% of treated patients develop gastrointestinal symptoms including nausea, vomiting, diarrhea, or abdominal pain. Such side effects are usually dose related, and doses required to normalize serum phosphate levels in affected patients may not be tolerable. Alternates to oral phosphate therapy are limited. The use of parenteral phosphate therapy has been reserved for life-threatening situations because of concerns over metastatic calcification, hypocalcemia, cardiac arrythmia, and electrolyte disturbances. Yeung *et al.* (2000) describe a case of oncogenic osteomalacia caused by an inoperable mesenchymal tumor in the cervical spine. The patient could not tolerate the oral phosphate supplements to restore serum phosphate levels to the normal range. The course of oncogenic osteomalacia was complicated by secondary hyperparathyroidism requiring parathyroidectomy. On three occasions, the patients was given intravenous phosphate infusions via a central venous catheter as well as oral calcitriol and calcium supplementation. These therapies contributed to improvement in biochemical and clinical parameters. The authors point out that such therapy should be used only when oral phosphate therapy is intolerable and requires meticulous monitoring (Yeung *et al.,* 2000).

In conclusion, oncogenic osteomalacia, which was once considered a rare metabolic bone disease, may be less of a rarity than previously believed. When the diagnosis suspected, an aggressive search for the offending neoplasm should be undertaken. When a tumor cannot be found, the search should be reinitiated every 3–5 years since occult growths may be discovered at a later date. Therapy for the disorder with oral phosphate supplementation and $1,25(OH)_2$ improves abnormal biochemistries but more importantly bone pain and function in patients in whom the offending neoplasm cannot be eradicated. Further studies into the pathosiciology of oncogenic ostemalacia and the heritary renal phosphate wasting disorders x-linked hypophosphatemic osteomalacia/rickets and autosomal domi-

nant hypophosphatemic rickets will provide new understanding of regulation of phosphate as well as vitamin D metabolism.

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CHAPTER 69

Osteopetrosis

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Introduction

Osteopetrosis results from a reduction in bone resorption relative to bone formation, leading to an accumulation of excessive amounts of bone. The relative decrease in resorption is a consequence of inadequate osteoclastic bone resorption. This imbalance leads to a thickening of the cortical region and a decrease in the size of the medullary space in the long bones, with sclerosis of the base of the skull (Elster *et al.,* 1992a,b) and vertebral bodies. There are a number of serious consequences resulting from the excessive accumulation of bone. A reduced marrow space results in decrease in hematopoiesis even to the point of complete bone marrow failure. Extramedullary hematopoiesis occurs but is unable to compensate for the reduction in medullary blood cell production. A decrease in the caliber of the cranial nerve and vascular canals leads to nerve compression and vascular compromise. Dense, poorly vascularized bones are subject to fracture and, being vascularized, are predisposed to necrosis and infection.

An understanding of osteopetrosis in humans has been intertwined with the description and creation of a variety of animal mutations in osteoclastic function (Seifert *et al.,* 1993). While the precise genetic defect in most patients remains to be established [one exception being the deficiency in carbonic anhydrase type II (Fathallah *et al.,* 1994; Whyte, 1993a)], the animal models have contributed substantially to the understanding of osteoclastic function and dysfunction. The osteoclast biology learned from these mutants has led to a variety of treatment strategies that have been used in patients with osteopetrosis. An understanding of the genetic basis of the animal mutations has generated a list of candidate genes that may prove to be the basis for discovering the genetic defects in humans.

Clinical Description

Classically, osteopetrosis has been divided into a fatal infantile malignant form, Albers–Schönberg Disease, and a milder adult form of osteopetrosis with long-term survival (Grodum *et al.,* 1995; Key, 1987; Shapiro, 1993; Whyte, 1993b). Recently, a variety of intermediate forms have been described. Without knowledge of the genetic defect explaining the osteoclastic dysfunction, it has been difficult to delineate the mechanisms of these different forms (Table I).

Infantile Forms

A variety of presentations of osteopetrosis have been seen in infancy. In general, patients have had sporadic forms that appear to have an autosomal recessive inheritance. While the severe, malignant form predominates, milder forms and autosomal dominant inheritance patterns have also been observed in individuals diagnosed in the neonatal period (Manusov *et al.,* 1993; Whyte, 1993b). In the absence of a known genetic defect in osteoclastic function, histomorphometric and clinical parameters have been established to describe the degree of severity.

MALIGNANT

Patients with osteopetrosis presenting at birth or in early infancy are usually referred to as having the severe, malignant form. The implication is that these patients will have severe sequelae and will die during the first decade of life. This has been the justification for using treatment modalities, such as bone marrow transplantation, that carry a high risk of mortality and morbidity (Gerritsen *et al.,* 1994b; Key and Ries 1993; Schroeder *et al.,* 1992). Patients with this form are characterized by a diffusely sclerotic skeleton with little or no bone

Table I Human Osteopetrosis Classification

Form	Genetic characterization
Infantile	
Malignant	Autosomal recessive
Subgroups	
Hematological impairment	
Neurodegenerative disease	
Intermediate	Autosomal recessive or dominant
Subgroup	
Carbonic anhydrase II deficiency	Autosomal recessive
Transient	?
Adult	
Severe	Autosomal dominant
Mild (benign)	
Subtype I	?
Subtype II	Autosomal dominant

marrow space evident, even at birth (Fig. 1) (Gerritsen *et al.,* 1994a). In addition, there is evidence of a severe defect in bone resorption, leading to the presence of the "bone in bone" appearance on radiographs (Fig. 2) and in cartilaginous islands within mineralized bone on histology (Fig. 3).

However, some patients with this phenotype will not have a fatal outcome. Indeed, up to 30% of patients diagnosed with severe, malignant osteopetrosis are still alive at age 6 years, with rare patients surviving into the second or third decade (Gerritsen *et al.,* 1994a). While the quality of life is reported to be poor, up to half of the surviving patients, despite a variety of skeletal and neurologic impairments, have normal intelligence and are capable of attending school (J. Charles, pers. commun.).

A subgroup of patients have an extremely malignant form of the disease. In a group of 33 patients reported by Gerritsen *et al.,* 8 patients had hematological and visual impairment before 3 months of age (Gerritsen *et al.,* 1994a). All 8 of these patients died before the age of 12 months. Hematological impairment before 6 months of life is prognostic of a markedly reduced survival rate; however, visual impairment alone does not correlate with an early fatal outcome.

Cytological evidence of large osteoclasts with increased numbers of nuclei and a markedly increased amount of ruffled border membrane correlates with a poor prognosis for cure with bone marrow transplantation (Schroeder *et al.,* 1992; Shapiro *et al.,* 1988). While there is no exact explanation for the reduced success in cure by transplantation, the defect in bone resorption in these patients could be explained if the osteoclast itself were normal but some other aspect of the bone rendered it less resorbable. Thus, histologic and electron microscopic analysis of osteoclasts is recommended before a transplantation is undertaken.

Another form of osteopetrosis that has not responded to any therapeutic modality is associated with a neurodegenerative disease (Whyte, 1993b). In some patients, a neuronal storage disease has been suggested by cytoplasmic inclusions. In most patients, seizures, poor neurologic development, abnormalities in the cerebral cortex seen on magnetic resonance imaging, and early development of central apnea characterize this specific presentation. No therapy that has been tried in these patients (bone marrow transplantation, calcitriol, or interferon-γ) has resulted in any significant improvement of the disease. It is likely that even when the bony disease can be reversed (as has occurred using each of the modalities), the underlying neurologic disorder remains unaffected. Death usually occurs before 2 years of age.

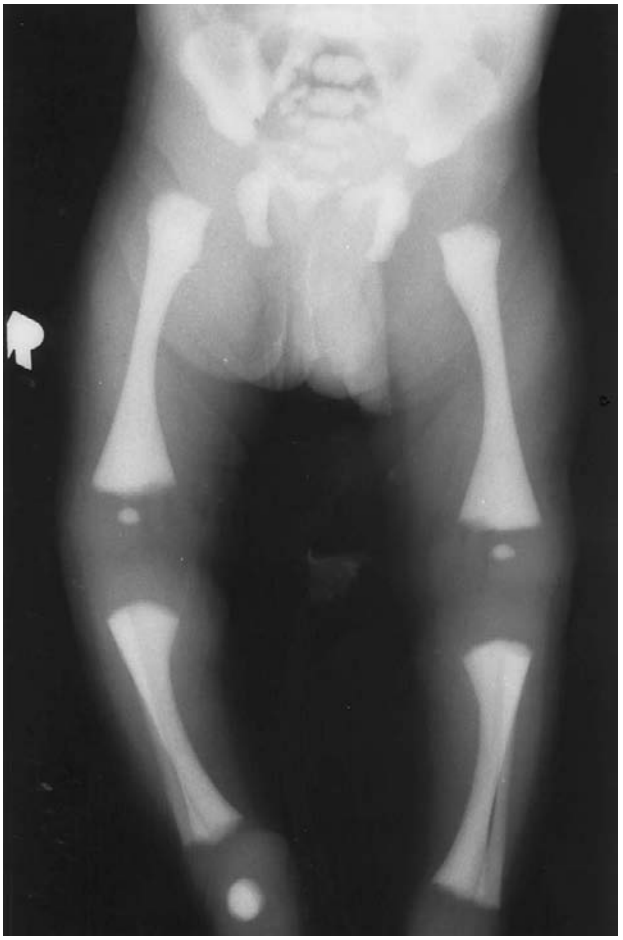

Figure 1 The skeleton, both the long bones and the pelvis, are shown to be sclerotic in this radiograph of a 5-day-old infant with anemia and optic nerve compression. Patients with this severe presentation have a 100% chance of death before age 1 year, if untreated. Note that no intramedullary space is seen in the long bones or the pelvis.

INTERMEDIATE

The intermediate form of osteopetrosis is frequently "silent" at birth with few or no obvious clinical abnormalities (Key, 1987; Whyte, 1993b). Some cases are diagnosed in infancy, when suspected, suggesting that the defect is present at a subclinical level even from birth. Of interest, the radiographs frequently demonstrate severe manifestation of diffuse sclerotic bone in this form of the disorder, quite similar to those seen in the malignant form (Fig. 4). These patients tend to have fractures toward the end of the first decade and frequently have repetitive fractures with very minor trauma. Infections of the bone can be difficult to eradicate, especially

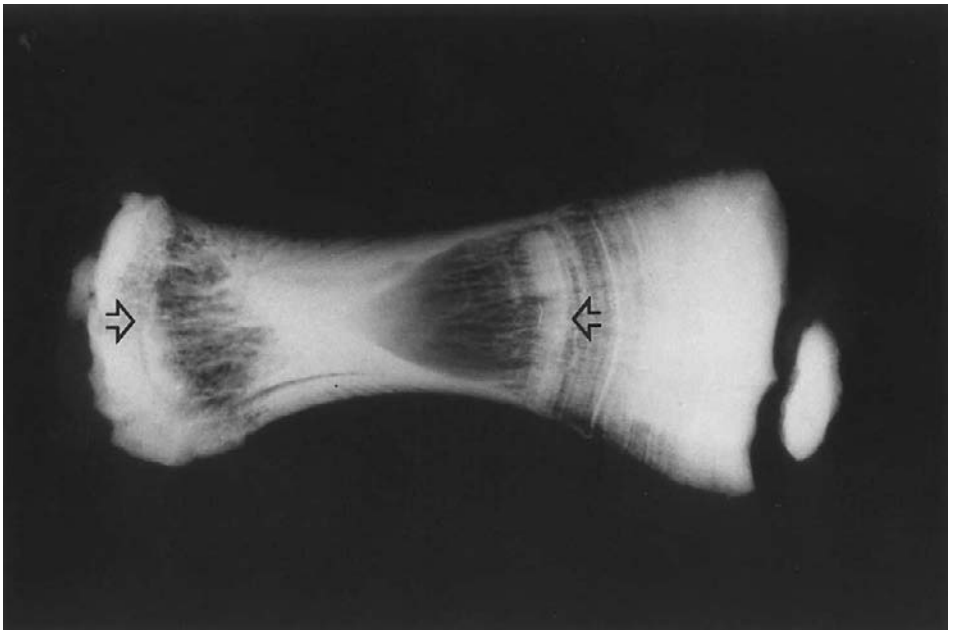

Figure 2 This radiograph shows a phalangeal bone, obtained at autopsy from a 7-year-old with malignant osteopetrosis treated with calcitriol. Note the presence of the original bony template (endobone, between the arrows) which was present at birth and never remodeled. The radiograph shows the presence of mineralized bone which has been laid down outside of this original bone/cartilage template without the underlying template having been resorbed.

if the mandible is involved. Most patients survive into adulthood. While anemia and hepatosplenomegaly are rare, at least one patient has developed anemia and thrombocytopenia so severe that he was transfusion dependent. His anemia and thrombocytopenia were eliminated by splenectomy.

A subgroup of patients with the intermediate form of osteopetrosis have a carbonic anhydrase II deficiency (Fathallah *et al.,* 1994; Whyte, 1993a). These patients have a hyperchloremic metabolic acidosis. Several different mutations have been described in the 50 cases diagnosed.

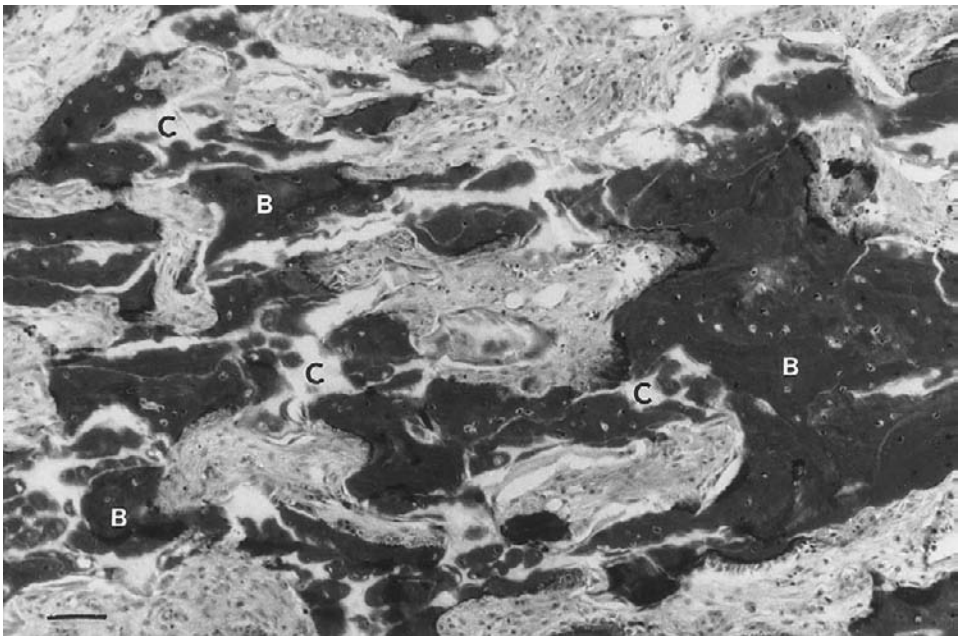

Figure 3 The pathognomonic feature of osteopetrosis at the histological level is the presence of unresorbed cartilage (denoted at "C"). Around the cartilage, a highly cellular, embryonic bone (designated as "B") has been laid down. This combination of nonresorbed cartilage and poorly formed bone is the microscopic feature which results in the macroscopic appearance seen in the "bone in bone" appearance on radiographs (see Fig. 2). The bar denotes 80

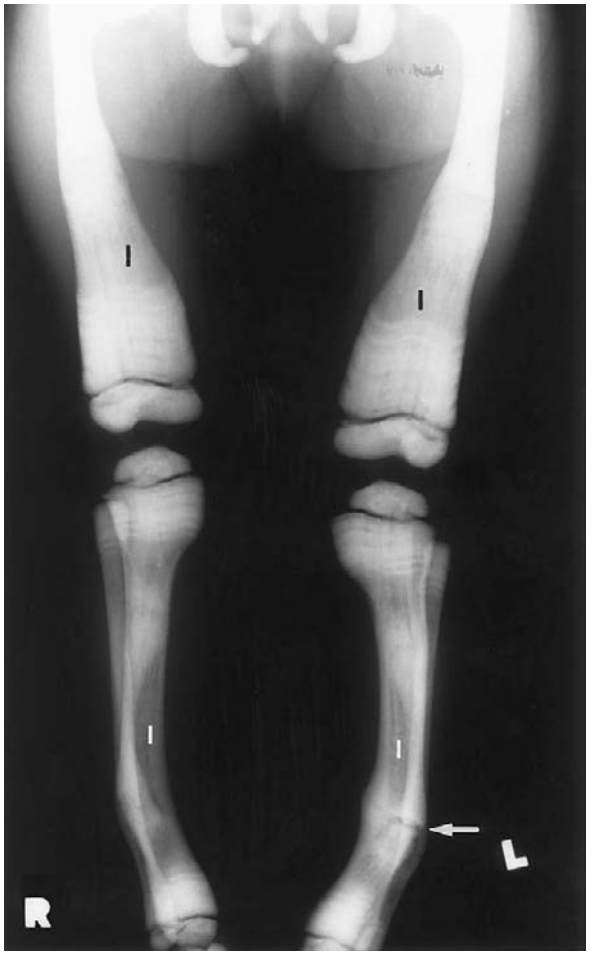

Figure 4 A radiograph of the long bones of a 6-year-old with an intermediate form of osteopetrosis shows the presence of some intramedullary space (denoted as "I"). The deformities of modeling result in bowing and thickening of the shaft and metaphyseal regions of the bone. Note a fracture, which was clinically not apparent, is present in the left tibia (arrow).

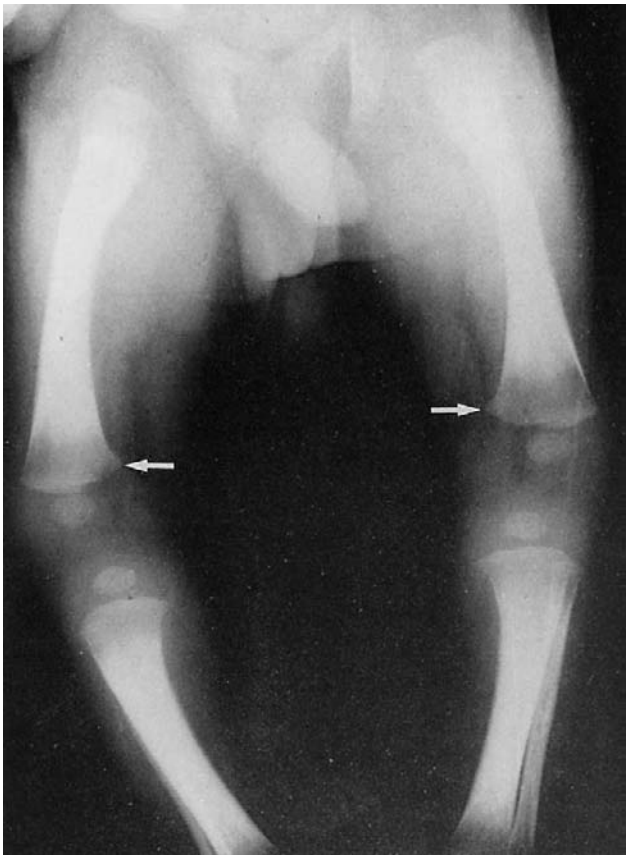

Figure 5 This radiograph of a 1 month old with craniometaphysial dysplasia demonstrates sclerotic long bones with little marrow space seen in the diaphyses. It should be noted that there is already some clearing of the bone in the distal metaphyseal region of both femurs (arrows, which had been sclerotic at birth).

Patients tend to have delayed development with a reduction in intelligence as adults, short stature, fractures, cranial nerve compression, dental malocclusion, and cerebral calcifications. Patients usually have no defects in hematological function and no increased risk of infection.

TRANSIENT

Some patients with severe radiographic abnormalities and with anemia have had severely sclerotic bone early, which resolves without specific therapy (Monaghan *et al.,* 1991; Whyte, 1993b, personal observation by Key). While long-term follow up is not available in these patients, no known sequelae resulted from the condition. The patients had no visual impairment, but did have anemia and thrombocytopenia. In one patient (personal experience), there was a history of acetazolamide administration. While osteopetrosis has not been widely associated with acetazolamide administration, the existence of osteopetrosis with naturally occurring mutations in carbonic anhydrase suggests that the therapy may be related to the osteoclastic dysfunction. In both cases, the resolution in the bone disease was apparent early (within 1 month). In addition, patients with other milder sclerosing bony dysplasias are frequently considered to have osteopetrosis in infancy. A classic case is seen in the natural history of severe craniometaphysial dysplasia where early radiographs are nearly indistinguishable from osteopetrosis (Fig. 5); however, there is no involvement of the vertebral bodies.

Adult Forms

In general patients with the adult forms of the disease have a family history suggesting an autosomal dominant inheritance pattern (Whyte, 1993b). Anemia is not a common manifestation; however, fractures and cranial nerve dysfunction are frequently observed (Bollerslev and Mosekilde, 1993; Bollerslev *et al.,* 1994). When defects have been sought for in infancy, radiographic abnormalities have been found which define the presence of the disease and portend the onset of symptoms in later life.

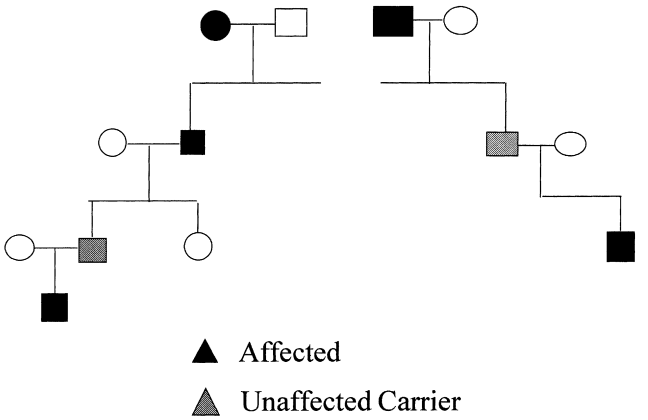

Figure 6 This is the family tree of two patients with adult forms of osteopetrosis. The presentation of these patients was severe enough (sclerosis of the long bones in one, anemia, sclerosis of the long bones, and blindness in the second) to result in an initial diagnosis of the severe malignant form.

SEVERE

We have observed two patients who presented with severe neonatal disease that had a family history suggestive of the presence of an autosomal dominant disorder. In each of these cases, the children were diagnosed with the malignant form of the disease due to anemia and cranial nerve dysfunction presenting in infancy. In one patient, there was a history of severe infections and failure to thrive. Family members in each case had survived into the fifth and sixth decade (Fig. 6). In each case, the parent transmitting the disease was unaffected.

MILD

The "benign" adult form of osteopetrosis is frequently "silent" until later in life (Bollerslev and Mosekidle, 1993; Whyte, 1993b). Two distinct subtypes have been described based upon radiographic appearance, symptoms and biochemical characteristics. Each of these subtypes have been inherited in a variety of kindreds. Each subtype has a distinct natural history, resulting in differing symptomatic presentations. Both types have universally sclerotic bones, primarily involving the axial skeleton. Little or no modeling defects are seen in the long bones. Both types have been observed in some children in the identified families. Approximately 40% of patients with the adult form of osteopetrosis are symptom-free regardless of type. Anemia is rare in either type. Bone pain is common to both types.

Type I is characterized radiographically, by massive sclerosis of the skull with increased thickness of the cranial vault. There is diffusely sclerotic bone in the spine and pelvis. Cranial nerve compression is common in type I. There are few abnormalities seen in the remodeling of bony trabeculae. Indeed, the strength of bone in type I is increased compared to normal. Thus, pathologic fractures are rarely observed.

Radiographs from patients with type II disease demonstrate massive sclerosis of the base of the skull, hypersclerotic endplates of the vertebrae resulting in the "rugger jersey" spine, and subcristal sclerotic bands diagnostic of "endobones." Bone turnover is decreased, leading to a reduction in bone tensile strength and resulting in frequent fractures. In type II, creatine phosphokinase, especially the BB isoenzyme, is increased. Histomorphometric analysis shows a defect in trabecular remodeling, resulting in bone that had not been replaced, yielding weakness.

In summary, the adult forms of osteopetrosis are usually diagnosed symptomatically in the second decade. These disorders rarely significantly alter hematopoiesis. Defects in type I disease result in cranial nerve compression, but fractures are rare. In type II disease, fractures are common, but nerve compression is rare.

Pathophysiology

Osteoclast Dysfunction

While animal models and a few patients have been found with a profoundly reduced number of osteoclasts, defective function rather than a reduction of formation appears to be the primary pathogenesis (Key and Ries, 1993). This holds true for malignant, intermediate, and benign forms. In the malignant form, there is the possibility of replacing the defective cell and curing the disease with bone marrow transplantation. However, defects in the osteoclasts' environment have also been suspected and may be the explanation for the greater-than-predicted rate of failure in bone marrow transplantation therapy in this disorder (Gerritsen *et al.,* 1994a; Key, 1987). However, to date, no patient has been definitively diagnosed with the one clear-cut stromal defect, a defect in producing macrophage colony-stimulating factor (M-CSF) seen in the *op/op* (osteopetrotic) mouse model (Key *et al.,* 1995a). One patient was reported with a decreased level of M-CSF after a failed transplantation; however, no data were available before the immunomodulation necessary for the transplantation.

A defect in white cell and osteoclastic superoxide production has been documented in the majority of patients with osteopetrosis (Key and Ries, 1993). Although the defect in superoxide generation in osteoclasts may represent a more generalized decrease in the ability of the osteoclast to resorb bone, therapy with interferon γ-1b, designed to increase superoxide production, increases white blood cell superoxide production, thereby reducing infections, and increases bone resorption, enhancing hematopoiesis and enlarging cranial nerve foramina. The result is a reduction in the need for intravenous antibiotics and transfusions and in a deceleration of cranial nerve damage. The net result is an improved survival with reduced morbidity.

GENETIC DEFECTS

The search for genetic abnormalities that result in osteopetrosis has recently identified two genetic defects that are related to the osteopetrotic condition. In the first report of a gene localization based upon linkage analysis, van Hul *et al.* reported a 8.5-cM region on chromosome 1*p*21 that is associated with the autosomal dominant adult form, type II (van Hul *et al.,* 1997). Although this region was chosen for study because of the presence of the *CSF-1* gene, a precise defect has not been identified. Indeed, a survey of 20 patients with osteopetrosis found no evidence of defects in the c-*src,* c-*fos,* c-*cbl,* and MITF (human equivalent of the mi/mi) genes (Yang *et al.,* 1998).

Two studies have identified precise mutations in a subunit of the V-type H^+-ATPase protein, OC116 (Kornak *et al.,* 2000; Frattini *et al.,* 2000). A total of 15 different genetic defects have been reported (Table II). All of these patients appear to have a classic severe osteopetrosis with severe osteosclerosis, hematologic failure secondary to reduced bone marrow space, and visual impairment in all but one patient. A history of consanguinity was a common theme for four patients.

Functionally, the defect causes the absence of the α3 subunit, OC116, of the V-type proton pump that is present along the ruffled border and responsible for acidification next to the bone surface. In the absence of this acidification, calcium cannot be removed from the surface of the bone. Of interest, a knockout animal was previously described (Li *et al.,* 1999) providing the first example of an animal model predicting a human osteopetrotic genetic defect (details of animal model below). This makes the existing animal models even more exciting as a repository of defects that may potentially explain human disease.

Animal Models

Classic

A variety of animal models for osteopetrosis have been explored. The M-CSF deficiency in the *op*/*op* mouse seems to be the most clearly related defect (Begg *et al.,* 1993; Lowe *et al.,* 1993; Marks *et al.,* 1992; Nilsson and Bertoncello, 1994; Philippart *et al.,* 1993; Wiktor-Jedrezejczak *et al.,* 1994); however, replacing the M-CSF with exogenous cytokine does not result in a complete remission (Sundquist *et al.,* 1995). Several possible explanations have been suggested. The timing of the administration may not be the most advantageous (Hofstetter *et al.,* 1995; Lee *et al.,* 1994; Sundquist *et al.,* 1995). Alternatively, there may be other related factors that must interact. One of the most plausible ideas is that in addition to circulating levels of M-CSF, there is also the need for membrane-bound M-CSF (Stanley *et al.,* 1994) on the osteoblast or embedded in the bone surface (Ohtsuki *et al.,* 1995) to be presented to the osteoclast or its precursors. A less clear-cut defect, but a demonstrated role for M-CSF therapy in improving the phenotypic abnormalities in the *tl*/*tl* (toothless) rat (Aharinejad *et al.,* 1995; Marks *et al.,* 1993), has suggested some involvement with the M-CSF production or the M-CSF receptor in the genesis of this mutant phenotype as well. To date the precise defect has not been reported. Thus, animals or humans with few macrophages and osteoclasts may be found to have a defect in M-CSF production or response.

In the mi/mi mouse (microphthalmic), there is a defect in the production of a transcription factor (Steingrímsson *et al.,* 1994). This has led to the possibility that a variety of defects, all related to the presence of a defective transcription factor,

Table II Genetic Defects in Autosomal Recessive Severe Congenital Osteopetrosis

Study	Patient	Location in DNA	Mutation	Effect	Allele
Kornak *et al.,* 2000					
	B	11/13*q TCIRG1* Exon 10	1024G→T	Abnormal splicing	Heterozygous
	K	11/13*q TCIRG1* Exon 20	2412G→A	Stop mutation	Heterozygous
	M	11/13*q TCIRG1* Exon 15	1787G→A	Unknown	Homozygous
	P	11/13*q TCIRG1* Intron 2	IVS2+4A→T	14 aa deletion	Homozygous
	T	11/13*q TCIRG1* Exon 12	1438–1439del	Stop mutation	Homozygous
Frattini *et al.,* 2000					
	1	11/13*q TCIRG1* Exon 9	C5272del	Frameshift	(M) Heterozygous
		11/13*q TCIRG1* Exon 15,-1	G10106A	Abnormal splicing	(F)
	2	11/13*q TCIRG1* Exon 12	C8759A	Stop mutation	Heterozygous
		11/13*q TCIRG1* Exon 15,-1	G10106A	Abnormal splicing frameshift	
	3	11/13*q TCIRG1* Exon 15,-1	G10106A	Abnormal splicing frameshift	Homozygous
	4	11/13*q TCIRG1* Exon 6,-6	C4391A	Abnormal splicing	(M) Heterozygous
		11/13*q TCIRG1* Exon 7	G4637Adel 14aa	Abnormal splicing	(F)
	5	11/13*q TCIRG1* Exon 19	11647– 11650del	Frameshift	(F) Heterozygous
		11/13*q TCIRG1*			

Note. M, mother; F, father.

could exist in the osteoclast and/or in other cells as well. One suggested defect is a decrease in the c-*kit* receptor production, which is necessary for stimulation of tyrosine kinase activity with the stem-cell factor (Ebi *et al.,* 1992). Stem-cell factor and M-CSF activate similar receptor populations, c-*kit* and c-*fms,* which are quite similar in their binding regions. There are also data suggesting that possibly there is a defect in the ability of mi/mi stromal cells, failing to support osteoclastic function (Key, 1987) or possibly even inhibiting osteoclastic function *in vitro.*

Both M-CSF and/or interferon-γ have been shown to improve the defect in the mi/mi mouse (Key *et al.,* 1995a). Since these cytokines are not deficient in these animals, the studies suggest that these therapies circumvent the specific defects rather than reversing the defects directly. Similar effects of these cytokines have been observed in patients with osteopetrosis where defects are unknown and not directly related to deficiencies in cytokine production or response elements.

Knockout

Perhaps the greatest interest has been in the analysis of man-made knockout mutations, which have been found to yield an osteopetrotic phenotype. The effects of a targeted disruption of *Atp6i,* a gene encoding a subunit of the vacuolar pump in the C57BL/6J inbred mouse strain have been described (Li *et al.,* 1999). The mutant animals resulting from this mutation have extremely dense bones with cartilaginous islands, reduced marrow space (decreased by 80% in the long bones), absent tooth eruption, decreased growth, and severely deformed bones. The condition was lethal in all animals by the fifth week. These animals appear to be quite similar to the patients described with defects in the *TCIRG1* gene of the human vacuolar proton pump described by Frattini *et al.* and Kornak *et al.* (Frattini *et al.,* 2000; Kornak *et al.,* 2000) The results of this animal mutation prove that a defect in the proton pump can cause osteopetrosis, confirming the speculation that the genetic mutations in the vacuolar pump documented in humans is responsible for the associated osteopetrotic phenotype.

The most well studied of these mutants is the c-*src* knockout mutation (Boyce *et al.,* 1993; Lowe *et al.,* 1993). In this mutation, the c-*src* tyrosine kinase is defective and yields a mutant with osteoclasts lacking ruffled borders. This appears to be due to the lack of production of phosphorylated proteins necessary to allow fusion of the membrane of endosomes with the cellular membrane within the sealed attachment of the osteoclast to bone. The result is an inactive cell with none of the machinery present at the osteoclast–bone interface necessary to resorb bone. The precise mechanism is not well understood and seems to be more complex than originally thought.

In the c-*fos* knockout mutation (Jacento, 1995; Grigoriadis *et al.,* 1994; Johnson *et al.,* 1992, Wang *et al.,* 1992), there are few mature osteoclasts formed and there is a reduction in the number of osteoclasts and an increase in the number of macrophages. Thus, it appears that c-*fos* is related to the osteoclast lineage's "switch point," which determines whether the progenitor moves in the direction of the osteoclast or the macrophage (Jacento, 1995).

The knockout of PU.1 (Tondravi *et al.,* 1997), an ETS-domain transcription factor essential for the development of myeloid and B-lymphoid cells, is thought to regulate the c-*fms* receptor (receptor for CSF-1). A mutation in this gene results in a nearly complete absence of osteoclasts, resulting in severe osteopetrosis. Bone marrow transplantation with PU.1 competent stem cells rescued the animals, completely reversing the defects. No human correlate has been found. Knocking out the gene for cathepsin K generated an additional osteopetrotic mouse model (Saftig *et al.,* 1998; Gowen *et al.,* 1999). This mutant was found to have dense bones with a reduction in bone marrow space, and extramedullary hematopoiesis. While these animals shared features of osteopetrosis, it was noted that the defective bone resorption varied from bone to bone. Recently, a similar mutation has been identified in humans with pycnodysostosis, a disorder resembling osteopetrosis, but having osteoacrolysis, poor fusion of the sagittal suture, micrognathia, and a predisposition for fracture (Ho *et al.,* 1999). The osteoclastic defect appears to be an inability of the osteoclast to degrade matrix, although removal of calcium appears to be normal. Thus, it is likely that some of the knockout mutations may be genes responsible for other sclerosing bony dysplasias. It appears that mutations resulting in osteopetrosis are those that completely inactivate the osteoclastic bone resorption by either leading to a failure to generate the osteoclast itself or the failure to form the ruffled border. This suggests that there is some redundancy of function in the osteoclast's bone resorbing process such that knocking out a single enzyme system does not completely inactivate bone resorption.

At the present time, knockout mutations have been much more important in understanding osteoclastic function than in explaining the human osteopetrotic condition. However, these studies do provide a variety of candidate genes and are beginning to suggest genetic defects that will allow us to diagnose certain types of osteopetrosis.

Avian Osteopetrosis (Increased Osteoblastic Function)

Osteopetrosis has been described in the chicken and appears to result from infection with the avian leukosis virus. This sclerosing condition is characterized by an increase in bone formation by infected osteoblasts (Smith and Ivanyi, 1980) with normal osteoclastic function. Avian osteopetrosis, while similar to sclerosis seen in a variety of human diseases, has not been shown to occur in humans with one possible exception. In "transient osteopetrosis," Ozsoylu and Besim have suggested that the etiology may be bony sclerosis secondary to excessive osteoblastic function (Ozsoylu and Besim, 1992).

Therapy of Human Disease

Even with the marked advances in understanding the osteoclastic defects that could result in osteopetrosis, we are still left today with the need to treat somewhat blindly in trying to combat this disorder. Transplantation has been the mainstay of treatment; however, somewhat disappointing results and, at present, the lack of available donors have made it difficult to rely on transplantation as the sole therapy for this disease. Thus, a variety of other therapies have been attempted with some success. Because the mortality associated with failed transplantations is high, other treatment alternatives and/or stopgap measures remain important despite the inability of these therapies to completely cure the condition in most patients. These therapies may be the sole therapy of the patient or may result in a milder course of the disease while awaiting a definitive cure.

Transplantation

Despite the fact that at present transplantation is available in only ~50% of children with osteopetrosis and is

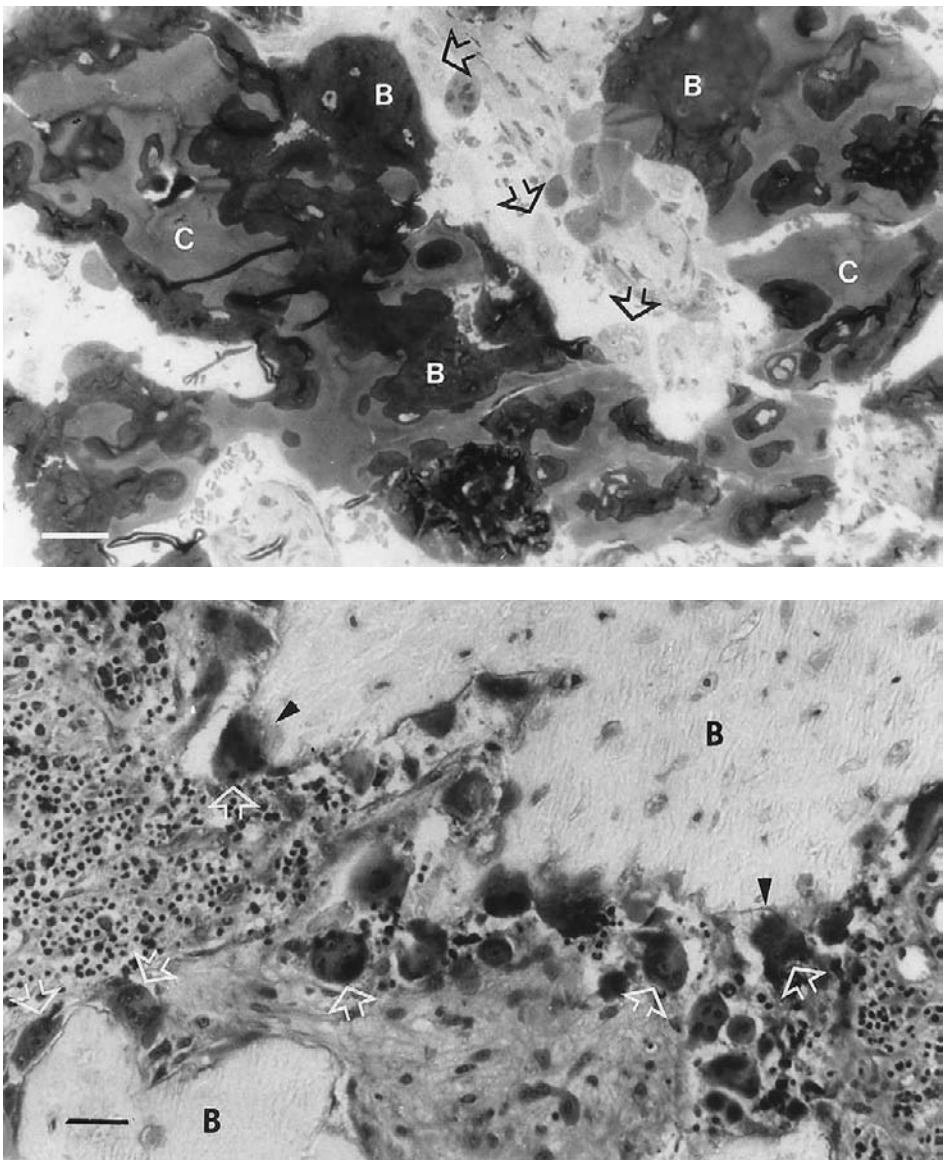

Figure 7 Bone biopsies from age 11 and 29 months in a patient with severe infantile osteopetrosis. The top panel contains a 1-μm Epon-embedded section obtained from the patient at 11 months of age, prior to therapy with interferon γ-1b and calcitriol. Coarse trabecular bone (denoted as "B") with cartilaginous streaks (denoted as "C") is evident. Lacunae are absent even though osteoclasts (arrows) adjoin the bone surface. Bone marrow cells are absent from this section. (Bottom) A 5-μm-thick methyl-methacrylate-embedded section obtained by biopsy of the same patient after 18 months of therapy. A combination of mineralized coarse trabecular bone and porous cortical bone (designated by "B") lined with numerous osteoclasts (open arrows) with brush borders and adjoining lacunae (arrowheads) is visible. Islands and streaks of cartilaginous tissue are absent. Bar = 40 μm.

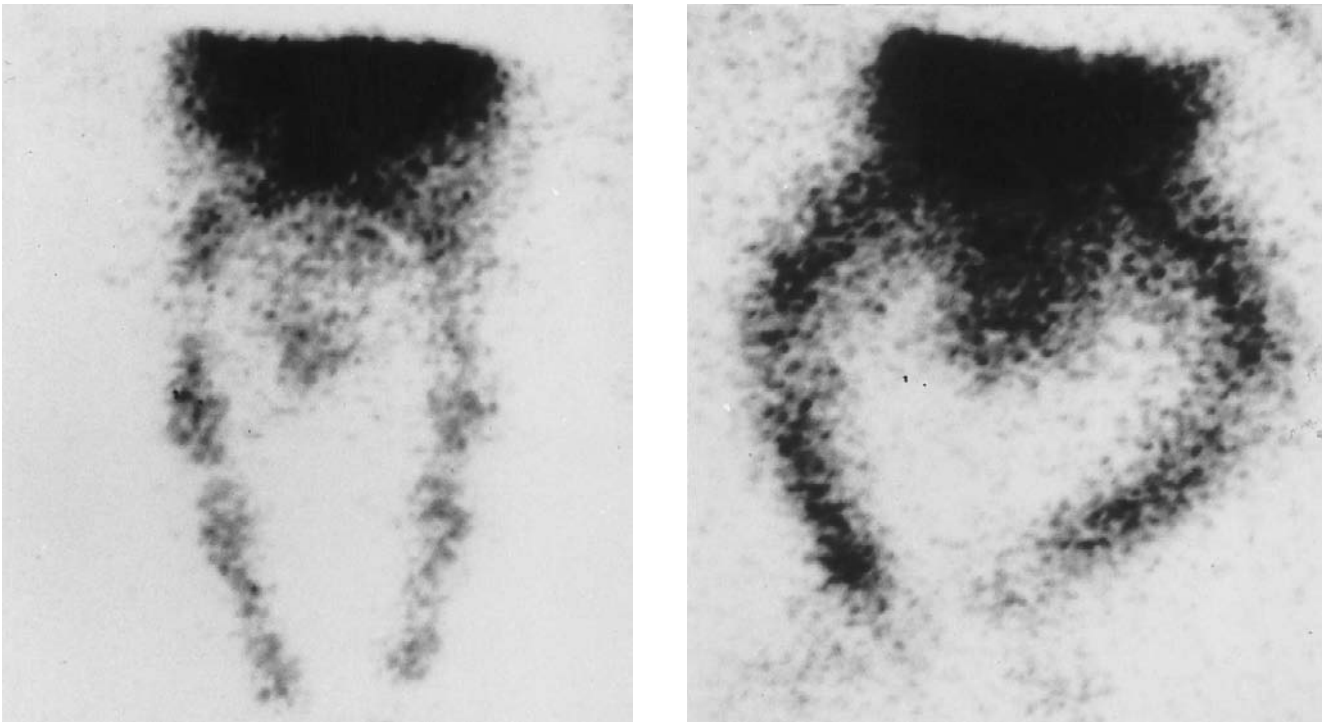

Figure 8 ^{999}Tc-labeled sulfur colloid bone marrow scans are shown from the same patient whose biopsies are shown in Fig. 7. The initial scan (left) shows abnormal uptake of label in the long bones of the lower extremities. Areas with little or no radionuclide are seen in the diaphyses with the uptake of the radionuclide being primarily concentrated to the subepiphyseal regions. (Right) After interferon gamma-1b therapy, there is an increase in the amount of radionuclide labeling and there are no longer any discreet areas with reduced uptake

successful in ~45% of patients who receive grafts; the allure of this therapy is that a successful transplantation yields a nearly complete cure (Gerritsen *et al.,* 1994b). If this cure can be obtained before there are too many physical disabilities, children with osteopetrosis tend to grow and develop normally. In transplantations where the donor graft is harvested from young siblings who have a favorable HLA match, there appears to be 70%, or slightly greater, survival. Despite the fact that not all engraftments are maintained, even the retention of a small number of donor cells seems to correct the defect significantly and stave off recurrence of symptomatic disease. However, beyond this group, the success rate is less favorable. In transplantations where the graft is obtained from HLA haplo-identical (mismatched) relatives, the engraftment and survival rates for transplants are only ~15%. Given the natural history of a survival of almost 30% for the malignant form of osteopetrosis as a whole, this is considered by most transplantation experts to be an unacceptable rate of failure. However, it should be noted that in the 15% of patients where transplantations have been successful, cures have been achieved. The great hope for the future are transplants with matched unrelated donors and possibly, cord blood stem cells (Information on cord blood transplantation in children communicated personally by M. Klemperer, St. Petersberg, FL). With the advent of DNA typing to ensure the adequacy of the matches, success rates of $\leq$80% are being achieved in most diseases. However, success rates of only ~40% have been found in osteopetrosis. The exact reason for this discrepancy is not clear at this time. The possibility that cord blood or placental transplants may improve chances for engraftments are just beginning to be explored. Nonetheless, at present, in the absence of a matched sibling donor, survival can be expected to be well below 50%.

Other Therapies

Many families have found transplantation to carry an unacceptable risk and thus, there has been a search for alternative therapies. The use of high dose calcitriol has been tried in a large number of children with some period of stabilization, and in some instances, a cure (Key and Ries, 1993; van Lie Peters *et al.,* 1993). Nonetheless, long-term cures have been achieved in 25% of patients treated.

Interferon-γ has been used as a treatment of osteopetrosis (Key *et al.,* 1995b; Kubo *et al.,* 1993). The intent of the therapy was to enhance the production of oxygen radicals by white blood cell phagocytes as a means of fighting off life-threatening infections. In addition, therapy with interferon-γ-1b along with calcitriol increases both bone remodeling and the amount of bone marrow space in patients (Fig. 7 and 8). To determine if interferon γ-1b (1.5 μg/kg/dose subcutaneously, three times per week, Actimmune, Intermune Pharmaceuticals, Inc., Palo Alto, CA) plus calcitriol (1 μg/kg/day orally) or calcitriol alone is effective in delaying the time to treatment failure, 16 patients with congenital osteopetrosis were recruited from all over the world to participate in a randomized, controlled trial. Patients were

randomized in a 2:1 ratio onto interferon γ-1b plus calcitriol or calcitriol alone, respectively. The drug was administered subcutaneously to patients three times a week. The patients receiving interferon γ-1b plus calcitriol had their first evidence of failure at a mean of 452 days compared to those on calcitriol alone, who failed at 130 days ($P = 0.016$). Only 10% of the patients treated with interferon γ-1b experienced a serious infection compared to 67% of the patients on calcitriol alone ($P < 0.0001$). In limited data, biopsies obtained from patients treated with interferon γ-1b had a 50% reduction in bone mass; while no reduction was seen in one patient treated with calcitriol alone. The size of both the optic nerve foramina and auditory canals increased in subjects treated with interferon γ-1b, but not in those treated with calcitriol only.

Summary

In summary, significant progress has been made in classifying osteopetrosis. There are at least three major categories (malignant, intermediate, benign) of the disease with a variety of subtypes. This classification allows prognostic information to be provided to the parent or patient, improving decision making concerning the appropriate therapy. The genetics of osteopetrosis have not been worked out, but the explosion of animal mutations expressing osteopetrotic phenotypes promises to lead to the identification of some, if not all, of the mutations in the near future. Finally, treatment is largely still directed toward enhancing osteoclastic function in general rather than specific defects. Bone marrow transplantation remains the only hope of complete cure. The introduction of calcitriol, M-CSF, and interferon γ-1b provides promise for the future survival and hopefully, the cure of osteopetrosis. As in the past, this very interesting family of disorders continues to lead the way in developing our understanding of osteoclastic function.

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CHAPTER 70

Hypophosphatasia

Nature's Window on Alkaline Phosphatase Function in Man

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Introduction

Alkaline phosphatase (ALP) was discovered in 1923 (McComb *et al.,* 1979). For most of the past eight decades, physicians have universally recognized the important clinical insight that can come from measuring ALP activity in serum. Detection and monitoring of hepatobiliary and skeletal disease are generally possible (Wolf, 1978). In fact, since the 1930s, quantitation of serum ALP activity has been routine in hospital laboratories, and this test may still be the most frequently performed enzyme assay (McComb *et al.,* 1979). Nevertheless, the physiological function of ALP, which is ubiquitous in nature, is largely unknown (Harris, 1990; McComb *et al.,* 1979; Moss, 1992; Whyte, 1994). However, as reviewed in this chapter, identification of ALP gene mutations in hypophosphatasia, a rare heritable metabolic bone disease, has confirmed in humans the hypothesis dating from its discovery that ALP functions importantly in skeletal mineralization (Whyte, 1994). The role for ALP was clarified by the observation that three phosphocompounds accumulate endogenously in hypophosphatasia. Elevated plasma levels of pyridoxal 5′-phosphate, due to alteration in vitamin B_6 metabolism, reveal that ALP acts as a cell surface enzyme (Whyte, 1994). The increased plasma and urinary concentrations of inorganic pyrophosphate, an inhibitor of hydroxyapatite crystal growth, provide a compelling explanation for the defective mineralization of bone and cartilage (Whyte, 1994). However, a great many intriguing questions remain.

Following a brief review of the history and the proposed physiological roles of ALP, an overview of hypophosphatasia is presented, and the biological insight gained from this "experiment-of-nature" is then discussed.

History and Proposed Physiological Roles of Alkaline Phosphatase

In 1923, Robert Robison (1883–1941) discovered in young rats and rabbits considerable phosphatase activity within ossifying bone and cartilage. He proposed that this enzyme functioned in skeletal mineralization by hydrolyzing some unknown phosphate ester to locally increase the concentration of inorganic phosphate (P_i) (Robison, 1923). One year later, Robison and Soames (1924) reported that this phosphatase precipitated mineral into rachitic rat bone when monophosphate esters were the only source of P_i and demonstrated its distinctly alkaline pH optimum. These studies represent the discovery of ALP (McComb *et al.,* 1979). Nevertheless, Robison never referred to this enzyme as "alkaline" phosphatase; the term was introduced by others (McComb *et al.,* 1979).

However, soon after the discovery of ALP, evidence that contradicted Robison's hypothesis emerged (McComb *et al.,*

1979). ALP was found to be abundant in tissues that normally do not calcify (e.g., liver, intestine, placenta), suggesting a more global physiological purpose. Furthermore, his theory was challenged because extracellular fluids were actually supersaturated with calcium and P_i and because he had not identified the enzyme's natural substrate(s) (Neuman and Neuman, 1957). Additional biological functions were proposed (see below).

At the end of the 1960s, electron microscopy helped to rejuvenate Robison's hypothesis when the earliest site of hydroxyapatite crystal deposition in the developing skeleton was noted by H. C. Anderson to be within novel extracellular structures called matrix vesicles (Anderson, 1969). These features were rich in ALP activity (Ali, 1986). We now know that matrix vesicles are replete with many enzymes, including inorganic pyrophosphatase (PP_i-ase) and ATPase, and may also contain phospholipids, polysaccharides, and glycolipids among numerous constituents (Anderson, 1992). During the earliest ("primary") phase of skeletal mineralization, hydroxyapatite crystals appear and grow within these structures. Soon after, the vesicles rupture and extravesicular ("secondary") mineralization occurs as crystal propagation continues (Ornoy *et al.,* 1985).

Following the discovery of matrix vesicles, *in vitro* model systems based upon inhibition of ALP with stereospecific compounds such as L-tetramisole (levamisole) demonstrated that calcification was blocked when ALP activity was inhibited (Fallon *et al.,* 1980). Nevertheless, these findings too proved controversial because subsequent controlled experiments using the noninhibitory stereoisomer sometimes also showed impaired mineralization.

Currently, the proposed biological roles for ALP in mammals are numerous (Harris, 1990; McComb *et al.,* 1979; Moss, 1992; Whyte, 1994) and include hydrolysis of phosphate esters to supply the *non*phosphate moiety, transferase action for the synthesis of phosphate esters, regulation of P_i metabolism, maintenance of steady-state levels of phosphoryl-metabolites, and action as a phosphoprotein phosphatase (Alpers *et al.,* 1990; Harris, 1990; McComb *et al.,* 1979; Moss, 1992; Muller *et al.,* 1991; Simko, 1991; Whyte, 1994). At cell membranes, it has been hypothesized that ALP conditions not only the active transport of P_i, but also the movement of calcium, fat, protein, carbohydrate, and Na^+/K^+ (Muller *et al.,* 1991; Simko, 1991). Furthermore, an interesting physical property of ALP in the placenta, binding to the Fc receptor of IgG, suggests that ALP transcytoses this immunoglobulin (Makiya *et al.,* 1992). ALP can also adhere to collagen (Wu *et al.,* 1992) (see below). In fact, sequence analyses of ALPs suggest that they may couple to other proteins, and it has been recommended that this physical property be considered when a potential physiological role of ALP is examined (e.g., phosphoprotein phosphatase action in skeletal matrix) (Tsonis *et al.,* 1988). Finally, although we now understand that ALP works primarily on cell surfaces (see below), at some stages of embryogenesis ALPs may act intracellularly (Narisawa *et al.,* 1992).

Table I Suggested Roles for ALP in Skeletal Mineralization

Locally increase Pi levels
Destruction of inhibitors of hydroxyapatite crystal growth
Transport of Pi
Ca^{2+}-binding protein (Ca^{2+} uptake by cells)
Ca^{2+}/Mg^{2+}-ATPase
Tyrosine-specific phosphoprotein phosphatase

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A variety of hypotheses also explain how ALP could act specifically in skeletal mineralization (Table I) (Whyte, 1994). In 1975, nucleoside phosphate (liberated by dying cells) was proposed as the ALP substrate source of P_i necessary to fulfill Robison's theory (Majeska and Wuthier, 1975). An early and important alternative suggestion, however, offers that ALP hydrolyzes an inhibitor of calcification (Moss, 1992; Neuman and Neuman, 1957; Whyte, 1994). As will be reviewed, at least three findings favor the "inhibitor hypothesis" and implicate inorganic pyrophosphate (PP_i) (Caswell *et al.,* 1991; Whyte, 1994): high concentrations of PP_i impair the growth of hydroxyapatite crystals, ALP can function as a PP_i-ase (Moss *et al.,* 1967), and urine and plasma levels of PP_i are increased in patients with hypophosphatasia (Russell, 1965; Russell *et al.,* 1971). Alternatively, it has been suggested that ALP is a plasma membrane transporter for P_i, an extracellular Ca^{2+}-binding protein that stimulates calcium phosphate precipitation and orients mineral deposition into osteoid (deBarnard *et al.,* 1986), a Ca^{2+}/Mg^{2+}-ATPase, or a phosphoprotein phosphatase that conditions the skeletal matrix for ossification (Lau *et al.,* 1985) (Table I). In fact, as mentioned, a particular ALP structural domain could enable it to bind to types I, II, and X collagen in cartilage and bone (Tsonis *et al.,* 1988; Wu *et al.,* 1992).

Approaching nearly a century after its discovery, the most commonly used methods for assaying ALP continue to reflect ignorance of this enzyme's physiological role(s) (Harris, 1990; McComb *et al.,* 1979; Moss, 1992; Whyte, 1994). In clinical and research laboratories, ALP in diluted biological specimens is typically assayed with high concentrations (millimolar) of artificial substrates (e.g., *P*-nitrophenylphosphate) at distinctly nonphysiological alkaline pHs (e.g., pH 9.2 – 10.5) (McComb *et al.,* 1979). Such simple assays possess sensitivity but were developed primarily for clinical medicine (Wolf, 1978). In fact, for certain ALP substrates at low concentrations, although hydrolytic rates are reduced, the pH optimum is considerably less alkaline (McComb *et al.,* 1979; Moss, 1992). For years, the significance of this observation was uncertain (Harris, 1990; McComb *et al.,* 1979; Moss, 1992; Whyte, 1994).

To understand hypophosphatasia and appreciate how this disorder teaches us about the physiological role of ALP, it is first necessary to briefly review the molecular biology and biochemistry of this enzyme.

Molecular Biology and Biochemistry of Alkaline Phosphatase

ALPs (orthophosphoric-monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1) are found in nearly all plant and animal species (McComb *et al.,* 1979). In humans, four ALP isoenzymes are encoded by four separate genes (Harris, 1990; Millan, 1988; Moss, 1992). Three of the isoenzymes are expressed in a tissue-specific distribution and are called intestinal, placental, and germ cell (placental-like) ALP. The fourth ALP isoenzyme is ubiquitous, but especially abundant in hepatic, skeletal, and renal tissue (liver / bone / kidney ALP) and is called tissue-*non*specific ALP (TNSALP) (Harris, 1990; Moss, 1992; Stigbrand and Fishman, 1984). The distinctive physicochemical properties (e.g., heat stability, electrophoretic mobility, etc.) among ALPs purified from liver, bone, and kidney are lost upon exposure to glycosidases (Moss and Whitaker, 1985). In fact, the TNSALPs are a family of "secondary" isoenzymes (isoforms), with the same polypeptide sequence, encoded by one gene (*TNSALP*) and differ only by posttranslational modification involving carbohydrate (Harris, 1980).

The TNSALP gene is located near the end of the short arm of chromosome 1 (1*p*36.1–*p*34); the genes for intestinal, placental, and germ-cell ALP are found near the tip of the long arm of chromosome 2 (2*q*34–*q*37) (Harris, 1990; Whyte, 2000). Unfortunately, the human gene mapping symbol for the *TNSALP* locus is *ALPL* ("ALP-liver") although the function of the liver isoform of TNSALP is unknown (*vide infra*).

The TNSALP gene is greater than 50 kb and contains 12 exons, 11 of which are translated to form the 507 amino acid nascent enzyme (Weiss *et al.,* 1988). The promoter region for *TNSALP* is located within 610 nucleotides 5′ to the major transcription start site (Kiledjian and Kadesch, 1990). TATA and Sp1 sequences may act as regulatory elements. Apparently, basal levels of *TNSALP* expression reflect inherent "housekeeping" promoter effects, whereas differential expression in various tissues may be mediated by a posttranscriptional mechanism (Kiledjian and Kadesch, 1990); the 5′ untranslated region differs between the bone and liver TNSALP isoforms (Nosjean *et al.,* 1997).

The tissue-specific ALP genes are smaller than the TNSALP gene, primarily reflecting shorter introns. Amino acid sequences deduced from the cDNAs suggest 87% positional identity between placental and intestinal ALP, but only 50 to 60% identity between TNSALP and the tissue-specific ALPs (Harris, 1990). Nevertheless, the active site of TNSALP, which is encoded by six exons and composed of 15 amino-acid residues (Zurutuza *et al.,* 1999), has a nucleotide sequence that has been conserved in ALPs found throughout nature (Henthorn and Whyte, 1991). *TNSALP* seems to represent an ancestral gene, whereas the tissue-specific ALPs likely originated from a series of gene duplications (Harris, 1990).

The ALPs are Zn^{2+}-metalloenzymes (McComb *et al.,* 1979). Catalytic activity requires a multimeric configuration of identical subunits ranging from 40 to 75 kDa. Each monomer has one active site and binds two Zn^{2+} atoms that stabilize its tertiary structure (Kim and Wycoff, 1991). The cDNA sequence of TNSALP predicts five potential N-linked glycosylation sites (Weiss *et al.,* 1988). In fact, *N*-glycosylation is necessary for enzymatic activity. *O*-glycosylation involves the bone, but not the liver, isoform of TNSALP (Nosjean *et al.,* 1997).

The ALPs are generally regarded as homodimeric in the circulation (McComb *et al.,* 1979). TNSALP, in symmetrical dimeric form, has α/β topology for each subunit with a 10-stranded β-sheet at its center (Hoylaerts and Millan, 1991). However, in tissues, ALPs are attached to cell surfaces probably as homotetramers (Fedde *et al.,* 1988).

ALP isoenzymes have broad substrate specificities and pH optima, which depend on the type and concentration of phosphocompound undergoing catalysis (McComb *et al.,* 1979). Catalytic activity requires Mg^{2+} as a cofactor (McComb *et al.,* 1979). Hydrolytic activity cleaves phosphoesters and PP_i (Xu *et al.,* 1991). The reaction involves phosphorylation–dephosphorylation of a serine residue, and dissociation of covalently linked P_i seems to be the rate-limiting step. In fact, P_i is a potent competitive inhibitor of ALP activity (McComb *et al.,* 1979). Of interest, however, P_i may also stabilize the enzyme (Farley, 1991).

Relatively little is certain about the biosynthesis of ALP in higher organisms. Analysis of the human ALP isoenzyme gene sequences indicates that the nascent polypeptides have a short signal sequence of 17 to 21 amino acid residues (Harris, 1990) and a hydrophobic domain at their carboxy-termini (Weiss *et al.,* 1988). ALPs become tethered to the plasma membrane surface, where they are bound to the polar head group of a phosphatidylinositol-glycan moiety and can be released by phosphatidylinositol-specific phospholipase (Fedde *et al.,* 1988). However, the precise interaction with phosphatidylinositol may differ among the ALP isoenzymes (Seetharam *et al.,* 1987). Intracellular degradation of ALPs can involve proteosomes (Cai *et al.,* 1998).

Actually, the ALP isoenzymes manifest broad pH optima and variable substrate specificity *in vitro* that reflect the concentration and type of phosphocompound being hydrolyzed (McComb *et al.,* 1979). ALPs can cleave PP_i or phosphoesters (Xu *et al.,* 1991). Catalysis involves phosphorylation–dephosphorylation of a serine residue. Dissociation of the covalently linked phosphate seems to be the rate-limiting step. P_i is a potent competitive inhibitor of ALP (Kim and Wyckoff, 1991; McComb *et al.,* 1979).

ALPs are coupled to cell surfaces by phosphatidylinositol (Whyte, 1994). Lipid-free enzyme comprises ALP normally found in the circulation, however, the mechanism(s) for ALP release from cell surfaces *in vivo* is unknown but

could involve a phosphatidase of the C or D type, detergent action, proteolysis, membrane fractionation, or lipolysis (Alpers *et al.,* 1990).

Clearance of circulating ALP, as for many other glycoproteins, is assumed to occur via uptake by the liver (Young *et al.,* 1984). In healthy men and women, the majority of ALP in serum reflects approximately equal amounts of the TNSALP isoforms from liver and bone (Millan *et al.,* 1980). In healthy infants and children (and particularly during the growth spurt of adolescence), serum is especially rich in the bone isoform of TNSALP (McComb *et al.,* 1979). The remaining ALP in serum is normally intestinal ALP, but this isoenzyme usually contributes just a few percent (maximum 20%) (McComb *et al.,* 1979; Mulivor *et al.,* 1985). Some individuals (with B and O blood types who are "secretors") modestly increase serum levels of intestinal ALP after ingesting a fatty meal (Langman *et al.,* 1966; McComb *et al.,* 1979). Placental ALP typically circulates only during the last trimester of pregnancy (Birkett *et al.,* 1966). Various cancers, however, put placental ALP or germ cell ("placental-like") ALP into the bloodstream (Millan, 1988). In 2001, the detailed crystal structure at 1.8Å resolution was reported for human placental ALP and deemed consistent with phosphorylated protein target (Le Due *et al.,* 2001).

Hypophosphatasia

Subnormal extracellular levels of calcium and/or P_i engender almost all types of rickets or osteomalacia. Hypophosphatasia is an instructive exception (Whyte, 1994, 2001). In the author's opinion, defended here, characterization and study of hypophosphatasia has best elucidated the physiological role of ALP. Now, as discussed below, with identification of numerous mutations in the TNSALP gene in hypophosphatasia (Weiss *et al.,* 1988; Whyte, 2000), unequivocal evidence demonstrates that TNSALP is essential for proper mineralization of our skeletons and development of our teeth (Whyte, 1994). However, the apparently undisturbed function of other organs/tissues in hypophosphatasia questions the biological significance for TNSALP elsewhere.

History

John Campbell Rathbun (1915–1972), a Canadian pediatrician, coined the term hypophosphatasia in 1948 when he reported an infant boy who developed and then died from severe rickets with epilepsy, yet whose ALP activity in serum, bone, and other tissues was paradoxically subnormal (Rathbun, 1948). About 300 patients have been described (Whyte, 2001).

Discussed later, the metabolic basis for hypophosphatasia and the physiological function of TNSALP were clarified by the discoveries in patients of elevated endogenous levels of three phosphocompounds. In 1955, increased urinary concentrations of phosphoethanolamine (PEA) provided a useful biochemical marker for the disease (Fraser *et al.,* 1955; McCance *et al.,* 1955). In 1965 and 1971, supranormal urinary and blood levels of PP_i (Russell, 1965; Russell *et al.,* 1971, respectively), suggested a mechanism for the defective mineralization of hard tissues. In 1985, high plasma concentrations of pyridoxal 5′-phosphate (PLP) indicated an ectoenzyme function for TNSALP (Whyte *et al.,* 1985).

Clinical Features

Hypophosphatasia (McKusick 146300, 171760, 241500, 241510) has been reported throughout the world and apparently affects all races. However, this condition is especially prevalent in inbred Mennonite families from Manitoba, Canada, where about 1 in 25 individuals is a carrier and 1 : 2500 newborns manifests severe disease (Greenberg *et al.,* 1993). In Toronto, Canada the incidence of severe forms was estimated by Fraser to be 1 per 100000 live births (Fraser 1957). In my experience, the disorder is particularly rare in blacks in the United States.

Despite the presence of relatively high levels of TNSALP in bone, liver, kidney, and adrenal tissue (and at least some TNSALP ubiquitously) in healthy individuals (McComb *et al.,* 1979), the impact of hypophosphatasia centers on the skeleton and dentition (Whyte, 2001). In 1953, premature loss of deciduous teeth was recognized to be a major clinical feature (Sobel *et al.,* 1953). However, the severity of expression is extraordinarily variable, ranging from death *in utero* with essentially an unmineralized skeleton, to early shedding of primary dentition only (Fraser, 1957; Whyte, 2001). In fact, some individuals with characteristic, albeit mild, biochemical abnormalities may never suffer symptoms or complications (Whyte *et al.,* 1982a).

Because the many TNSALP gene defects that cause hypophosphatasia are now rapidly being elucidated (Weiss *et al.,* 1988; Henthorn *et al.,* 1992; Mornet *et al.,* 1998; Mumm *et al.,* 1999; Whyte, 2000), a useful mutation-based nosology may emerge (Whyte, 2001). Nevertheless, the current classification of patients for prognostication, etc., remains a clinical one (Whyte, 2001). Six forms of hypophosphatasia have been identified. The age at which lesions in bone are discovered distinguish the perinatal, infantile, childhood, and adult types (Fraser, 1957; Whyte, 2001). Patients who do not have skeletal disease but develop only dental manifestations are regarded as having "odontohypophosphatasia" (Whyte, 2001). The exceedingly rare hypophosphatasia variant called "pseudohypophosphatasia" in all ways (i.e., clinical, radiographic, and biochemical) resembles infantile hypophosphatasia, except that serum ALP activity is not subnormal in the clinical laboratory (see below) (Whyte, 2001). The prognoses for these six forms of hypophosphatasia generally depend upon the degree of skeletal disease which, in turn, reflects the age at presentation. Typically, the earlier a patient manifests skeletal symptoms, the more severe the disorder (Fraser, 1957; Whyte, 2001). However, this classification scheme does not unambiguously distinguish all patients.

PERINATAL HYPOPHOSPHATASIA

This most pernicious form of hypophosphatasia is expressed *in utero* and can cause stillbirth. Profound skeletal hypomineralization results in *caput membraneceum* and limbs that are shortened and deformed during gestation and at birth. Some affected neonates survive several days or weeks, but then suffer from increasing respiratory compromise due to rachitic disease of the chest and due to lungs that are hypoplastic (Silver *et al.,* 1988). Epilepsy can occur and prove lethal *(vide infra)*. Myelophthisic anemia may be present, perhaps from encroachment on the marrow space by excessive osteoid (Terheggen and Wischermann, 1984). Rarely, there is long-term survival.

Radiographic examination of the skeleton readily distinguishes perinatal hypophosphatasia from even the most severe forms of osteogenesis imperfecta and congenital dwarfism. In fact, the X-ray appearance can be considered diagnostic. Nevertheless, there is considerable patient-to-patient variability and the radiographic findings are diverse (Shohat *et al.,* 1991). In some stillborns, the skeleton is nearly without mineralization (Fig. 1). In others, one sees marked bony undermineralization and severe rachitic changes. Occasionally, there can also be peculiar complete or partial absence of ossification of one or more vertebrae (Shohat *et al.,* 1991). In the skull, individual membranous bones may calcify only at their centers. Thus, the areas of unossified calvarium give the illusion that cranial sutures are widely separated, although they may be functionally closed. Other unusual radiographic features include bony spurs that protrude laterally from the midshafts of the ulnae and fibulae (Whyte, 1988).

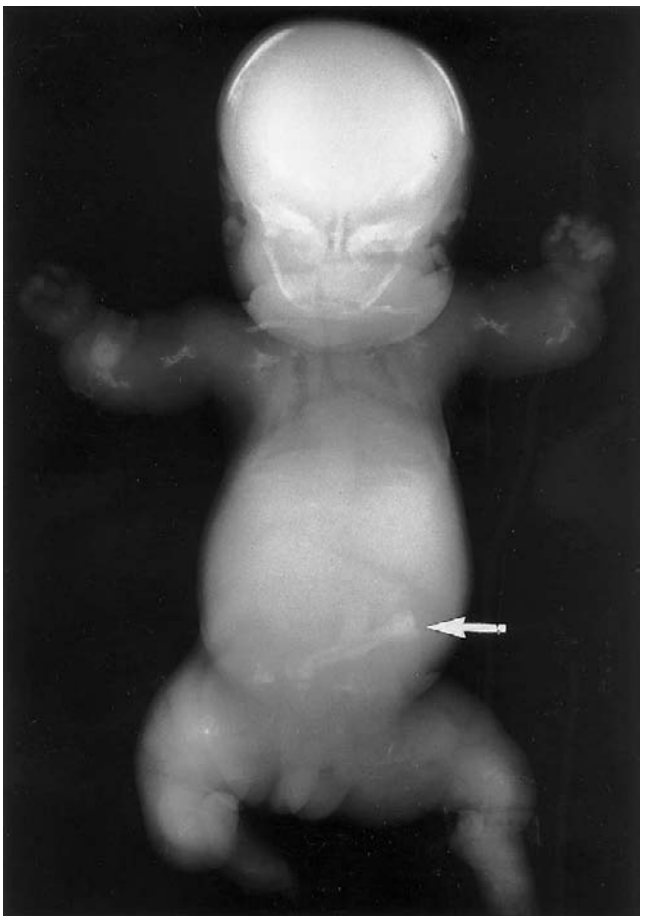

Figure 1 Perinatal hypophosphatasia. Profound skeletal hypomineralization is obvious; umbilical clip (arrow) is more dense than the pelvis. The ends of the long bones in the upper limbs show severe rachitic changes.

INFANTILE HYPOPHOSPHATASIA

This form of hypophosphatasia is the type described by Rathbun (Rathbun, 1948). It presents before the age of 6 months (Fraser, 1957; Whyte, 2001). Postnatal development often seems normal until the onset of poor feeding and inadequate weight gain and the clinical manifestations of rickets are recognized. The cranial sutures appear to be wide, but actually reflect hypomineralization of the skull and often there is a "functional" craniosynostosis. Furthermore, true premature bony fusion of these sutures can occur if the patient survives infancy. A flail chest from rib fractures, rachitic deformity, etc., often lead to pneumonia. Hypercalcemia and hypercalciuria are common and may explain nephrocalcinosis, renal compromise and episodes of recurrent vomiting (Fraser, 1957; Whyte *et al.,* 1982b).

The striking radiographic features are characteristic but generally less severe than in the perinatal form of hypophosphatasia. In some newly diagnosed patients, abrupt transition from relatively normal-appearing diaphyses to uncalcified metaphyses is of interest, suggesting that a metabolic change occurred suddenly (Fraser, 1957). Serial radiographic studies may reveal not only persistence of impaired skeletal mineralization (i.e., rickets), but gradual, generalized demineralization of osseous tissue as well (Whyte *et al.,* 1982b).

CHILDHOOD HYPOPHOSPHATASIA

This form of hypophosphatasia is especially variable in its clinical expression (Fallon *et al.,* 1984; Whyte, 2001). Premature loss of deciduous teeth (i.e., earlier than age 5 years) results from aplasia, hypoplasia, or dysplasia of dental cementum, which should connect the tooth root with the periodontal ligament (Lundgren *et al.,* 1991). The incisors are frequently shed first, but occasionally almost the entire primary dentition is exfoliated prematurely. There may be delayed walking and a characteristic waddling gait. Patients can complain of stiffness and pain and have appendicular muscle weakness (especially in the thighs) consistent with a nonprogressive myopathy (Seshia *et al.,* 1990).

Radiographs typically show characteristic focal bony defects near the ends of major long bones; i.e., "tongues" of radiolucency that project from the rachitic growth plate into the metaphysis (Fig. 2). Functional synostosis of cranial sutures occurs not uncommonly in infants and young children who are severely affected, although widely "open" fontanels appear to be present on radiographic study. This illusion results from large areas of hypomineralized calvarium. Subsequently, true premature bony fusion of cranial

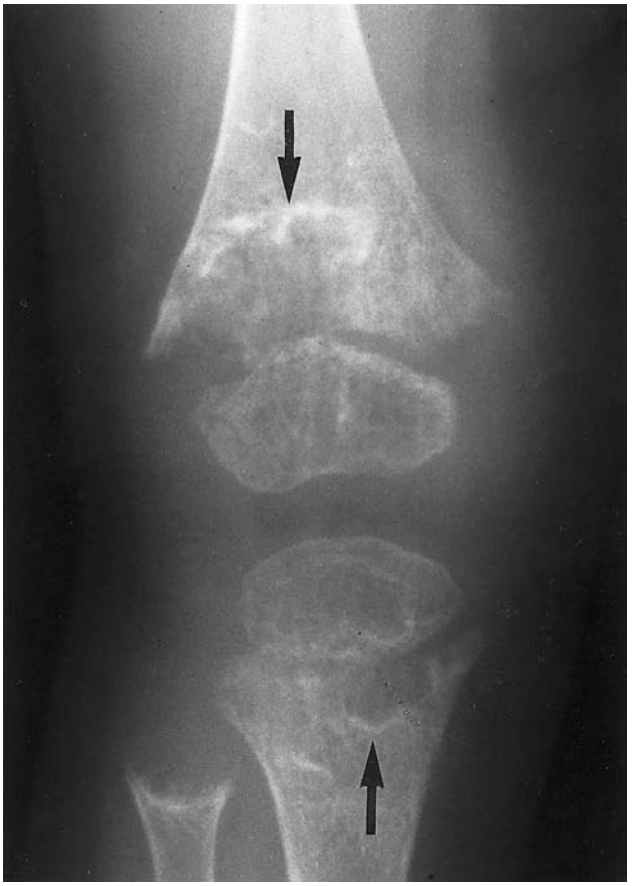

Figure 2 Childhood hypophosphatasia. Characteristic tongues of radiolucency (arrows) project from growth plates into frayed metaphyses.

sutures may also cause raised intracranial pressure. Dental radiographs sometimes show enlarged pulp chambers and root canals that characterize the "shell teeth" of rickets.

ADULT HYPOPHOSPHATASIA

This form of hypophosphatasia usually presents during middle age (Whyte, 2001; Whyte *et al.,* 1982a). Not infrequently, however, remote histories of rickets and/or premature exfoliation of deciduous teeth precede relatively good health after adolescence and during young adult life. Subsequently, osteomalacia often first manifests with painful feet due to poorly healing metatarsal stress fractures that become recurrent. With advanced disease, aching or tenderness in the thighs or hips is often explained by femoral pseudofractures that may progress to completion. Early loss or extraction of the secondary dentition is not uncommon (Whyte *et al.,* 1982a). Calcium pyrophosphate dihydrate (CPPD) crystal deposition, occasionally with overt attacks of arthritis (pseudogout), troubles some patients. Apparently, this complication stems from increased endogenous levels of PP_i (see below) (O'Duffy, 1970; Whyte *et al.,* 1982a). Affected individuals may suffer degeneration of articular cartilage and "pyrophosphate arthropathy" (Whyte *et al.,* 1982a).

In some families with hypophosphat*asemia,* calcium phosphate deposition manifests as "calcific periarthritis" with ossification of ligaments (syndesmophytes) resembling spinal hyperostosis (Forestier's disease) (Lassere and Jones, 1990).

Radiographs may also reveal osteopenia and chondrocalcinosis (Lassere and Jones, 1990; Whyte *et al.,* 1982a).

ODONTOHYPOPHOSPHATASIA

This form of hypophosphatasia is diagnosed when the only clinical abnormality is dental disease (radiographic or histologic studies reveal no evidence of rickets or osteomalacia).

PSEUDOHYPOPHOSPHATASIA

This is a particularly interesting, but especially rare, variant of hypophosphatasia. The disorder has been documented convincingly in two infants (Whyte, 2001). Clinical, radiographic, and biochemical findings of pseudohypophosphatasia recapitulate infantile hypophosphatasia, with the important exception that serum total ALP activity is consistently normal or increased (Whyte, 1994, 2001). The enzymatic defect involves a TNSALP with diminished activity toward PEA, PP_i, and PLP at physiological pH, but normal or even increased activity in the highly artificial conditions of the ALP assays used in clinical laboratories (*see below*) (Fedde *et al.,* 1990).

Laboratory Diagnosis

BIOCHEMICAL FINDINGS

Hypophosphatasia can be diagnosed confidently when the patient has a consistent clinical history, physical findings, and radiographic changes together with serum ALP activity that is clearly and reproducibly subnormal (Whyte, 2001). Even patients with odontohypophosphatasia are distinguishable from healthy individuals by their low serum ALP activity, although values do approach the lower end of carefully established, age-adjusted reference ranges (Fig. 3). Usually, the more severe the disease, the lower the age-appropriate serum ALP activity. In perinatal and infantile hypophosphatasia, hypophosphat*asemia* is detectable in umbilical cord blood at birth (Whyte, 2001).

Hypophosphat*asemia* occurs under a variety of conditions (starvation, hypothyroidism, scurvy, severe anemia, Wilson disease, celiac disease, hypomagnesemia, Zn^{2+} deficiency), from certain drugs (glucocorticoids, clofibrate, chemotherapy, intoxication levels of vitamin D, milk–alkali syndrome), and with exposure to radioactive heavy metals or massive transfusion of blood or plasma (Macfarlane *et al.,* 1992). These clinical situations, however, should be readily apparent. Of note, especially rare cases of lethal osteogenesis imperfecta (Royce *et al.,* 1988) and infants with cleidocranial dysplasia can have low levels of serum ALP activity from osteoblast dysfunction (personal observation). Finally, transient increments in serum ALP activity (probably the bone isoform of TNSALP) occur in hypophosphatasia patients after orthopedic surgery or fracture. In theory, conditions that increase serum levels of any form of ALP (e.g., pregnancy, liver disease) could mask the diagnosis. Thus, in confusing cases, documentation that serum ALP activity is low on more than one occasion during

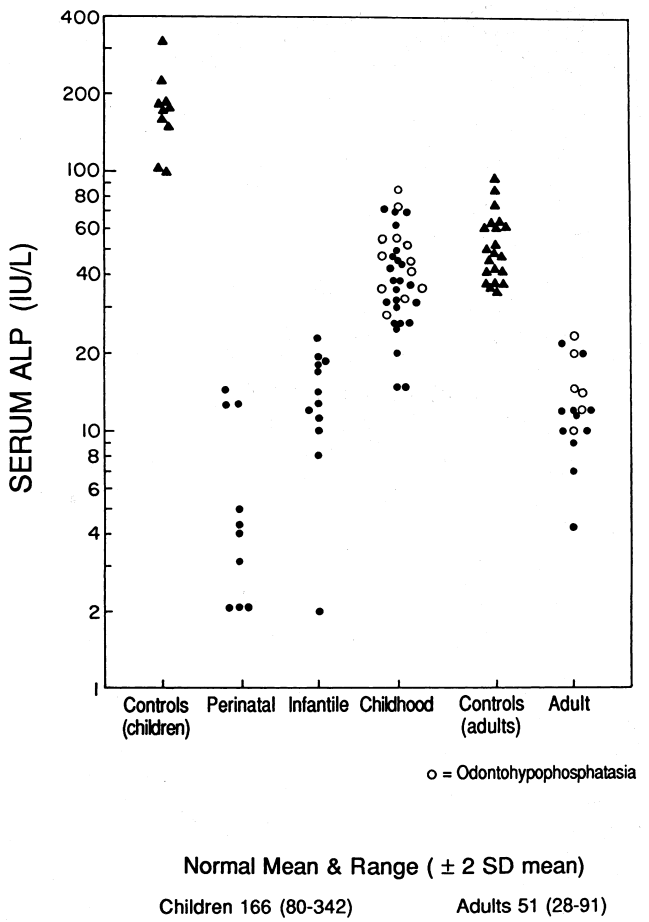

Figure 3 Serum ALP activity in hypophosphatasia. Serum ALP activity in normal children and normal adults and in 52 patients from 47 families with the various clinical forms of hypophosphatasia (note the logarithmic scale). All ALP assays performed at the Metabolic Research Unit, Shriners Hospitals for Crippled Children, St. Louis, MO. [Reproduced with permission from Whyte, M. P. Hypophosphatasia. *In* "The Metabolic and Molecular Bases of Inherited Disease" (C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, eds.), 2001, 8th ed., pp. 5313–5329. McGraw-Hill, New York.]

clinical stability seems advisable. Quantitation of specific ALP isoenzymes (Mulivor *et al.,* 1985), isoforms of TNSALP (Whyte *et al.,* 1996), or leukocyte ALP (Iqbal *et al.,* 2000) may also be helpful.

Neither calcium nor P_i levels in serum are subnormal in hypophosphatasia. In fact, there may be hypercalcemia and hyperphosphatemia. Hypercalcemia and hypercalciuria occur frequently in infantile hypophosphatasia (Fraser, 1957; Shohat *et al.,* 1991; Whyte *et al.,* 1982b). The disturbed calcium homeostasis is poorly understood but appears to reflect defective uptake of mineral by a slowly growing skeleton together perhaps with progressive skeletal demineralization. In childhood hypophosphatasia, severely affected patients occasionally manifest hypercalciuria but without hypercalcemia. Serum levels of 25(OH)D and parathyroid hormone (PTH) are typically unremarkable, unless there are aberrations in serum calcium levels or renal failure (Fallon *et al.,* 1984). Several patients have had elevated serum PTH levels, but impaired renal function from hypercalcemia (causing retention of immunoreactive PTH fragments) may have explained this finding (Whyte, 1994). Low circulating levels of PTH, potentially from the feedback of hypercalcemia on the parathyroid glands, have also been reported.

Individuals with the childhood or adult forms of hypophosphatasia have serum P_i levels above the average values for controls. Indeed, 50% of these patients are distinctly *hyper*phosphat*emic.* Enhanced renal reclamation of P_i (increased TmP/GFR) is the mechanism for this poorly understood finding (Whyte and Rettinger, 1987). Conversely, especially rare patients with hypophosphat*asemia* have been reported who have *hypo*phosphat*emia* from kidney P_i wasting (Juan and Lambert, 1981).

Phosphoethanolamine Elevated urinary levels of PEA support a diagnosis of hypophosphatasia (Rasmussen, 1968), but are not pathognomonic. Licata and coworkers have shown that phosphoethanolaminuria occurs under other conditions, including several metabolic bone diseases (Licata *et al.,* 1978). For diagnosing mild cases, the clinician must recognize that urinary PEA levels are influenced by patient age and somewhat by diet, follow a circadian rhythm, and reportedly can be unremarkable in mildly affected patients. Age-adjusted normal ranges (expressed as micromoles PEA per gram of creatinine) are: <15 years, 83–222; 15–30 years, 42–146; 31–41 years, 38–155; and >45 years, 48–93 (Licata *et al.,* 1978).

Pyridoxal 5′-phosphate An increased plasma concentration of PLP is a specific and sensitive marker for hypophosphatasia (including pseudohypophosphatasia) (Coburn *et al.,* 1988; Coburn and Whyte, 1988) (Fig. 4). Commercial laboratories now perform this assay. Even patients with odontohypophosphatasia manifest this biochemical finding (Whyte *et al.,* 1985). However, vitamin supplements containing pyridoxine should not be taken for 1 week before testing. In general, the more severely affected the patient, the greater the elevation in the plasma PLP concentration. Nevertheless, there is overlap among the plasma PLP levels for the different clinical phenotypes (Fig. 4). Assay of plasma PLP levels after oral challenge with pyridoxine seems to reveal affected individuals especially well (Whyte, 1994), and this procedure can identify Canadian Mennonite carriers of severe hypophosphatasia (Chodirker *et al.,* 1990).

Inorganic Pyrophosphate Assay of urinary PP_i remains a research technique. PP_i levels are increased in most patients with hypophosphatasia, but are occasionally unremarkable in mildly affected subjects (Caswell *et al.,* 1991). Nevertheless, this biochemical parameter has been reported to be sensitive for carrier detection (Whyte, 2001).

RADIOLOGIC FINDINGS

The radiographic features have been described above for each form of hypophosphatasia.

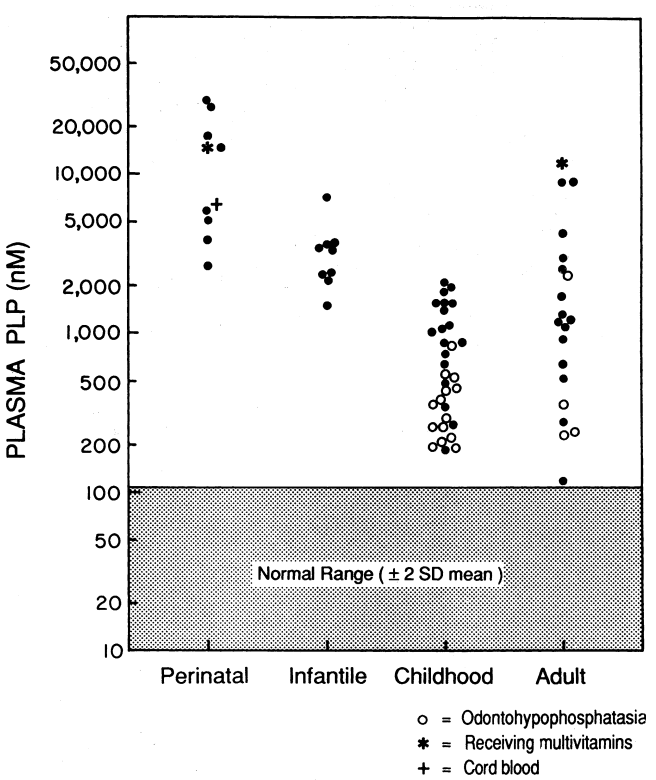

Figure 4 Plasma PLP levels in hypophosphatasia. PLP levels in plasma in various clinical forms of hypophosphatasia (hatched area is the normal range for children and adults). Note, the logarithmetic scale with some overlap between clinical forms (assays performed courtesy of Dr. Stephen P. Coburn, Fort Wayne State Developmental Center, Fort Wayne, IN). [Reproduced with permission from Whyte, M. P. Hypophosphatasia. *In* "The Metabolic and Molecular Bases of Inherited Disease" (C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, eds.), 2001, 8th ed., pp. 5313–5329. McGraw-Hill, New York.]

HISTOPATHOLOGIC FINDINGS

Histopathologic changes are limited essentially to hard tissues in hypophosphatasia. In severe cases, additional abnormalities—such as extramedullary hematopoiesis (Fallon *et al.,* 1984; Ornoy *et al.,* 1985)—may be secondary to the skeletal disease itself. The reason for pulmonary hypoplasia, however, is not clear (Silver *et al.,* 1988).

Skeleton In all but the mildest cases (i.e., odontohypophosphatasia), nondecalcified sections of bone reveal evidence of defective mineralization (Fallon *et al.,* 1984; Ornoy *et al.,* 1985). The degree of osteomalacia generally reflects the clinical severity of hypophosphatasia (Fallon *et al.,* 1984). Nevertheless, features of secondary hyperparathyroidism (present in rickets or osteomalacia associated with hypocalcemia) are generally absent (Fallon *et al.,* 1984).

In growth plates and osseous tissue, sources of the bone form of TNSALP, chondrocytes and osteoblasts, as well as matrix vesicles, are observed, but levels of TNSALP activity are subnormal (Fallon *et al.,* 1984; Ornoy *et al.,* 1985). However, the histopathologic changes cannot be distinguished from those of most other forms of rickets or osteomalacia unless ALP activity is quantitated. ALP activity in bone inversely reflects the amount of osteoid accumulation (Fallon *et al.,* 1984).

Electron microscopy of perinatal hypophosphatasia has shown normal distribution of matrix vesicles, proteoglycan granules, and collagen fibers in the extracellular space of cartilage (Ali, 1986; Fallon *et al.,* 1984), yet matrix vesicles lack ALP activity. Early reports described only isolated or tiny groups of hydroxyapatite crystals (calcospherites) frequently not associated with vesicular structures (Ali, 1986; Shohat *et al.,* 1991). More recently, Anderson and coworkers showed that matrix vesicles contain hydroxyapatite crystals that are not seen enlarging after these structures rupture (Anderson *et al.,* 1997).

Dentition Premature exfoliation of primary teeth occurs under a variety of conditions. In hypophosphatasia, a paucity of cementum due to aplasia, hypoplasia, or dysplasia (despite the presence of cells that look like cementoblasts) seems to be a major factor engendering this complication (Fig. 5) (el-Labban *et al.,* 1991; Lundgren *et al.,* 1991). If cementum is present, it may appear to be afibrillar (el-Labban *et al.,* 1991). Desiccated teeth that were shed years earlier may still be useful for examination (Fig. 5). The severity of this defect varies from tooth to tooth, but typically reflects the extent of

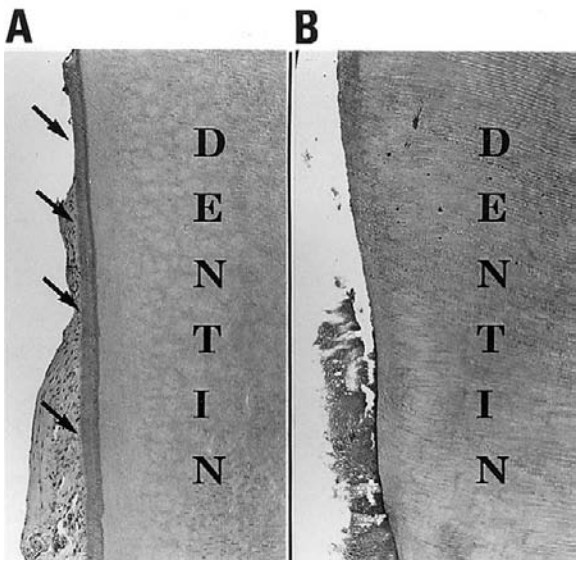

Figure 5 Dental findings in hypophosphatasia. (A) Decalcified section of part of the root of a maxillary incisor from a child with *X*-linked hypophosphatemia is essentially normal and shows primary cementum (delineated by arrows) at its surface (original magnification × 150). (B) In hypophosphatasia, cementum is absent. Original magnification × 150. [Reproduced with permission from Whyte, M. P. Hypophosphatasia. *In* "The Metabolic and Molecular Bases of Inherited Disease" (C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, eds.), 2001, 8th ed., pp. 5313–5329. McGraw-Hill, New York.]

skeletal disease. Incisors are usually the most notably affected and are the first to be lost.

In addition to defects in cementum, retarded dentinogenesis seems to occur as suggested by big pulp chambers. Dentin tubules may be enlarged although reduced in number. The excessive width of predentin, increased amounts of interglobular dentin, and impaired calcification of cementum are analogous to the osteoidosis found in bone. The enamel is not impacted directly (Lundgren *et al.,* 1991). The histopathologic changes observed in the permanent teeth are similar to those in the deciduous teeth (el-Labban *et al.,* 1991), but their prognosis is better (Lepe *et al.,* 1997).

Biochemical and Genetic Defect

TNSALP DEFICIENCY

Early on, postmortem investigations of the perinatal and infantile forms of hypophosphatasia were critical for clarifying the underlying biochemical defect, but they also intimated the disorder's genetic basis. Profound deficiency of ALP activity was discovered in liver, bone, and kidney tissue, yet ALP activity was not decreased in intestine or placenta (fetal trophoblast) (Vanneuville and Leroy, 1981). This observation was consistent with amino acid sequence analyses of ALP purified from various healthy human tissues (Stigbrand and Fishman, 1984) and argued for a selective decrease in the enzymatic activity of all isoforms comprising the TNSALP family.

Postmortem studies of children or adults with hypophosphatasia have not been reported. Nevertheless, indirect findings indicated that these clinically milder forms also reflect globally diminished TNSALP activity. In such patients, TNSALP activity can be deficient in circulating granulocytes (Fallon *et al.,* 1984), bone tissue (Fallon *et al.,* 1984), and cultivated skin fibroblasts (Vanneuville and Leroy, 1981).

Exploration of the biochemical hallmark, hypophosphat*asemia,* corroborated this hypothesis and provided further insight. Hypophosphatasemia did not involve enhanced loss of TNSALP from the circulation (Gorodischer *et al.,* 1976). Purified placental ALP as well as the bone form of TNSALP (contained in plasma of patients with Paget bone disease) showed normal circulating half-lives of several days when infused intravenously into severely affected infants during attempted enzyme-replacement therapy (*vide infra*) (Whyte *et al.,* 1982b). Furthermore, coincubation experiments with mixtures of serum and coculture or heterokaryon studies with fibroblasts from severely affected patients excluded an inhibitor or absence of an activator of TNSALP (Fraser, 1957; O'Duffy, 1970; Whyte and Vrabel, 1985). Thus, the hypophosphatasemia of hypophosphatasia is due to low levels of TNSALP activity throughout the body.

In *tissues,* ALP immunoreactivity has been quantitated in liver, bone, and kidney as well as in skin fibroblasts obtained from patients with severe hypophosphatasia (Fallon *et al.,* 1989; Fedde *et al.,* 1996). The preliminary report by Fallon and coworkers, based upon a polyclonal antibody to the human liver form of TNSALP, revealed normal amounts of immunoreactive TNSALP in bone, liver, and kidney in five such individuals (Fallon *et al.,* 1989). Different investigators, using isoelectric focusing and enzyme inhibition studies, suggested that the low ALP activity in a similar patient reflected intestinal ALP (Mueller *et al.,* 1983). In fibroblasts from severely affected individuals, the ALP has somewhat different physicochemical and immunological properties compared with the enzyme from controls, but seems to be a form of TNSALP (Fedde *et al.,* 1996).

In the *circulation,* the nature of ALP in hypophosphatasia requires further study. Leukocyte ALP activity can be subnormal in any type of the disease except perhaps pseudohypophosphatasia and, therefore, likely represents TNSALP (see below) (Fallon *et al.,* 1984). Monoclonal antibody-based IRMAs for polymeric TNSALP demonstrate low levels of the bone and liver isoforms of TNSALP in sera from patients with all clinical types of hypophosphatasia except pseudohypophosphatasia, in which levels are normal (Whyte *et al.,* 1996). Upon release from plasma membranes, TNSALP in hypophosphatasia seems to be altered in such a way that immunoreactivity diminishes or clearance is accelerated (Whyte *et al.,* 1996). The observations of Fedde and coworkers indicate that the precise impact of TNSALP gene mutations (see below) on the enzyme's structure in tissues must be understood to fully appreciate the disease expression of hypophosphatasia (Fedde *et al.,* 1996).

INHERITANCE

Although it is apparent that perinatal and infantile hypophosphatasia represent autosomal recessive traits, the mode of transmission of milder forms requires investigation. Vertical propagation of mild disease seems unusual (Whyte *et al.,* 1982a). Nevertheless, multigeneration biochemical (Whyte *et al.,* 1982a) and occasionally clinical (Eastman and Bixler, 1983) abnormalities argue that less severe types of hypophosphatasia sometimes represent autosomal dominant traits. In fact, clinical studies of exceptional families are consistent with mildly affected individuals possessing a gene defect that causes severe disease in homozygotes or compound heterozygotes (Eastman and Bixler, 1982).

However, identifying carriers for hypophosphatasia using conventional biochemical testing has been difficult. The utility of quantitating several parameters, including urinary PP_i, has been discussed (Sorensen *et al.,* 1978). Pyridoxine (vitamin B_6) loading followed by assay of plasma PLP levels helps to detect Mennonite carriers in Canada (Chodirker *et al.,* 1987) (*vide infra*). Now, mutation analysis of *TNSALP* is rapidly clarifying the patterns of inheritance for hypophosphatasia. All clinical forms can be transmitted as autosomal recessive traits, but autosomal dominant inheritance of mild disease due to certain *TNSALP* mutations also occurs (Henthorn, *et al.,* 1996; Whyte, 2000) (*vide infra*).

GENE DEFECTS

In 1988, proof that hypophosphatasia can involve the TNSALP (candidate) gene came with the discovery by Weiss and coworkers of a homozygous *TNSALP* missense mutation in a consanguineous patient with perinatal hypophosphatasia (Weiss *et al.,* 1988). Site-directed mutagenesis and transfection analysis confirmed that the single base transition compromised the enzyme's catalytic activity, perhaps by altering metal ligand binding to an important arginine residue at the catalytic pocket (Weiss *et al.,* 1988).

In 1992, Henthorn and colleagues reported eight additional missense mutations in four unrelated patients with perinatal or infantile hypophosphatasia (Henthorn *et al.,* 1992). Allele-specific oligonucleotide screening disclosed 23 individuals, among 50 unrelated patients manifesting all clinical forms of hypophosphatasia, with one of these base changes (the other TNSALP allele remained uncharacterized). Two siblings with typical childhood hypophosphatasia and one unrelated elderly woman with classic adult hypophosphatasia proved to be compound heterozygotes for a novel combination of two of these molecular defects. This observation confirmed that some cases of nonlethal hypophosphatasia reflect autosomal recessive inheritance and the childhood and adult forms can, as predicted, be the same entity (Weinstein and Whyte, 1981; Henthorn *et al.,* 1992).

In 1993, Greenberg and coworkers discovered that homozygosity for a 10th TNSALP missense mutation accounts for the high incidence of severe hypophosphatasia in Canadian Mennonites, presumably reflecting a "founder effect" (Greenberg, *et al.,* 1993).

Now, all clinical forms of hypophosphatasia (except pseudohypophosphatasia) have been shown to involve mutations in *TNSALP* that compromise the enzyme's structure (Whyte, 2000). Other gene defects have not been encountered. However, a genetic nosology for hypophosphatasia must currently consider 79 specific *TNSALP* mutations (Mornet *et al.,* 1998) and in the future this classification scheme will likely involve many more (Mumm, *et al.,* 1999), thereby creating significant challenges for phenotype/genotype correlation. Perhaps information from heterozygous carriers of hypophosphatasia concerning the quantitative impact of individual *TNSALP* defects *in vivo* will prove helpful in predicting disease severity (Whyte, 2000). A web site now records *TNSALP* mutations (http://www.sesep.uvsq.fr/Database.html).

TNSALP STRUCTURAL DEFECTS

Many of the 79 different mutations identified to date in hypophosphatasia (Taillander *et al.,* 2001) alter an amino acid residue that is conserved in mammalian TNSALPs (Henthorn and Whyte, 1992; Mornet *et al.,* 1998; Whyte, 2000). In fact, several of the changes are conserved in bacteria (Henthorn *et al.,* 1992).

Revelation of the three-dimensional structure of *Escherichia coli* ALP by X-ray crystallography (Kim and Wyckoff, 1991) has underpinned further progress in our understanding of the biochemical basis for hypophosphatasia (Henthorn *et al.,* 1992; Kim and Wyckoff, 1991). Missense mutations in *TNSALP* can be examined for their impact on dimeric TNSALP [Chemscape Chime version 1.02 by MDL Information Systems, Inc. (San Leandro, CA) at *http://www.mdli.com* and RasWin Molecular Graphics, Windows version 2.6 by Roger Sayle, Glaxo-Wellcome Research and Development (Stevenage, Hertfordshire, UK) at *http://www.umass.edu/microbio/rasmol/index.html*]. Some alterations in *TNSALP* appear to disturb the enzyme's catalytic pocket or structurally important metal ligand binding sites; others could affect dimer formation (Mornet *et al.* 1998). Nevertheless, how all *TNSALP* mutations identified to date are deleterious is far from understood (Whyte, 2000). In fact, site-directed mutagenesis with the Ala^{161}–Thr substitution, first discovered to cause hypophosphatasia (Weiss *et al.,* 1988), does not compromise catalytic activity of *E. coli* ALP (Chaidaroglou and Kantrowitz, 1993). It seems that some mutations alter intracellular movement of TNSALP (Mornet *et al.,* 1998; Shibata *et al.,* 1998; Fukushi *et al.,* 1998).

Treatment

MEDICAL

There is no established medical regimen for hypophosphatasia, although several therapies have been studied (Fraser *et al.,* 1955; Whyte *et al.,* 1982, 1986a). Some patients with infantile hypophosphatasia manifest progressive skeletal demineralization (Whyte *et al.,* 1982b); others show spontaneous improvement (Whyte *et al.,* 1986a).

Hypercalcemia in infantile hypophosphatasia can be treated by restriction of dietary calcium and/or glucocorticoid therapy (Whyte *et al.,* 1982b). However, progressive skeletal demineralization may follow (Whyte *et al.,* 1982b, 1986a). Consequently, subcutaneous injections of salmon calcitonin could be helpful (Barcia *et al.,* 1997).

Hypothetically, treatment with drugs that would stimulate TNSALP biosynthesis or enhance its activity in the skeleton might be beneficial for hypophosphatasia. Administration of cortisone to a few patients with severe disease reportedly was associated with periods of normalization of serum ALP activity and radiographic improvement (Fraser and Laidlaw, 1956), but this has not been a consistent finding (Fraser, 1957). Brief treatment with Mg^{2+}, Zn^{2+}, or an active fragment of PTH to stimulate ALP activity has been unsuccessful. Hypophosphat*asemia* seems to be quite refractory in hypophosphatasia. The author is aware of serum ALP activity remaining subnormal in one patient despite viral hepatitis and in two patients suffering end-stage kidney disease, perhaps with renal osteodystrophy.

Enzyme replacement therapy for hypophosphatasia has been attempted by intravenous infusion of several types of ALP. Generally, the results have been disappointing. In 1972, serum from a patient with Paget bone disease, given by Macpherson and colleagues to an affected infant, was associated with radiographic improvement (Macpherson *et al.,* 1972). However, subsequent trials of this treatment

showed no significant clinical or radiographic benefit for four patients with infantile hypophosphatasia (Whyte *et al.,* 1984). In 1982, intravenous infusions weekly of fresh plasma from healthy subjects was followed by clinical and radiographic advances in one child (Albeggiani and Cataldo, 1982). Furthermore, plasma from several healthy individuals that had been pooled and then administered intravenously was followed by correction of hypophospha*tasemia* and marked temporary clinical, radiographic, and histologic improvement in a boy with the infantile form (Whyte *et al.,* 1986a). However, subsequent trials of combined plasma infusions did not produce this response (Whyte *et al.,* 1988). In follow-up of a brief report in 1989 which suggested that intravenous administration of ALP purified from liver improved the histologic appearance of bone and decreased urinary PEA levels in an infant with hypophosphatasia (Weninger *et al.,* 1989), a vigorous trial was conducted with intravenous infusions of purified placental ALP. Elevated concentrations of PLP in plasma and increased PEA and PP_i levels in urine were found to normalize during the third trimester of pregnancy in women who are hypophospha*tasemic* carriers for hypophosphatasia (Whyte *et al.,* 1995). Accordingly, placental ALP seems to recognize the same substrates as TNSALP; the presence of placental ALP in the circulation, or more likely *in situ,* could reflect a form of "endogenous" enzyme replacement therapy. Nevertheless, when purified placental ALP was infused intravenously into a severely affected infant in repeated doses that caused *hyper*phosphatasemia, there were only modest decrements of plasma PLP and urinary PEA concentrations, no change in urinary PP_i levels, and no clinical or radiographic improvement (Whyte *et al.,* 1992).

These discouraging cumulative observations from attempted ALP replacement therapy may reflect the fact the *tissue* levels of ALP are normally greater than those achieved in the circulation by such treatments. Alternatively, the findings are consistent with a requirement for ALP *in situ* in the skeleton (and/or perhaps bound to a lipid bilayer; e.g., placenta) for physiologic activity (Whyte *et al.,* 1995). In this regard, extreme skeletal disease occurs in perinatal hypophosphatasia despite abundant circulating placental ALP in the mother; here the *in utero* environment is clearly not protective (Whyte, 2001).

There is now 4-year follow-up of one patient with infantile hypophosphatasia which indicates that marrow cell transplantation, especially a stromal cell boost, can treat potentially lethal disease, perhaps by engendering sufficient numbers of TNSALP-replete osteoblasts (Whyte *et al.,* 1998).

Because patients with hypophosphatasia are often hyperphosphatemic, I speculate that dietary P_i restriction and/or P_i binding may be therapeutic by diminishing competitive inhibition of TNSALP by P_i (Coburn *et al.,* 1999) and by inducing TNSALP gene expression (Goseki-Sone *et al.,* 1999).

SUPPORTIVE

Symptoms from CPPD crystal deposition or perhaps periarticular calcium phosphate precipitation ("calcific periarthritis") may respond to nonsteroidal anti-inflammatory agents.

Prognosis

Perinatal hypophosphatasia is almost always a fatal condition. With intensive support, life may be extended for a short time. Rarely, there is long-term survival. Infantile hypophosphatasia has a less certain outcome. Often, there is clinical and radiographic deterioration. Approximately 50% of these patients die from the pneumonia and respiratory compromise that follows worsening skeletal involvement in the chest (Fraser, 1957). The prognosis, however, seems to improve after infancy. Sometimes, there is remarkable spontaneous improvement following a diagnosis of infantile hypophosphatasia. In fact, a preliminary report from Canada (Ish-Shalom *et al.,* 1986) suggests that, in their patient population, the stature of adult survivors of infantile hypophosphatasia may be normal (the author, however, is aware of significant exceptions in the United States) (Whyte, 2001). Childhood hypophosphatasia may also spontaneously improve, but usually after adolescence. Unfortunately, recurrence of symptoms in adulthood is possible, if not likely (Fraser, 1957; Weinstein and Whyte, 1981). Adult hypophosphatasia causes lingering and recurrent orthopedic difficulties following the onset of skeletal symptomatology (Whyte *et al.,* 1982a). Worsening osteomalacia, associated with osteopenia and fractures, can occur in affected women at menopause, but was not prevented by estrogen replacement therapy in two patients (personal observation).

Prenatal Diagnosis

The perinatal form of hypophosphatasia has been diagnosed reliably *in utero.* During the first trimester, chorionic villus samples from 15 pregnancies were assessed using a monoclonal antibody that captures TNSALP for assay of its catalytic activity (Brock and Barron, 1991). During the second trimester, perinatal hypophosphatasia has been diagnosed by ultrasonography (van Dongen *et al.,* 1990), radiographic study, and assay of ALP activity in amniotic fluid cells by an experienced laboratory (Kousseff and Mulivor, 1981). Combined use of these techniques, with preference for serial fetal ultrasonography, was deemed best before DNA-based studies became possible (Henthorn and Whyte, 1995). Indeed, we now know that mild forms of hypophosphatasia can cause *in utero* bowing of the lower extremities detected by ultrasound, suggestive of a potentially lethal form of skeletal dysplasia. Postnatally, the deformity corrects spontaneously and the phenotype is childhood hypophosphatasia (Moore *et al.,* 1999; Pauli *et al.,* 1999).

Currently, *TNSALP* mutation detection is proving especially reliable (Henthorn and Whyte, 1995), including for the infantile form. However, the great number and some variety of specific defects in *TNSALP* complicates the search.

Physiological Role of ALP Explored in Hypophosphatasia

Demonstration by Weiss and colleagues in 1988 that a missense mutation within the TNSALP gene can inactivate this ALP isoenzyme and that homozygosity for the defect causes severe hypophosphatasia (Weiss *et al.,* 1988) provided proof that Robert Robison (Robison, 1923; Robison and Soames, 1924) was correct; i.e., ALP functions importantly in mineralization of the skeleton (Whyte, 1994). Elucidation of the dental manifestations of hypophosphatasia showed that this is also true for dentition. Description of many *TNSALP* structural mutations in all forms of hypophosphatasia (Henthorn *et al.,* 1992; Greenberg *et al.,* 1993; Mornet *et al.,* 1998; Zurutuza, *et al.,* 1999; Whyte, 2000) confirms these physiological roles for TNSALP in man.

Subsequently, *in vitro* models showed that transfection of ALP cDNA conferred both catalysis against P_i esters and mineralization in a calcification system (Farley *et al.,* 1994) (Yoon *et al.,* 1989). In fact, there has been skepticism that this type of experiment accurately reflects biomineralization, because increases in P_i concentration in metastable solutions are expected to precipitate calcium phosphate.

Furthermore, in 1995, Waymire and coworkers described a *tnsalp* "knockout" mouse (Waymire *et al.,* 1995). This murine model manifests a disturbance in vitamin B_6 metabolism that causes lethal seizures. With pyridoxine treatment, the epilepsy is ameliorated and the mice survive long enough to develop skeletal and dental disease. In 1999, Fedde and coworkers demonstrated that this murine model and the *tnsalp* knockout mouse developed by Narisawa and colleagues (Narisawa *et al.,* 1997) recapitulate the infantile form of hypophosphatasia remarkably well (Fedde *et al.,* 1999).

Several specific roles for TNSALP in skeletal formation have been proposed (Table I). Investigation of hypophosphatasia is a "window of opportunity" to explore each function in man. The *tnsalp* knockout mouse compliments and can expand these patient studies. Recently, the mineralization defect was reproduced with osteoblasts in culture lacking TNSALP (Wennberg *et al.,* 2000). The disturbance does not seem to be related to the aberration in vitamin B_6 metabolism (Narisawa *et. al,* 2001).

Electron microscopy of skeletal tissue from severely affected patients indicates that deficient TNSALP activity causes a fundamental disturbance in biomineralization at the very earliest sites of calcification. In 1985, Ornoy and coworkers reported that the process of "primary mineralization" is deranged. Hydroxyapatite crystals were found near, but not within, patient matrix vesicles (Ornoy *et al.,* 1985). However, in 1997, Anderson and colleagues discovered that secondary (extravesicular), not primary, mineralization is deranged (Anderson *et al.,* 1997). There appeared to be a failure of hydroxyapatite crystal growth after matrix vesicles ruptured.

The defects in the dentin and the cementum of teeth seem to be analogous to those in the skeleton (Lundgren *et al.,* 1991). A prominent role for TNSALP during two critical phases of dental biomineralization, initiation, and completion was proposed recently (Hotton *et al.,* 1999).

Although the liver, kidneys, and adrenal glands are normally rich in TNSALP activity (McComb *et al.,* 1979), they are not dysfunctional in hypophosphatasia (*see below*). It has been suggested that TNSALP deficiency might disturb the biosynthesis of phospholipids causing atelectasis, however, the respiratory problems of patients likely reflect rib cage deformities, hypoplastic lungs, etc. (Silver *et al.,* 1988). Accordingly, it may be that TNSALP is critically important in humans only for mineralization of hard tissues (Whyte, 1994). Why patients with severe hypophosphatasia experience idiopathic seizures is not clear, but this complication has not responded to vitamin B_6 treatment. Conversely, the observation of epilepsy in the murine model is significant, because low levels of γ-aminobutyric acid occur in the central nervous system (Waymire *et al.,* 1995) and pyridoxine supplementation is helpful (Fedde *et al.,* 1999).

As reviewed below, discovery that PEA, PLP, and PP_i accumulate endogenously in hypophosphatasia (and are inferred therefore to be natural substrates for TNSALP) has helped elucidate the physiological role of TNSALP in humans.

Phosphoethanolamine

The reports in 1955 by McCance and colleagues (McCance *et al.,* 1955) and Fraser and coworkers (Fraser *et al.,* 1955) that PEA levels are elevated in urine and plasma in hypophosphatasia provided both a useful biochemical marker for this disorder and the first evidence from this inborn error of metabolism for a natural substrate for TNSALP. In 1968, detailed evaluation by Rasmussen of the renal handling of PEA showed that this phosphocompound is excreted when plasma levels are scarcely detectable; i.e., essentially no renal threshold exists for PEA excretion (Rasmussen, 1968).

Although the metabolic origin of PEA is uncertain, it is thought not to be a derivative of phosphatidylethanolamine, i.e., from plasma membrane phospholipid breakdown. The principal source of circulating PEA has been reported to be the liver, which metabolizes PEA to ammonia, acetaldehyde, and P_i in a reaction catalyzed by O-phosphorylethanolamine phospholyase (Gron, 1978). Indeed, in one family with adult hypophosphatasia, urinary levels of PEA correlated inversely with the circulating activity of the liver (but not the bone) isoform of TNSALP (Millan *et al.,* 1980). PEA is now known to be a constituent of the phosphatidylinositol–glycan linkage apparatus (Low and Zilversmit, 1980). Hence, the source of circulating and urinary PEA could be the degradation of this tether for a variety of cell-surface (ecto-) proteins (*vide infra*).

Pyridoxal 5′-Phosphate

Discovery in 1985 that plasma levels of PLP are elevated in hypophosphatasia helped to clarify the physiological role of TNSALP (Whyte *et al.,* 1985). As reviewed in Fig. 6, the

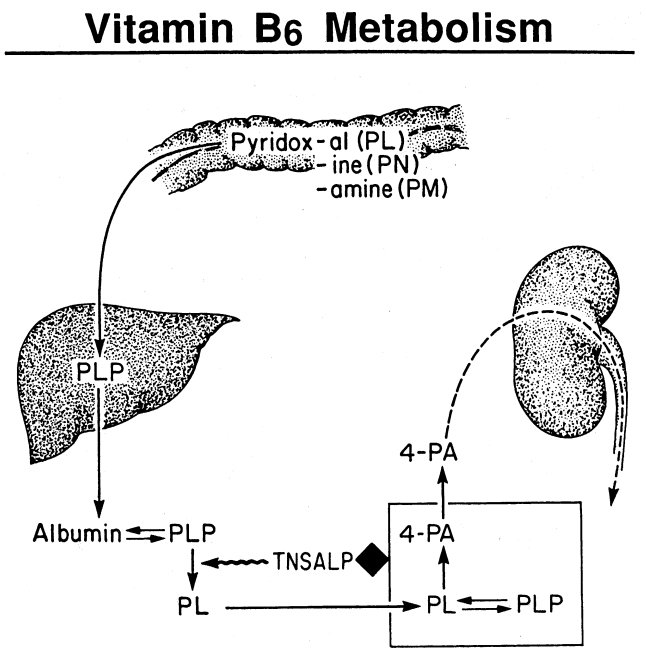

Figure 6 Role of TNSALP in vitamin B_6 metabolism. The various vitameric forms of vitamin B_6 in the diet are absorbed into the hepatic portal circulation (phosphorylated forms are first dephosphorylated in the gut). In the liver, they are converted to PLP, which is secreted bound to albumin into the plasma. Before entering tissues, plasma PLP must be dephosphorylated to PL which can traverse membranes. 4-Pyridoxic acid (4-PA), the major degradation product of vitamin B_6, is excreted in the urine. High plasma levels of PLP in hypophosphatasia, yet normal plasma concentrations of PL, are consistent with an ectoenzyme role for TNSALP in the extracellular dephosphorylatin of PLP to PL. [Reproduced with permission from Whyte, M. P. Hypophosphatasia. *In* "The Metabolic and Molecular Bases of Inherited Disease" (C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, eds.), 2001, 8th ed., pp. 5313–5329. McGraw-Hill, New York.]

dietary forms of vitamin B_6 (pyridoxine, pyridoxal, and pyridoxamine and their phosphorylated derivatives) are converted to PLP in the liver (Dolphin *et al.,* 1986). Organ ablation studies indicate that the liver is the principal source of plasma PLP in mammals. PLP is released from hepatocytes into the circulation where $>95\%$ is coupled to albumin (Dolphin *et al.,* 1986). Some additional PLP in plasma is bound to various enzymes; only a small amount of PLP circulates freely.

Similar to many phosphorylated compounds, PLP cannot cross plasma membranes, but first must be dephosphorylated to pyridoxal (PL) before it can enter tissues. Inside cells, PL is rephosphorylated to PLP or converted to pyridoxamine 5′-phosphate. These phosphocompounds act intracellularly as cofactors for a considerable variety of enzymatic reactions. Ultimately, vitamin B_6 is degraded to a 4-pyridoxic acid, primarily in the liver, and is then excreted into the urine (Dolphin *et al.,* 1986; Coburn *et al.,* 2001).

In disorders characterized by elevations in circulating bone and liver TNSALP activity, plasma PLP concentrations are decreased (Anderson *et al.,* 1980). The diminished plasma PLP levels have been attributed to vitamin B_6 deficiency. However, the discovery of elevated plasma levels of PLP in hypophosphatasia explains the reciprocal relationship between PLP concentrations and TNSALP activity. Importantly, hypophosphatasia indicates that such changes may not be physiologically significant because TNSALP acts on extracellular, not intracellular, dephosphorylation of PLP. Cell membranes are impermeable to PLP. The elevated plasma level of PLP in hypophosphatasia does not seem to reflect enhanced PLP synthesis, but failure of PLP hydrolysis outside cells (Whyte *et al.,* 1985). Accordingly, TNSALP (known to be associated with plasma membranes) seems to function as an ectoenzyme (Fedde *et al.,* 1988). Consonant with this conclusion is the observation that patients with hypophosphatasia typically do not have symptoms of vitamin B_6 excess. Peripheral neuropathy, a complication of vitamin B_6 toxicity (Dolphin *et al.,* 1986), is not a feature of this disorder (*see below*). Similarly in hypophosphatasia, there does not seem to be deficiency of vitamin B_6. Stomatitis, dermatitis, peripheral neuritis, anemia, or depression—clinical hallmarks of vitamin B_6 deficiency (Dolphin *et al.,* 1986)—are not characteristics. Furthermore, a variety of biochemical findings indicate that intracellular levels of vitamin B_6 are normal in hypophosphatasia. First, urinary concentrations of 4-pyridoxic acid were unremarkable in all of four patients examined with the childhood form of the disease (Whyte *et al.,* 1988). Second, children with hypophosphatasia respond normally to L-tryptophan loading—a test for vitamin B_6 deficiency (Whyte and Coburn, unpublished observation). Third, in homogenates of TNSALP-deficient fibroblasts obtained from patients with infantile hypophosphatasia, the levels of PLP and the total amount of the various other forms of vitamin B_6 are the same as in controls (Whyte *et al.,* 1986b). Finally, tissues obtained at necropsy from three perinatal cases of hypophosphatasia (plasma PLP concentrations 50- to 900-fold elevated) contained essentially normal levels of PLP, PL, and total vitameric B_6 (Whyte *et al.,* 1988).

Because TNSALP seems to condition dephosphorylation of PLP to PL extracellularly, plasma levels of PL could be low in hypophosphatasia. However, patients with hypophosphatasia usually have normal or somewhat supranormal plasma PL levels (Whyte *et al.,* 1985). Whether extracellular levels of PL are intrinsically normal, or there is sufficient extracellular dephosphorylation of excess PLP to PL to account for the normal vitamin B_6 status, is uncertain.

Of note, vitamin B_6 deficiency has been associated with renal stone disease and epilepsy. Nephrocalcinosis in infants with hypophosphatasia is likely due to hypercalciuria. However, the possibility of altered oxalate metabolism (a consequence of vitamin B_6 deficiency) has not been studied (Dolphin *et al.,* 1986). The epilepsy of severely affected patients may be related to cranial deformity, hemorrhage, periodic apnea, etc. Of interest, however, PEA seemed to cause seizures when given intravenously to a severely affected infant during a study of PEA metabolism (Takahashi *et al.,* 1984). In two patients with perinatal hypophosphatasia and epilepsy, both of whom had plasma PL levels below assay sensitivity, administration of vitamin B_6 as pyridoxine did

not correct the seizure disorder (personal observation). The *tnsalp* knockout mouse model for hypophosphatasia, however, manifests seizures that respond to pyridoxine administration (Waymire *et al.,* 1995; Fedde *et al.,* 1999).

The clinical and biochemical observations concerning vitamin B_6 metabolism in hypophosphatasia indicate an ectoenzyme role for TNSALP (Whyte *et al.,* 1985). Characterization of liver ALP as a plasma membrane-bound glycoprotein, covalently linked to the polar head group of phosphatidylinositol–glycan anchors, is consistent with this hypothesis (Low and Saltiel, 1988). In fact, Fedde and coworkers used cultivated human osteosarcoma cells (Fedde *et al.,* 1988) and dermal fibroblasts from patients with infantile hypophosphatasia (Fedde *et al.,* 1990) exposed to PLP and PEA in the medium to demonstrate that TNSALP is primarily plasma membrane-associated with ectotopography (see below).

Inorganic Pyrophosphate

The discoveries of Russell and colleagues in 1965 and 1971 that PP_i levels are increased in patient urine (Russell, 1965) and plasma (Russell *et al.,* 1971) constitute a tenable explanation for the disordered calcium metabolism and defective skeletal mineralization in hypophosphatasia (Caswell *et al.,* 1991). At low concentrations, PP_i can enhance calcium and P_i precipitation from solution to form amorphous calcium phosphate yet PP_i can prevent transformation of such precipitate to hydroxyapatite crystals. Additionally, absorption of PP_i to hydroxyapatite crystals will impair their growth and dissolution (Caswell *et al.,* 1991).

Caswell and coworkers, using cultivated TNSALP-deficient fibroblasts from perinatal and infantile patients, demonstrated that nucleoside triphosphate pyrophosphatase (NTP-PP_i-ase) activity is unremarkable in hypophosphatasia. Generation of PP_i extracellularly from ATP by these cells is normal (Caswell *et al.,* 1986). Consequently, NTP-PP_i-ase seems to be different from TNSALP. This conclusion is supported by recent studies of osteoblasts from the *tnsalp* knockout mouse (Johnson *et al.,* 2000). Conversely, the clearance of $^{32}PP_i$ administered into the circulation of two adults with hypophosphatasia was markedly delayed, supporting the hypothesis that endogenous accumulation of PP_i reflects defective degradation of PP_i (Caswell *et al.,* 1991).

Consonant with the *in vitro* effects of PP_i, several disturbances of mineralization are observed in hypophosphatasia that probably reflect local concentrations of PP_i together with other factors. Low concentrations of PP_i cause precipitation of amorphous calcium phosphate and may explain the associated calcific periarthritis (Lassere and Jones, 1990). Furthermore, ALP has been shown to dissolve CPPD crystals *in vitro* (Xu *et al.,* 1991). This PP_i-ase activity seems to be unrelated to its capacity to hydrolyze phosphoesters. Thus, CPPD crystal deposition leading to chondrocalcinosis, pseudogout, and pyrophosphate arthropathy would be explained by failure of TNSALP not only to destroy PP_i but to hydrolyze CPPD crystals as well. High concentrations of PP_i inhibit hydroxyapatite crystal formation and growth; rickets or osteomalacia would be the cardinal clinical feature. As discussed, electron microscopy of bone shows aberrations in secondary mineralization of skeletal tissue surrounding matrix vesicles (Anderson *et al.,* 1997).

Circulating TNSALP

Several observations suggest that ALP in the circulation is physiologically inactive (Whyte *et al.,* 1982b). Among the most compelling evidence is the observation that infants with hypophosphatasia who received intravenous infusions of plasma from patients with Paget bone disease, or who were given purified placental ALP (engendering elevated serum ALP levels), demonstrated no clinical or radiographic improvement. In fact, such therapy failed to normalize urinary PEA or PP_i levels or plasma PLP concentrations (Whyte *et al.,* 1982b). Hence, deficiency of TNSALP activity intrinsic to the skeleton seems to account for the rickets and osteomalacia of hypophosphatasia. In fact, in studies reminiscent of Robison's work, Fraser and Yendt reported in 1955 that rachitic rat cartilage would calcify in serum obtained from an infant with hypophosphatasia, yet slices of the patient's costochondral junction would not mineralize in synthetic calcifying medium or in the pooled serum from healthy children (Fraser and Yendt, 1955). Although this observation appears to support an *in situ* role for TNSALP, such models have been criticized as merely reflecting P_i liberation followed by mineral precipitation (Khouja *et al.,* 1990). Experience with marrow cell transplantation for one patient with infantile hypophosphatasia, who had clinical and radiographic improvement despite unaltered biochemical abnormalities, suggests that even small increases in ALP activity within the skeleton can improve mineralization in hypophosphatasia (Whyte *et al.,* 1998). In fact, transfection studies indicate that small differences in ALP activity reflecting various *TNSALP* mutations can account for lethal versus nonlethal hypophosphatasia (Mornet *et al.,* 1998).

Hypophosphatasia Fibroblast Studies

Much of our insight concerning the physiologic role of TNSALP has come from investigation of *patients* with hypophosphatasia. Although dermal fibroblasts are not rich in ALP activity (Fedde *et al.,* 1990; Whyte and Vrabel, 1985; Whyte *et al.,* 1986b), these cells express some TNSALP-like activity which peaks at confluence in culture (Whyte and Vrabel, 1987). Hypophosphatasia fibroblasts in monolayer obtained from severely affected patients are profoundly deficient ($< 5\%$ control) in ALP activity (Whyte *et al.,* 1983). What ALP activity is present seems to be a form of TNSALP (Whyte *et al.,* 1987).

Preliminary studies using patient fibroblasts indicate that phospholipid composition and rates of $^{32}P_i$ accumulation are normal (Tsutsumi *et al.,* 1986; Whyte and Vrabel, 1983). In 1990, Fedde and colleagues demonstrated that TNSALP is present primarily at the surface of these cells (Fedde *et al.,* 1990). The ectophosphatase activity hydrolyzes extracellular PEA and PLP (Fedde *et al.,* 1990). Although some reports suggest that ALP conditions cell growth and differentiation by influencing the phosphorylation of nucleotide pools, hypophosphatasia fibroblasts proliferate at normal rates (Whyte *et al.,* 1983). Furthermore, TNSALP does not seem to be a phosphoprotein phosphatase at plasma membranes (Fedde *et al.,* 1993).

Hypophosphatasia as a Model for TNSALP Function

Observations from hypophosphatasia indicate how TNSALP might act physiologically in man (Fig. 7): (1) Increased endogenous levels of PEA, PP_i, and PLP indicate that TNSALP is catalytically active toward a variety of substrates with fairly variable chemical structure and can act not only as a phosphomonoester phosphohydrolase but also as an inorganic pyrophosphatase (Fig. 7). (2) Because in healthy individuals the putative natural substrates for TNSALP (PEA, PP_i, and PLP) occur in extracellular fluid at nanomolar or micromolar concentrations, TNSALP must act at levels of these compounds that are much lower than the millimolar concentrations of artificial substrates used in routine clinical assays. (3) Clearly, TNSALP must function at physiologic pH. Accordingly, the term "alkaline phosphatase" is a misnomer. (4) Clinical and biochemical investigations of vitamin B_6 metabolism, supported by cell culture studies, reveal that TNSALP acts primarily as an ectoenzyme. Extracellular accumulation of PEA, PP_i, and PLP is a consequence of deficient ecto-TNSALP activity. Increments of membrane-impermeable PLP in plasma, but not in tissues, explains the absence of vitamin B_6 deficiency or toxicity. (5) The origin of plasma PLP appears to be the liver. The source of PEA is unclear, but excesses might reflect incomplete degradation of the phosphatidylinositol–glycan moiety that anchors proteins to cells surfaces. PP_i is formed extracellularly by a separate enzyme, NTP-PP_i-ase. (6) Calcium *phosphate* crystal deposition causes calcific periarthritis and calcium *pyrophosphate* dihydrate crystal formation results in chondrocalcinosis, pseudogout, and/or pyrophosphate arthropathy. Calcific periarthritis reflects the regional effect of PP_i at low concentrations to stimulate amorphous calcium phosphate formation. Rickets and osteomalacia reflect the effect of high extracellular concentrations of PP_i to inhibit hydroxyapatite crystal formation and growth at sites of mineralization. (7) Failure of enzyme replacement therapy by intravenous infusion of various forms of ALP indicates that TNSALP is physiologically active in tissues, but not in the circulation.

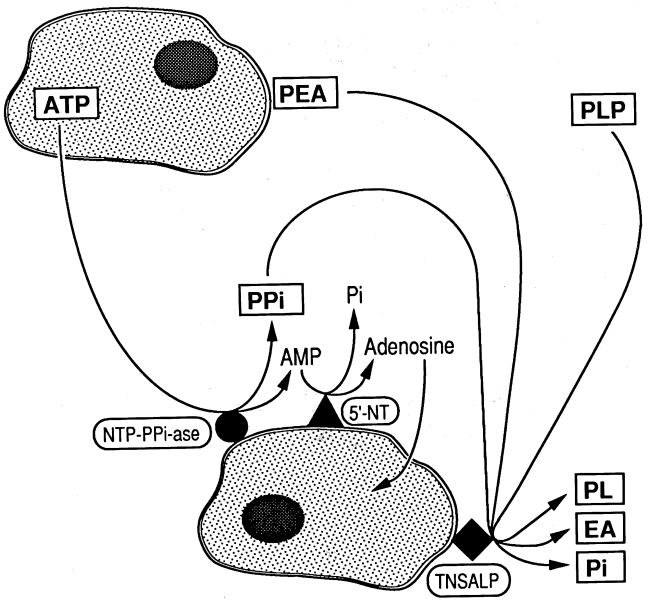

Figure 7 Metabolic basis for hypophosphatasia (hypothesis). Extracellular generation of PPi, presumably by the action of nucleoside triphosphate pyrophosphatase (NTP-PP_i-ase), is normal in hypophosphatasia, but extracellular degradation of PEA, PP_i, and PLP is diminished because of deficient ecto-TNSALP activity. Accumulation of PP_i extracellularly accounts for the CPPD precipitation and associated calcium phosphate crystal deposition. The inhibitory effect of PP_i on hydroxyapatite crystal growth accounts for the rickets/osteomalacia. [Reproduced with permission from Whyte, M. P. Hypophosphatasia. *In* "The Metabolic and Molecular Bases of Inherited Disease" (C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, eds.), 2001, 8th ed., pp. 5313–5329. McGraw-Hill, New York.]

Conclusions

Hypophosphatasia is a rare but remarkably instructive inborn error of metabolism that establishes a critical role for ALP in mineralization of the skeleton and formation of the dentition in humans. Subnormal serum ALP activity (hypophosphat*asemia*), the biochemical hallmark, reflects a generalized deficiency of activity of the tissue-*nonspecific* ("liver / bone / kidney") ALP isoenzyme (TNSALP). Activities of the three tissue-specific ALP isoenzymes in humans—intestinal, placental, and germ cell (placental-like) ALP—are not diminished.

Hypophosphatasia is characterized clinically by defective skeletal mineralization that manifests as rickets in infants and children and osteomalacia in adults. Clinical expressivity is, however, extremely variable. Stillbirth can occur from *in utero* onset in the perinatal form, which is apparent in newborns and is associated with the most severe skeletal hypomineralization and deformity as well as epilepsy in some patients. The infantile form presents as through a developmental disorder by age 6 months. It can cause craniosynostosis and nephrocalcinosis from hypercalcemia and hypercalciuria and is often fatal due to worsening rachitic disease. Premature loss of deciduous teeth, rickets, and muscle weakness are the cardinal clinical features of childhood hypophosphatasia. Adult hypophosphatasia typically results in

recurrent metatarsal stress fractures and pseudofractures in long bones of the lower limbs and occasionally manifests with arthritis from CPPD crystal deposition and rarely with calcific periarthritis from precipitation of calcium phosphate. Odontohypophosphatasia refers to especially mild disease with dental but no skeletal manifestations. Pseudohypophosphatasia is an extremely rare variant that resembles infantile hypophosphatasia except that serum ALP activity is normal in routine clinical assays.

Perinatal and infantile hypophosphatasia are transmitted as autosomal recessive traits and can be due to homozygosity or compound heterozygosity for a great number of different mutations in *TNSALP* that affect the structure of the encoded enzyme. Most are missense mutations. Individuals with even the mildest forms of hypophosphatasia can also be compound heterozygotes for *TNSALP* defects. In some kindreds, however, mild disease is transmitted as an autosomal dominant trait.

There is no established medical treatment for hypophosphatasia. Enzyme replacement therapy by intravenous infusion of ALP from various tissues has generally been disappointing, suggesting that circulating TNSALP is physiologically inactive. Marrow cell transplantation has been followed by considerable clinical and radiographic improvement, without biochemical changes, in one severely affected infant.

Prenatal diagnosis of *perinatal* hypophosphatasia has been successful. During the second trimester of pregnancy, assay of ALP activity in amniotic fluid cells, as well as fetal ultrasonography or radiography have proven helpful. During the first trimester, chorionic villus samples have been used for quantitation of TNSALP activity. Now, molecular studies of *TNSALP* are available, but must deal with a considerable number of different *TNSALP* mutations.

Three phosphocompounds [phosphoethanolamine (PEA), inorganic pyrophosphate (PP_i), and pyridoxal 5′-phosphate (PLP)] accumulate endogenously in hypophosphatasia and are inferred to be natural substrates for TNSALP. Preliminary studies using ^{31}P-NMR spectroscopy indicate that there are at least several more, but their identities have not yet been characterized. A variety of evidence shows that PLP, a cofactor form of vitamin B_6, collects extracellularly; intracellular levels of PLP are normal. This observation explains the absence of symptoms of deficiency or toxicity of vitamin B_6 and indicates that TNSALP functions as an ectoenzyme. Extracellular accumulation of PP_i, which at low concentrations promotes calcium phosphate deposition yet at high concentrations acts as an inhibitor of hydroxyapatite crystal growth, seems to explain the associated calcific periarthritis as well as the CPPD crystal deposition and defective mineralization of bones and teeth.

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CHAPTER 71

Paget's Disease of Bone

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The Patient

Paget's disease is a common disorder of the skeleton that is local in nature and extremely variable in its clinical manifestations (Singer and Krane, 1998). The majority of patients are without symptoms. When symptoms are present, skeletal deformity and pain are most common. Deformities are usually most apparent in the skull, face, and lower extremities. Pain is of several origins. Localized bone pain is surprisingly uncommon, but joint pain (hips and knees) is seen not infrequently due to degenerative arthritis. Pain of neural or spinal origin is unusual but the most severe.

A variety of complications may first bring the patient to medical attention. The complications depend both on the affected skeletal sites and the overall extent of the disease (six bones are commonly involved). Patients with Paget's disease in the skull often develop a hearing deficit if the temporal bone is affected. Massive enlargement of the cranium is associated with basilar impression and neurological impairment. Vertebral involvement may produce compression fractures, spinal stenosis, neurological impairment, and degenerative arthritis. Paget's disease in the pelvis and femurs commonly is associated with degenerative arthritis of the hips. Involvement of the femur and tibia may lead to pathological fractures of these long bones. Nonunion of a femoral fracture is relatively common. Degenerative arthritis in the knees is also a common feature when lower extremity long bones are extensively involved by Paget's disease. The most serious complication is the development of a sarcoma that fortunately occurs in only $<1\%$ of patients. It always arises in a pagetic lesion and not in unaffected bone. Systemic complications of Paget's disease generally occur with more extensive disease. Hypercalcemia, preceded by hypercalcuria, usually is noted with total bed rest and is related to the accelerated bone resorption induced by immobilization. Increased cardiac output, and less commonly, congestive heart failure, are a consequence of the great vascularity of bones affected by Paget's disease. Hyperuricemia has been observed in males with extensive disease and may reflect increased purine turnover.

Recent evidence suggests that Paget's disease occurs much more commonly in families than was previously reported. An extensive investigation of the relatives of 35 patients with Paget's disease in Madrid revealed that 40% of the patients had at least one first-degree relative with the disease (Morales-Piga *et al.,* 1995). All studies of familial Paget's disease suggest an autosomal dominant inheritance. There is considerable evidence for a predisposition gene on the long arm of chromosome 18 (Cody *et al.,* 1997; Haslam *et al.,* 1998). There is also evidence for genetic heterogeneity in that a majority of families with Paget's disease do not show linkage of the disease to markers on chromosome 18 (Hocking *et al.,* 2000). As yet, no predisposition gene has been identified in familial Paget's disease, but families with a rare variant of Paget's disease (Osterberg *et al.,* 1988) exhibit mutations in the gene coding receptor activator of nuclear factor-κ B (RANK) (Hughes *et al.,* 2000). This gene is located on 18*q* in the same region as the predisposition gene for Paget's disease, but mutations in the RANK gene have not been reported in patients with familial or sporadic Paget's disease.

Radiology and Nuclear Medicine

The diagnosis of Paget's disease is primarily accomplished by roentgenographic evaluation of the skeleton. Over the years it has become clear that the disease evolves through several stages as observed by serial roentgenograms.

The initial stage of the disease is represented by a localized area of reduced bone density often referred to as an osteolytic lesion. This is most readily detected in the skull where it is found as a discrete round or oval lesion in the frontal or occipital bones. It is called *osteoporosis circumscripta.* Paget's disease in the long bones almost always begins in the subchondral region of either epiphysis (uncommonly both may be affected simultaneously). The osteolytic process has then been seen to advance proximally or distally at ~1 cm/year in the untreated patient. The advancing front usually has a V-shaped or arrowhead appearance.

In the most advanced stage of Paget's disease, the areas of previous osteolytic dominance now are characterized by a chaotic sclerotic appearance, a phase that is called osteoblastic or osteosclerotic. In long bones the osteolytic phase is commonly seen preceding the osteosclerotic region when much of the bone has been affected by the disease. Another feature of this phase is considerable thickening of the sclerotic bone, which can reach monumental proportions in the skull. Osteolytic activity of a secondary nature often can be observed as clefts in the thickened bone. It is likely that the evolution of the disease into its most severe form occurs over much of the life span of the patient. Two other features of the disease have been noted through serial roentgenographic observations. Although the disease can slowly course through an entire bone, it does not cross a joint space to affect an adjacent bone. Also it is extremely rare for new lesions of Paget's disease to be detected at any site in the skeleton after the diagnosis and extent of the disease has been determined initially.

Bone scans are the most sensitive means of detecting pagetic lesions. Radiolabeled bisphosphonates accumulate in regions where blood flow and bone formation are increased and can outline early lesions that are not detectable on roentgenograms. Radioactive gallium can also define areas of Paget's disease activity because of uptake of gallium by osteoclasts.

Histopathology

Beginning with the observations of Schmorl in 1932, it has become appreciated that the osteoclast is the dominant cell in the pathogenesis of Paget's disease. Osteoclasts are increased in number in the Haversian canals of the cortex in the absence of other abnormalities and are in great number at the leading edge of the osteolytic front. The osteoclasts in Paget's disease may be far greater in size than osteoclasts in normal bone and contain as many as 100 nuclei in a cross section as compared with two or three nuclei in a normal osteoclast. Osteoclasts of Paget's disease have a characteristic ultrastructural abnormality (Gherardi *et al.,* 1980; Howatson and Fornasier, 1982; Mills and Singer, 1976; Rebel *et al.,* 1974). This consists of microfilaments, sometimes grouped in a paracrystalline array, located in the nucleus and sometimes in the cytoplasm of osteoclasts (Fig. 1). These microfilaments are not seen in nonpagetic bone or bone marrow cells. These inclusions closely

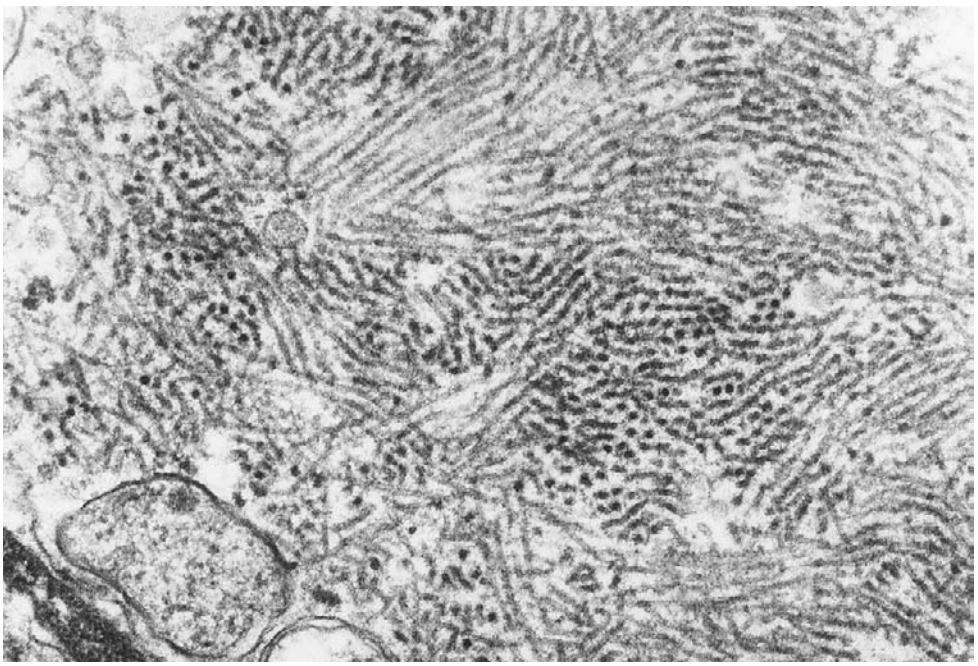

Figure 1 Electron micrograph of a nuclear inclusion in an osteoclast of a patient with Paget's disease. The microfilaments are seen both in longitudinal array and in paracrystalline array in cross section. Provided by Dr. Barbara G. Mills.

resemble nucleocapsids of viruses of the Paramyxoviridae family, a group of RNA viruses responsible for some of the most common childhood diseases. Despite the finding of these inclusions in the vast majority of patients studied, the budding off of an infectious virus from the osteoclasts has rarely been observed (Abe *et al.,* 1995).

Osteoblasts are another prominent feature of the cellular pathology of Paget's disease. Large numbers of osteoblasts are often found near areas of resorbed bone and may even be prominent in a lesion that appears purely osteolytic by X-ray. The osteoblasts are usually prism-shaped or polyhedral and contain abundant rough endoplasmic reticulum, mitochondria, and a well-developed Golgi zone. These signs of cellular activity are consistent with the increased bone formation in active lesions established by the use of double labeling with tetracycline.

In addition to the increased numbers of osteoclasts and osteoblasts, the marrow of pagetic lesions tends to be grossly abnormal. The normal hematopoietic elements are usually absent and replaced by mononuclear cells of indeterminate origin intermixed with highly vascular connective tissue.

The bone matrix in Paget's disease is highly abnormal in structure and arises as a consequence of disordered bone resorption and formation. The matrix consists of a "mosaic" of irregularly shaped pieces of lamellar bone with an erratic pattern of cement lines. The matrix is interspersed with numerous foci of woven bone, which, in adults, is ordinarily found associated with fracture healing.

Biochemistry

The biochemical findings in Paget's disease help to provide an integrated assessment of the cellular events occurring throughout the skeleton of affected patients.

Historically, the earliest index of bone matrix resorption was measurement of urinary hydroxyproline excretion while ingesting a low-gelatin diet. This index is well correlated with the extent of the disease despite the fact that hydroxyproline is a prominent component of extraskeletal connective tissue as well as skeletal collagen. Measurement of collagen cross-link degradation products in urine provides more specific measurements of skeletal matrix. Urinary *N*-telopeptide, pyridinoline, and deoxypyridinoline have all been reported to be more specific indices of skeletal matrix resorption and are not influenced by dietary gelatin (Alvarez *et al.,* 1995; *Garnero et al.,* 1994).

Serum tartrate-resistant acid phosphatase, presumably released by osteoclasts, appears to be another index of bone resorption in Paget's disease but is not routinely available (Kraenzlin *et al.,* 1990).

Osteoblast activity can be assessed by measurement in the serum of total alkaline phosphatase activity, bone-specific alkaline phosphatase activity, osteocalcin concentration, and type 1 carboxy-terminal procollagen peptide concentration. The most useful of these serum markers are the total alkaline phosphatase and bone-specific alkaline phosphatase (Alvarez *et al.,* 1995; Garnero and Delmas, 1993). For reasons that are not understood, serum osteocalcin is a poor index of disease activity; similarly, the type I carboxy-terminal procollagen assay is insensitive to increases in bone turnover and does not change reproducibly as the disease is suppressed by medical therapy.

Therapy

Salmon calcitonin by injection and etidronate disodium by the oral route are both effective medications for Paget's disease. They have been in use for ~20 years. They generally suppress biochemical parameters of the disease by 50%. Intravenous pamidronate disodium, oral alendronate, and risedronate sodium are more recent, more potent bisphosphonates that can reduce bone turnover to normal in the majority of patients with Paget's disease (Singer and Krane, 1998).

Surgery is sometimes used in Paget's disease in patients with associated degenerative arthritis of the hip (total hip replacement) and of the knee (high tibial osteotomy). Orthopedic and/or neurosurgical procedures may occasionally be necessary after fractures and when skull or vertebral complications are present.

Evidence for the Presence of Paramyxoviruses in Paget's Disease

In early studies, Mills and colleagues attempted to rescue an infectious virus from cells cultured from surgical specimens of Paget's disease (Mills and Singer, unpublished observations; Mills *et al.,* 1979). After trypsinization or after growing cells from explants of pagetic bones, cells were cultivated for months and then cocultivated with cell lines used to isolate Paramyxoviridae viruses. These studies failed to demonstrate an infectious virus in pagetic bone. Perhaps this is not surprising since little anatomic evidence of an infectious virus has been found in any specimen of Paget's disease, and the mononuclear cells cultured from surgical specimens rarely were found to exhibit nuclear inclusions. Mills and colleagues (1985) also attempted to develop an animal model of Paget's disease by injecting crushed bone extracts or lysates from cells cultured from pagetic bone into the tibiae of athymic nude mice. Nothing resembling Paget's disease was induced in the mouse bone, although one cell line did produce an osteosarcoma-like lesion reproducibly.

Despite the failure to find an infectious (mature) virus in specimens of Paget's disease, abundant evidence has been generated using immunohistological and molecular biological techniques that indicate the presence of viral antigens and mRNA in Paget's disease. Initially Rebel and colleagues (1980a) used a variety of antisera directed against measles virus to demonstrate measles antigen in the osteoclasts of 20 patients with Paget's disease, but not in one patient with fluorosis and another with a healing fracture. Positive results

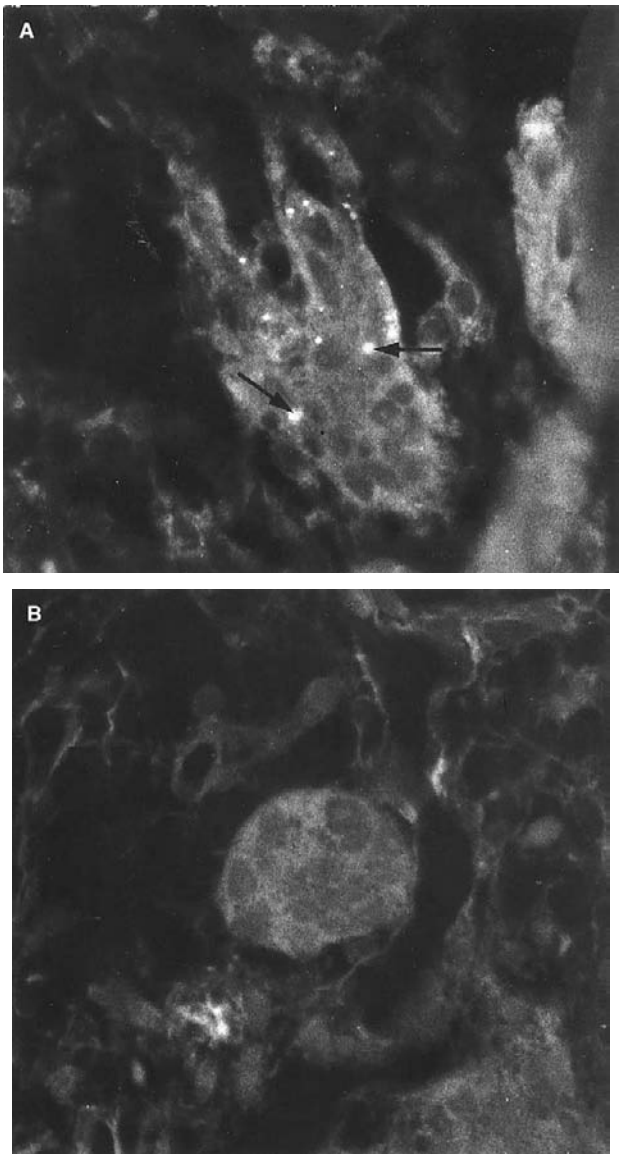

Figure 2 (A) A section of decalcified pagetic bone incubated with rabbit anti-LEC serum followed by goal antiserum coupled to fluorescein isothiocyanate. The LEC strain of measles virus was isolated from a patient with SSPE. The osteoclast shows a strong positive punctate (arrows) fluorescent reaction. (B) A section of decalcified pagetic bone was treated as in (A) except nonimmune rabbit serum was used instead of anti-LEC serum. There is no fluorescent reaction. Reproduced with permission from Rebel *et al.,* 1980b.

were obtained with both indirect immunofluorescence (Fig. 2) and immunoperoxidase techniques (Rebel *et al.,* 1980b). Mills and colleagues (1980, 1981) initially reported that cell cultures from pagetic bone, as well as bone sections from 12 patients, were positive when stained with an antiserum against respiratory syncytial virus but not with antisera against measles virus, parainfluenza viruses, influenza A and B, rubella, mumps, and herpes simplex. Subsequently, Mills and colleagues (1984) did observe that measles virus nucleocapsid protein antigens were present in the osteoclasts and/or cultured bone cells of most patients with Paget's disease. Of particular interest was their finding that in serial sections of pagetic bone, both measles virus and respiratory syncytial virus nucleocapsid antigens were demonstrable in the same osteoclasts. This observation could not be explained by cross-reactivity of the measles virus monoclonal antibodies used in this study with respiratory syncytial virus antigens and vice versa. In other immunohistological studies, Basle and colleagues (1985) demonstrated not only measles virus antigens in 6 of 6 specimens of Paget's disease but also simian virus 5 in 6 of 6 and parainfluenza 3 in 3 of 6. No reactivity with respiratory syncytial virus, mumps virus, influenza virus, or adenovirus type 5 antibodies was found in this study. Subsequently, Basle did find a positive response with a monoclonal antibody against respiratory syncytial virus provided by Mills (pers. commun.). He also noted the presence of measles virus and respiratory syncytial virus antigens in the same osteoclasts on serial sections of pagetic bone. Recently Mills and colleagues (1994) have studied immunohistological staining of long-term cultures of marrow from patients with Paget's disease and from normal subjects with respect to viral antigens. In 12 of 12 patients, measles virus and/or respiratory syncytial virus nucleocapsid antigens were found in 40–50% of the mononuclear cells and/or multinucleated cells in culture. In the control subjects, $< 5\%$ of cells were positive. Reddy and colleagues (1995) found measles virus transcripts in peripheral blood mononuclear cells in 4 of 5 patients and 0 of 10 normal subjects.

In the past 15 years, the techniques of molecular biology have increasingly been applied to the issue of the identity of the osteoclast inclusions of Paget's disease. In the first study, Basle and colleagues (1986) used *in situ* hybridization with a cloned measles virus cDNA probe specific for the nucleocapsid protein to search for measles virus RNA sequences in the bone of five patients. These sequences were detected in 80–90% of the osteoclasts in these specimens. Surprisingly, 30–40% of the mononuclear cells in these bone specimens also had detectable mRNA sequences of measles virus nucleocapsid protein. Evidence of the measles virus was found in osteoblasts, osteocytes, fibroblasts, lymphocytes, and monocytes. Negative results were obtained in three subjects who had fluorosis, fracture healing, and hyperparathyroidism, respectively.

Because an epidemiological study in England indicated that patients with Paget's disease were more likely to have had a pet dog in the past (O'Driscoll and Anderson, 1985), Gordon and colleagues (1991) used *in situ* hybridization to examine the possibility that canine distemper virus might be involved in Paget's disease. Canine distemper virus is a paramyxovirus with considerable structural homology to measles virus. Bone biopsies from 27 patients and 6 patients with other bone disorders (primary hyperparathyroidism, prostatic carcinoma, osteoporosis, osteomalacia, and fracture healing) were studied. Sense and antisense RNA probes to the nucleocapsid and fusion genes of the canine distemper virus were used as well as RNA probes to mRNA sequences of measles virus nucleocapsid protein. There was no demonstrable cross-reactivity of these probes with the heterologous

virus under the experimental conditions used by the investigators. Eleven of 25 patients showed hybridization with the antisense but not the sense canine distemper virus fusion protein probe. Ten of 26 patients also showed hybridization with the antisense but not the sense probe for canine distemper virus nucleocapsid protein. The canine distemper virus probes produced hybridization in 80% of multinucleated osteoclasts, 60% of osteoblasts, and in osteocytes as well as marrow mononuclear cells (monocytes, lymphocytes). No significant hybridization was found with the measles virus probe, and none of the control specimens reacted with any of the above probes. In a subsequent study, Gordon and colleagues (1992) obtained pagetic bone for RNA extraction, reverse transcribed the RNA, and specifically amplified for canine distemper virus and measles virus sequences using the polymerase chain reaction (PCR) technique. They found that 8 of 13 patients had canine distemper virus nucleic acid sequences, and 1 of 10 patients had measles virus nucleic acid sequences in the bone specimens. One patient had both. Dideoxy sequencing of the canine distemper virus PCR products revealed 2% base pair (bp) changes in a 187-bp fragment from within position 1231–1464 of the nucleocapsid gene as compared with the Onderstpoort strain of canine distemper virus. Further support of the hypothesis that canine distemper virus could be involved in the pathogenesis of Paget's disease was sought by examining the bones of dogs with distemper infections (Mee *et al.,* 1992). In 2 of 4 dogs studied, *in situ* hybridization of metaphyseal specimens revealed strong signals with sense and antisense probes for the nucleocapsid and phosphoprotein genes. The osteoclasts were strongly positive but osteoblasts, osteocytes, and bone marrow cells were also positive. Mee and colleagues (1993) also have found canine distemper virus transcripts in the bone cells of dogs with metaphyseal osteopathy. This is an acute disorder affecting young rapidly growing dogs whose signs include fever, anorexia, and painful swollen metaphyses. The histology does not resemble Paget's disease. Although the presence of canine distemper virus transcripts in dogs with distemper or metaphyseal osteopathy does not relate directly to Paget's disease, it does indicate that a paramyxovirus can infect mammalian bone cells. This has also been demonstrated *in vitro* by incubating canine distemper virus with canine bone marrow (Mee *et al.,* 1995).

Additional evidence of paramyxovirus nucleocapsid transcripts in Paget's disease has come from the studies of Reddy and colleagues. They studied bone marrow mononuclear cells obtained from aspirations of the iliac crests of 6 patients with radiologically demonstrable Paget's disease and from the aspirations of 10 normal subjects (Reddy *et al.,* 1995). Using the reverse transcriptase–PCR techniques, they observed that 5/6 patients had measles virus nucleocapsid transcripts, whereas none of the 10 normal subjects had detectable transcripts. Dideoxy sequencing of the PCR fragments revealed several mutations within the position 1360–1371 bp of the nucleocapsid gene as compared with the Edmonston strain of measles virus. These mutations are in the same region as the mutations reported in the canine distemper virus nucleocapsid gene. Since granulocyte macrophage colony-forming units (CFU-GM), the most likely osteoclast precursors, circulate in the peripheral blood, Reddy and colleagues (1996) examined peripheral blood samples for the presence of measles virus nucleocapsid transcripts by reverse transcriptase-PCR in Paget's disease and control subjects. In 9 of 13 patients, measles virus transcripts were detected. They were localized to peripheral blood monocytes (whose precursor is CFU-GM) by *in situ* hybridization. Studies were negative in 10 control subjects.

Three studies from the United Kingdom and one United States study have produced negative results with respect to detection of Paramyxoviridae mRNA in Paget's disease. In one study, RNA extracts of 10 bone specimens failed to exhibit measles virus, canine distemper virus, respiratory syncytial virus, or parainfluenza 3 virus transcripts after reverse transcriptase-PCR evaluation (Ralston *et al.,* 1991). In a second study, both bone cells cultured from pagetic explants and bone biopsies were studied similarly by reverse transcriptase-PCR techniques for the presence of measles virus and canine distemper virus transcripts (Birch *et al.,* 1994). Completely negative results were obtained. A second United Kingdom study using the same primers also failed to find measles virus or canine distemper virus transcripts in long-term bone marrow cultures from lesions of Paget's disease (Ooi *et al.,* 2000). In the United States study, Nuovo and colleagues (1992) also could not detect measles virus-specific cDNA in pagetic specimens using PCR and *in situ* hybridization in combination. The explanation for the disparate results may reflect the techniques used by these investigators. They did not use secondary PCR to try to demonstrate the presence of paramyxovirus nucleocapsid mRNA and may have used inappropriate primers. Measles virus transcripts in Paget's patients are in extremely low abundance, so that secondary PCR and very stringent PCR conditions and primers are required to demonstrate viral mRNAs.

Several studies have addressed the levels of circulating antibodies against various paramyxoviruses in patients with Paget's disease. Antibody titers have not been found to be greater in Paget's disease than in control subjects (Basle *et al.,* 1983; Gordon *et al.,* 1993: Pringle *et al.,* 1985).

Cellular and Molecular Biology of Paget's Disease

The development of *in vitro* techniques for the study of the ontogeny of human osteoclasts has made it possible to gain new insights into the pathogenesis of Paget's disease. Kukita and colleagues (1990) first established long-term cultures of marrow from involved bones from patients with Paget's disease and noted that the multinucleated cells that formed shared many of the characteristics of pagetic osteoclasts. As compared with osteoclast-like cells formed in normal marrow cultures, the pagetic osteoclast-like cells formed more rapidly and in much greater numbers (10- to 100-fold greater), had increased numbers of nuclei and had higher levels of tartrate-resistant acid phosphatase. Examination of these cells by electron microscopy did reveal many features

of osteoclasts found in pagetic bone biopsies, but the characteristic nuclear and cytoplasmic inclusions were not observed. As previously mentioned, the antigens of measles virus and respiratory syncytial virus nucleocapsids were detectable in these cells (Mills *et al.,* 1994); apparently the nucleocapsid structures do not form in this *in vitro* setting.

Because the increased numbers of osteoclasts in pagetic lesions are of obvious importance in the pathogenesis of the disease, it seemed logical to examine osteoclast precursors in the marrow aspirates to determine if they were abnormal or whether other cells in the marrow microenvironment were participants in the pathology. Demulder and colleagues (1993) examined CFU-GM in cultures of unfractionated marrow mononuclear cells and found that CFU-GM colony formation was significantly increased compared with that of normal cells. Using an antibody that recognizes the CD34 antigen present on most hematopoietic precursors, they also isolated enriched hematopoietic precursors and found similar numbers of osteoclast precursors in pagetic and normal marrow aspirations. Subsequent coculture experiments with highly purified hematopoietic precursors ($CD34^+$ cells) and nonhematopoietic marrow accessory cells ($CD34^-$ cells) demonstrated that the growth of pagetic precursors was significantly enhanced by both normal and pagetic $CD34^-$ cells. CFU-GM colony formation was also significantly increased when normal $CD34^+$ cells were cocultured with pagetic, but not normal, $CD34^-$ cells. CFU-GM colony-derived cells from pagetic patients also formed osteoclast-like multinucleated cells with 1,25-dihydroxyvitamin D_3 ($1,25\text{-}(OH)_2D_3$), concentrations 1/10th of that required for normal multinucleated cell formation. Thus, these experiments suggest that osteoclast precursors are abnormal in Paget's disease and that other cells in the pagetic marrow microenvironment may stimulate the growth and differentiation of these abnormal precursors (Fig. 3). Recently, Menaa and coworkers (2000a) have extended the original studies of Demulder *et al.* (1993) to further understand the enhanced sensitivity of osteoclast precursors from patients with Paget's disease to the marrow microenvironment and the enhanced osteoclastogenic potential of the marrow microenvironment from Paget's patients. Menaa and coworkers (2000a) showed that pagetic osteoclast precursors are hyperresponsive to RANK ligand, a recently described member of the tumor necrosis factor (TNF) gene family, which is absolutely required for osteoclast formation. The increased sensitivity to RANK ligand was due to the additive effects of interleukin-6 produced by the pagetic marrow and RANK ligand on osteoclast formation. Furthermore, Menaa and coworkers showed that marrow stromal cells from patients with Paget's disease expressed higher levels of RANK ligand than normal marrow stromal cells. Thus, in pagetic lesions the osteoclast precursors are hyperresponsive to RANK ligand, and increased amounts of RANK ligand are expressed in the marrow microenvironment, further enhancing the osteoclastogenic potential of the pagetic lesion. In areas of bone not affected by Paget's disease, enhanced expression of RANK ligand was not detected.

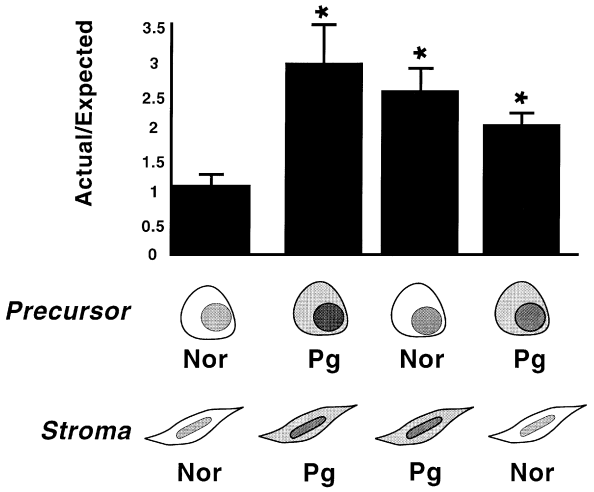

Figure 3 Summary of coculture studies using highly purified osteoclast precursors from normal and pagetic patients and stromal cells from normal and pagetic patients. Coculture of pagetic osteoclast precursors with normal or pagetic stromal cells produces a significantly increased number of CFU-GM-derived colonies over the expected number. When highly purified normal osteoclast precursors are cocultured with normal stromal cells, the expected number of CFU-GM-derived colonies are formed. In contrast, when normal osteoclast precursors are cocultured with pagetic marrow stromal cells, increased CFU-GM-derived colonies are formed. These data suggest that pagetic marrow stromal cells can increase the growth of both normal and pagetic osteoclast precursors and that the pagetic stromal cells can enhance the growth of normal osteoclast precursors above expected levels. The results are presented as the ratio of actual over the expected number of CFU-GM colonies formed. The asterisk denotes a significant difference from the normal cell combination. Adapted from Demulder *et al.,* 1993.

As noted above, a strong candidate for a significant autocrine/paracrine factor involved in the increased osteoclast formation in Paget's disease is interleukin-6. Roodman and colleagues (1992) found that conditioned media from long-term pagetic marrow cultures increased multinucleated cell formation in normal marrow cultures, and antibodies to interleukin-6 blocked the stimulatory activity. Antibodies to interleukin-1, GM-CSF, and TNF-α had no effect on the stimulatory activity. *In situ* hybridization studies demonstrated that the multinucleated cells in the pagetic marrow cultures were actively transcribing interleukin-6 mRNA. In addition, bone marrow plasma samples obtained from sites of Paget's disease had increased levels of interleukin-6 in 9 of 10 patients as compared with samples from normal subjects. Peripheral plasma also had elevated interleukin-6 levels in 17 of 27 patients. In another study, basal plasma interleukin-6 activity was increased in 19 of 22 patients (Schweitzer *et al.,* 1995). The concept that interleukin-6 may be an important autocrine/paracrine factor in Paget's disease is also supported by the studies of Hoyland and colleagues (1994), who used *in situ* hybridization to localize the expression of interleukin-6, interleukin-6 receptor, and interleukin-6 transcription factor in the bone of patients with Paget's disease in comparison with those with osteoarthritis. The osteoblasts in both disorders expressed all three mRNAs, but in Paget's disease interleukin-6 and its receptor mRNA showed higher levels of expression. In the

osteoclasts of both disorders, the receptor and transcription factor were expressed, but only in Paget's disease was interleukin-6 mRNA expressed in osteoclasts. Hoyland and Sharpe (1994) also examined the expression of c-*fos* protooncogene in the bone of 6 patients with Paget's disease by *in situ* hybridization. c-*fos* has been found to be important in the regulation of osteoclasts and was markedly upregulated in pagetic osteoclasts and, to a lesser extent, in the osteoblasts. It is possible that this is a consequence of interleukin-6 action (Korholz *et al.,* 1992).

Ralston and colleagues (1994) also studied cytokine and growth factor expression in bone explants of Paget's disease and in control subjects (postmenopausal women with and without osteoporosis and young bone graft patients) and could not find differences between pagetic and nonpagetic bone. Interleukin-6 mRNA was not detected in 40% of the pagetic specimens from severely affected individuals. There is no obvious explanation for their nonconfirmatory data.

Another abnormality of pagetic osteoclast precursors is that they are hyperresponsive to 1,25-$(OH)_2D_3$. Osteoclast precursors form osteoclasts *in vitro* with concentrations of 1,25-$(OH)_2D_3$ that are 1–2 logs less than that required to induce osteoclast formation by normal osteoclast precursors (Kukita *et al.,* 1990). Menaa and coworkers (2000b) have shown that this increased sensitivity to 1,25-$(OH)_2D_3$ is not due to increased numbers of vitamin D receptors in pagetic osteoclast precursors or to mutations in the vitamin D receptor. Recently, Kurihara and colleagues (2000a) have used a GST–vitamin D receptor fusion protein to further examine the increased sensitivity of pagetic osteoclast precursors to 1,25-$(OH)_2D_3$. These workers found that TAFII-20, a component of the TFIID transcription complex, was increased in osteoclast precursors from patients with Paget's disease compared to normals. This increase in expression of TAFII-20 did not require treatment of the cells with 1,25-$(OH)_2D_3$. These data suggest that pagetic osteoclast precursors express higher levels of TAFII-20 or have increased levels of a coactivator which can bind to TAFII-20 and the vitamin D receptor in the presence of lower concentrations of 1,25-$(OH)_2D_3$ to initiate transcription of VDR responsive genes.

The Etiology of Paget's Disease

As has been reviewed, Paget's disease is a focal disorder that often occurs in multiple family members. In most affected individuals, it progresses slowly over many years without extending to new sites of involvement. The underlying pathophysiology appears to reflect a localized increase in the numbers of osteoclasts followed by a secondary increase in osteoblastic activity. The osteoclasts have striking characteristics by both light and electron microscopy. In Paget's disease the osteoclasts may be far greater in size than osteoclasts in normal individuals or in patients with diseases in which osteoclasts are activated such as primary hyperparathyroidism. A correlate of this observation is the increased number of nuclei noted in the pagetic osteoclast. The nuclei and cytoplasm harbor microfilamentous inclusions that are structurally identical to the nucleocapsids of viruses of the Paramyxoviridae family. However, the structure of a mature Paramyxoviridae virus has rarely been reported in bone biopsy or cell culture specimens. Immunohistological and molecular techniques have indicated the presence of Paramyxoviridae proteins and mRNA in patients with Paget's disease. Recently, Kurihara and coworkers (2000b) have tried to determine the pathophysiologic role of the measles virus nucleocapsid gene in abnormal osteoclast activity in Paget's disease. These workers transfected normal human osteoclast precursors with the measles virus nucleocapsid gene or the matrix gene. They showed that cells transfected with the nucleocapsid gene but not the matrix gene formed osteoclasts that had many of the features of pagetic osteoclasts. The multinucleated cells that formed in these cultures were markedly increased in number and size, had increased numbers of nuclei per osteoclast, had increased bone-resorbing capacity, and were hyperresponsive to 1,25-$(OH)_2D_3$. All these are features of pagetic osteoclasts. Furthermore, these osteoclasts produced increased amounts of interleukin-6 compared to normal osteoclasts formed in marrow cultures. Similarly, Reddy and coworkers (1999b, 2000a) have produced transgenic mice in which the measles virus receptor, CD46, is targeted to cells in the osteoclast lineage. These workers have shown that bone marrow cells from these animals, when infected with measles virus, form osteoclasts which have the features of pagetic osteoclasts. Taken together, these recent studies support a pathophysiologic role for paramyxovirus in the abnormal osteoclast in Paget's disease and implicate the nucleocapsid gene as a potential mediator of these effects. Finally, interleukin-6 has been implicated as a potential important mediator of osteoclast function in Paget's disease. Based on this information, it seems reasonable to propose the hypothesis that Paget's disease represents a slow virus infection of bone.

Slow (or persistent) viral infections of the nervous system have been studied since the mid-1960s when Bjorn Sigurdsson, an Icelandic veterinary virologist, identified several viral disorders in sheep which exhibited a prolonged incubation period before the occurrence of symptoms. In man, the best studied disorder of this type is subacute sclerosing panencephalitis (SSPE). A small percentage of children experience an often fatal neurological syndrome ~5 years after a classical measles infection (ter Meulen *et al.,* 1983). It proved quite difficult to rescue an infectious virus from brain specimens from patients with SSPE although nucleocapsid-like structures similar to those in Paget's disease were abundant. Persistent infection of human glioma cell lines with measles virus has been shown to result in the induction of interleukin-6 (Schneider-Schaulies *et al.,* 1993). This observation may be relevant to Paget's disease. Examination of measles virus genes from a brain specimen of an SSPE patient revealed that nearly 2% of the nucleotides were mutated during persistence and that 35% of these mutations resulted in amino acid changes (Cattaneo *et al.,* 1988). This also appears analogous to findings in Paget's disease.

The existence of persistent viral infections of the nervous system provides a model for pursuing a similar pathogenesis of Paget's disease. An acute viral infection with one of several paramyxoviruses could result in the establishment of a persistent viral infection in one or more bone and/or bone marrow cells, particularly in genetically susceptible individuals. This could trigger the initial osteoclastic stimulus with interleukin-6 as an important intermediary. Subsequently, the normal osteoblastic response to bone resorption may become overzealous in response to the continuing pathologic osteoclastic activity.

Many issues need to be resolved before the viral hypothesis of Paget's disease can be accepted. The characteristic osteoclast nuclear and cytoplasmic inclusions of Paget's disease have also been noted in some patients with giant cell tumors of bone (Schajowicz *et al.,* 1985), pycnodysostosis (Beneton *et al.,* 1987), osteopetrosis (Mills *et al.,* 1988), and primary oxalosis (Bianco *et al.,* 1992). Does this mean that the inclusions are merely incidental findings in all these disorders or in the appropriate genetic setting does a persistent viral infection trigger the pathology?

It is of considerable importance to determine the complete sequences of the Paramyxoviridae proteins and genes found in the pagetic specimens. This would clarify whether there were two viruses in many lesions, whether gene recombination occurs, or whether mutations over time account for the finding of measles and respiratory syncytial virus antigens in the same osteoclasts.

In the past there has been no evidence that canine distemper virus is responsible for any human disorder. The finding of antibodies to canine distemper virus in the circulation of patients with Paget's disease and in control subjects is consistent with this possibility. These antibody titers did not correlate with antibodies to measles virus in these individuals (Gordon *et al.,* 1993).

Of particular interest is what accounts for the focal nature of Paget's disease when circulating mononuclear cells can be demonstrated to contain measles virus transcripts? Based on the studies of Menaa *et al.* (2000a), it seems that changes in the marrow microenvironment are critical for maintenance of the localized lesions. It remains for future studies to fully define the cytokines produced by the bone cells and marrow stroma which interact to produce the florid advancing pathology of a pagetic lesion.

Clearly, it will require many more investigations into the mechanism of the proliferation of osteoclasts in Paget's disease before the etiology of this common skeletal problem will be understood fully.

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CHAPTER 72

Genetic Determinants of Bone Mass and Osteoporotic Fracture

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Introduction

Osteoporosis is a common disease characterized by low bone mass, microarchitectural deterioration of bone tissue, and an increased risk of fracture. Bone mineral density (BMD) is an important risk factor for osteoporotic fracture and osteoporosis is defined to exist when BMD values fall more than 2.5 standard deviations below the young adult mean. Whilst 50% of all women will have osteoporosis by the age of 80 according to this definition, only a small proportion of these individuals will suffer a fragility fracture. This is because fall-related factors such as advancing age, muscle weakness, impaired vision, and cognitive impairment contribute to fracture risk (Dargent-Molina *et al.,* 1996), as well as other determinants of bone fragility such as bone quality, bone turnover, and femoral neck geometry (Faulkner *et al.,* 1993; Garnero *et al.,* 1996a; Bauer *et al.,* 1997). Over the past 5 years, increasing interest has focused on the role that genetic factors play in regulating BMD and other determinants of osteoporotic fracture risk. In this chapter, I will describe the strategies which have been used to identify and quantitate genetic influences on bone mass and other phenotypes relevant to the pathogenesis of osteoporotic fracture and discuss the candidate loci and genes that have been implicated in regulation of these processes.

Identifying and Quantitating Genetic Effects on Osteoporosis

Genetic contributions to diseases and continuous traits can be identified and quantitated by studies of twins and families (Martin *et al.,* 1997). Twin studies provide the classical method of dissecting out the relative importance of genes and environment to disease etiology by comparing the similarity of monozygotic (MZ) and dizygotic (DZ) twins. While MZ twins and DZ twins generally share similar environments, they differ genetically in the sense that MZ twins share 100% of their genes in common whereas DZ twins share only 50% of their genes in common. Assuming that a disease has a genetic component, it is expected to occur significantly more frequently in MZ as compared with DZ twins. The same is true for genetically determined quantitative traits such as BMD which are more closely correlated in MZ than DZ twin pairs (Fig. 1). Genetic influences on diseases and quantitative traits can also be assessed by studying the resemblance between other relatives such as parent–offspring pairs or sibling pairs. For qualitative traits (such as presence of absence of an event like fracture) familial resemblance is usually expressed in terms of a relative risk (denoted λ), which is derived from the proportion of relatives affected with a disease as compared with the population prevalance of the disease. The higher the λ value, the greater the genetic contibution to the disease. For quantitative traits, resemblance between relatives is usually expressed in terms of "heritability," which is defined as the ratio of genetic variance to total variance of the trait. Two types of heritability are recognized. Heritability in the narrow sense (h^2) is usually derived by comparing resemblance between parents and offspring and reflects the component of genetic variance which is directly transmissible from one generation to the next (this component of genetic variance is also called "additive genetic variance"). In practice, h^2 values are derived from the regression coefficient (slope) of mid-parent values (i.e., mean of father and mother) against offspring values (either one child or mean

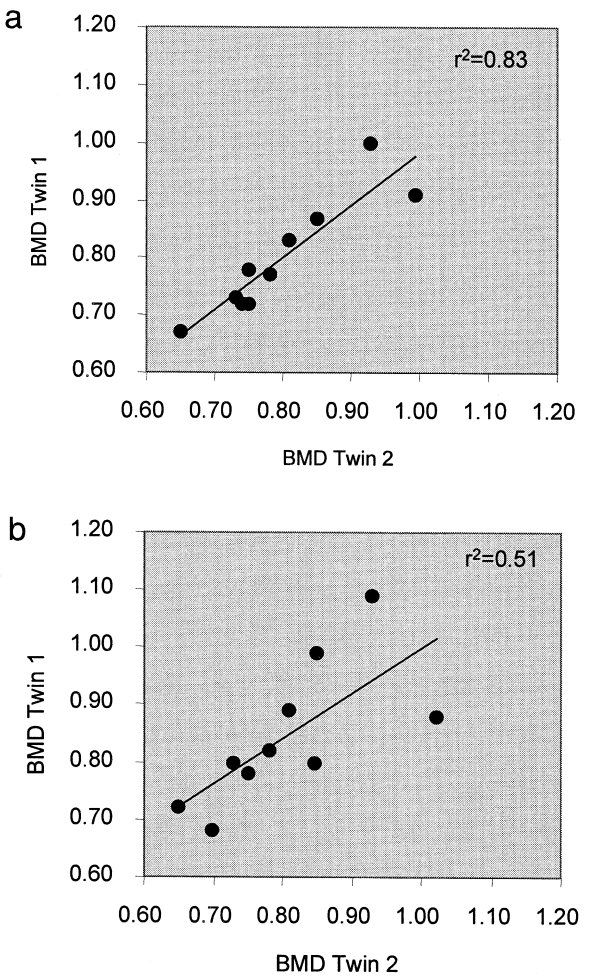

Figure 1 Analysis of heritability of quantitative traits in twins. Comparing values for quantitative traits such as BMD in MZ and DZ twin pairs can help define the role of genetic influences on the trait. In the example shown, the closer correlation between BMD values in the MZ twin pairs (a) as compared with the DZ (b) is consistent with a role for genetic factors in regulating BMD.

Evidence for a Genetic Contribution to BMD and Osteoporotic Fracture

Studies in twins and families have indicated that genetic factors play an important role in the regulation of BMD, other markers of fracture risk, and osteoporotic fracture itself. It has been estimated from twin studies, for example, that between 50 and 85% of the variance in BMD is genetically determined depending on the site examined (Smith *et al.,* 1973; Pocock *et al.,* 1987; Christian *et al.,* 1989; Slemenda *et al.,* 1991; Flicker *et al.,* 1995). Family studies have also shown evidence of strong genetic effects on BMD, with heritability estimates ranging from about 40 to 85% (Krall and Dawson-Hughes, 1993; Gueguen *et al.,* 1995). The data are more conflicting with regard to the influence of genetic factors on age-related bone loss; in one study of wrist BMD in elderly male twins, no evidence for a genetic effect on bone loss was found (Christian *et al.,* 1989), whereas in another study of younger female twins strong genetic effects on axial bone loss were observed (Kelly *et al.,* 1993). Recent interest has focused on the relationship between genetic factors and the clinically important outcome of osteoporotic fracture. A twin study in Finland (Kannus *et al.,* 1999) showed slightly higher rates of concordance for fracture in MZ as compared with DZ twins but the differences were not significant overall, leading the authors to conclude that environmental factors may be more important in the pathogenesis of fracture. A recent family based study demonstrated clear evidence for a genetic contribution to the pathogenesis of wrist fracture, however, with relative risks of 1.31 for women with a maternal history of wrist fracture and 1.88 for a woman whose sister had suffered a wrist fracture (Deng *et al.,* 2000). This study also showed that BMD values were reduced in women with Colles' fracture, suggesting that the genetic contribution to fracture was mediated in part, by differences in BMD. There is evidence from twin studies and population-based studies that family

of all children). Heritability in the broad sense (H_2) on the other hand, is usually derived by comparing resemblance between siblings. This reflects the component of variance derived from genes inherited from the parents (i.e., additive variance), *plus* the variance which occurs as the result of gene–gene interactions, *plus* the variance that results from common environment. In practice, heritability in the broad sense (H_2) estimates are derived from twice the correlation coefficient (r value) between siblings in a regression analysis. If values for heritability in the broad sense are higher than values for heritability in the narrow sense, it implies that gene-gene interactions and/or shared environment contribute substantially to the resemblance between siblings. Common environment is, however, very difficult to separate out from genetic components using family and sib-pair designs, leading to erroneous estimates of heritability.

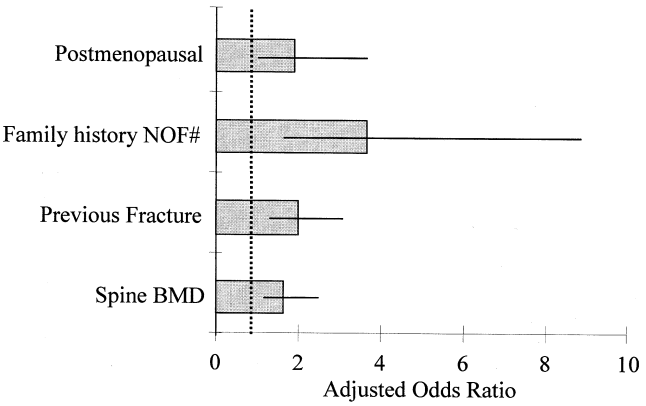

Figure 2 Family history of fracture predicts osteoporotic fracture independent of BMD. Comparison of relative risk for fracture (± 95% confidence intervals) in perimenopausal women according to menopausal status, history of previous fracture, family history of hip fracture (adjusted for BMD), and a 1 SD reduction in spine BMD. Data from Torgerson *et al.* (1996).

resemblance in BMD is not the only factor of importance in determining fracture risk, however. Twin studies have shown that determinants of osteoporotic fracture which are independent of BMD such as femoral neck geometry (Arden *et al.,* 1996; Flicker *et al.,* 1996); ultrasound properties of bone (Arden *et al.,* 1996) and biochemical markers of bone turnover (Garnero *et al.,* 1996b) also have a strong heritable component. Population-based studies support the potential importance of these and other variables in determining fracture risk in showing that family history of fracture is a significant risk factor for fracture by mechanisms that are partly independent of bone density (Cummings *et al.,* 1995; Torgerson *et al.,* 1996; Keen *et al.,* 1999a) (Fig. 2). In conclusion, there is evidence for a strong genetic contribution to the pathogenesis of osteoporotic fracture by genes which regulate several predisposing phenotypes including BMD, bone turnover, skeletal geometry, and bone quality.

Patterns of Inheritance for Regulation of Bone Mass

Several different genes each with modest effects, are thought to combine with environmental factors to regulate BMD and other determinants of fracture risk in most normal individuals (Gueguen *et al.,* 1995). In some cases, however, the influence of a single gene can predominate. Syndromes of low BMD and osteoporosis which are inherited in a Mendelian manner include osteogenesis imperfecta, due to mutations in the collagen genes (Rowe, 1991), osteoporosis–pseudoglioma syndrome (Gong *et al.,* 1998), and severe osteoporosis associated with inactivating mutations in the aromatase (Morishima *et al.,* 1995) and estrogen receptor alpha genes (Smith *et al.,* 1994). Increased BMD can also occur as the result of mutations in a single gene. The classic example of this is osteopetrosis, which is characterized by disordered osteoclast function and greatly increased BMD. A syndrome of unusually high BMD distinct from osteopetrosis has also been described which is inherited in an autosomal dominant manner (Johnson *et al.,* 1997). In all of the above examples, the consequences of the gene mutation on bone cell function are so profound as to overwhelm the effects of the many other genes which contribute to regulation of bone mass. Even in such extreme cases, it is sometimes possible to identify polygenic effects on disease severity. The best examples of this are in osteogenesis imperfecta, where disease severity can vary markedly within and between families who have identical mutations in the collagen genes, presumably due to the influence of other genes on bone mass and bone fragility (Willing *et al.,* 1990).

Strategies for Identifying Osteoporosis Genes

The approaches used to identify the genes responsible for diseases with a genetic component have been recently reviewed by Ott and Hoh (2000) and these are discussed in more detail below.

Linkage Analysis

The classical approach for gene discovery is linkage analysis which involves identifying a model of inheritance for the disease and looking for evidence of segregation of the disease within a family according to that model (for example, autosomal dominant or autosomal recessive). The results of linkage studies are reported by calculating the lodscore, which is defined as the logarithm of the odds that the disease locus and marker locus are linked (no recombination between loci) rather than unlinked (50% recombination between loci). Because lodscores are a function of the recombination fraction (θ), they are calculated over a range of recombination values and the maximum value (Zmax) is estimated. By convention, linkage is considered significant when the lodscore exceeds about +3.3, whereas linkage is considered suggestive when the lodscore exceeds +1.9. Conversely, linkage can be excluded by the finding of a lodscore below −2.0. Linkage studies are usually carried on a genome-wide basis, with a panel of markers approximately 10 cM apart, but they may also be conducted in relation to a specific locus or gene. Linkage analysis has been used widely in human genetic mapping and has been successful in identifying disease genes in hundreds of monogenic traits. It is less suitable for the study of complex diseases such as osteoporosis or complex traits such as BMD, where multigeneration families are more difficult to come by and the mode of disease inheritance is generally unclear.

Allele-Sharing Studies

Allele-sharing studies test the hypothesis that sibling pairs who are both affected by a disease inherit the same alleles in certain regions of the genome more often than would be expected by chance. As in the case of classical linkage analysis, these studies are generally performed by studying a panel of polymorphic markers on a genome-wide basis, but may also be performed by studying markers within specific candidate genes or candidate loci. Allele-sharing studies can also be used in the analysis of quantitative traits such as BMD. By calculating the difference between sib-pairs for the trait under study and relating this to the number of alleles shared identical by descent at a specific marker, it is possible to assess if there is evidence of linkage between that marker and the quantitative trait (Fig. 3). As in the case of classical linkage studies, statistical significance in sib-pair linkage studies is usually measured in terms of lodscore units. It should be noted, however, that the threshold values for significant linkage using sib-pair methods are between +0.5 and +1.3 lodscore units higher than with classical linkage methods, depending on which statistical program is used to analyze the data (Nyholt, 2000). Advantages of the allele-sharing approach include the fact that it does not require collection of large families and that it does not depend on knowing the mode of disease inheritance. A disadvantage of

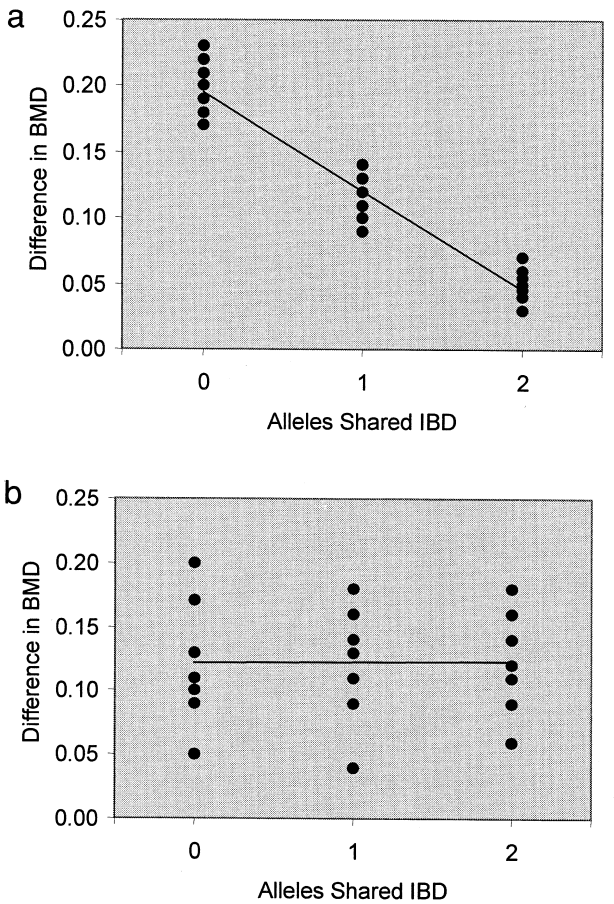

Figure 3 Assessing linkage between a genetic marker and quantitative trait in sib-pairs. Linkage studies of quantitative traits in sib-pairs are based on assessing whether the difference between sib pairs is related to number of alleles shared identical by descent with a series of polymorphic markers. (a) Clear evidence of linkage as reflected by the close concordance between BMD values in sib-pairs who share both alleles identical by descent (IBD) and marked discordance of BMD values in sib-pairs who share no alleles IBD. (b) There is no evidence of linkage since the difference in BMD values between sib-pairs is unrelated to alleles shared IBD.

the approach is that it requires large numbers of sib pairs to gain adequate statistical power for the detection of genes which have modest effects on disease susceptibility.

Association Studies

Association studies compare the frequency with which polymorphisms in or around a candidate gene of interest are present in a group of patients with a disease as compared with a group of controls. Association studies may also be used to study quantitative traits such as BMD by comparing the mean values of the trait in different genotype groups. Recent interest has focused on the possibility that association studies may also be used to search for novel loci on a chromosome-wide or genome-wide basis using the technique of linkage disequilibrium mapping (Collins *et al.,* 1999). Linkage disequilibrium (LD) refers to the phenomenon whereby genomic regions which are physically close to one another on the same chromosome tend to segregate together from one generation to the next. Linkage disequlibirum mapping exploits this fact in testing the hypothesis that unrelated individuals who have the same disease will also share in common those regions of the genome which predispose to that disease. Successful LD mapping depends on analyzing a dense set of markers, spaced no further apart than the average distance of LD in the population under study. It has been estimated on the basis of experimental data that LD extends on average about 300 kb (Collins *et al.,* 1999), but this varies depending on the chromosomal region and population under study (LD tends to extend further in isolated, genetically homogeneous populations).

Association studies have been successful in identifying a role for the HLA region in the pathogenesis of autoimmune diseases and the role of the ApoE gene in sporadic Alzheimer's disease. A potential pitfall with association studies is that they can give spurious results due to population stratification, particularly when the sample sizes are small and when insufficient care has been paid to matching cases and controls. These problems have probably been overestimated in the past and can be partly circumvented by careful study design with statistical correction for possible confounding factors. It should be noted, however, that positive associations between candidate gene polymorphisms and disease can occur as the result of two mechanisms; the first is that the polymorphism actually causes or contributes to the disease. In this case one would expect to find a similar association in all populations which carry the polymorphism. The second possibility is that the polymorphism is in linkage disequilibrium with the actual cause, which may be another polymorphism or mutation in the same gene, or in a gene nearby. Defining a causative role for any individual polymorphism and a complex disease is difficult. Approaches which may be employed include haplotype analysis, which involves genotyping several polymorphisms at the candidate locus in an attempt to identify those that are most strongly associated with the disease. Once these polymorphisms have been identified, functional studies are required to define the biological effects of putative disease-causing polymorphisms on protein function and/or gene transcription *in vitro* and *in vivo.*

The transmission disequilibrium test (TDT) if often used to confirm the results obtained from population based association studies (Spielman *et al.,* 1993). This examines the hypothesis that a polymorphism or allele contributes to disease by analyzing the frequency with which affected individuals inherit the allele from a heterozygous parent. If the given allele contributes to disease, then the probability that an affected person has inherited the allele from a heterozygous parent should vary from the expected mendelian ratio of 50:50 (Fig. 4). Because the transmitted allele acts as the "case" and the nontransmitted allele acts as the "control," the TDT is unaffected by confounding due to population stratification. These advantages of the TDT are offset by the fact that it is applicable only to the study of diseases where parental samples are available. This is a limiting factor in

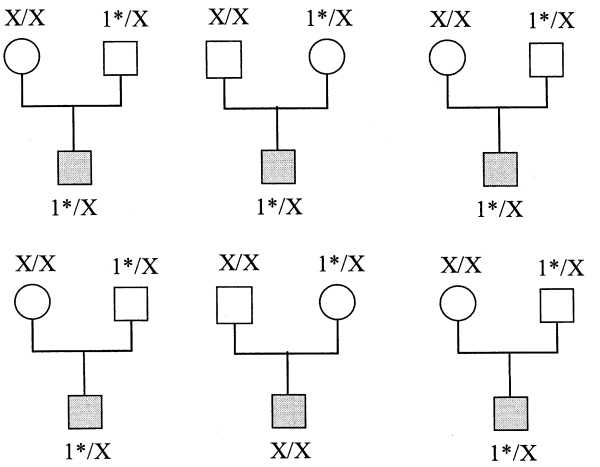

Figure 4 The transmission disequilibrium test. The transmission disequilibrium test is based on assessing if an allele suspected to increase the risk of disease (here designated 1*) is transmitted to affected children from heterozygous parents more frequently than would be expected by chance (i.e., more than 50% of the time). In the example shown the 1* allele is transmitted 5/6 possible times, whereas the expected value would be 3/6. Nonrisk alleles are represented by "X".

the study of late-onset diseases such as osteoporosis where fracture might be the phenotypic measure of interest.

Animal Studies

Studies in experimental animals provide an additional method of identifying the genes responsible for human disease, based on the assumption that key regulatory genes will be shared across species. These approaches are discussed in more detail in Chapter 96.

Linkage and Allele Sharing Studies

Relatively few classical linkage studies have been performed in the osteoporosis field. An exception is the study by Johnson *et al.* (1997), who described a large family in which unusually high bone mass (BMD *Z* score +3.0 or greater) was inherited in a simple autosomal-dominant fashion through several generations. A genome-wide search in this family resulted in the identification of a candidate locus for the syndrome on chromosome 11q12–q13 and excluded the rest of the genome. This locus is of interest since it harbors the gene responsible for the osteoporosis pseudoglioma syndrome (OPS), an autosomal recessive disease, characterized by ocular abnormalities and juvenile osteoporosis (Gong *et al.,* 1998) and with autosomal recessive osteopetrosis—a disease characterized by increased BMD and defective osteoclast function (Heaney *et al.,* 1998). Recently mutations of the TCIRG1 gene, encoding a subunit of the vacuolar proton pump have been identified as causing many cases of 11q-12–q13-linked autosomal recessive osteopetrosis in humans (Frattini *et al.,* 2000). Mutations of the murine homolog appear also to be responsible for autosomal recessive osteopetrosis in oc/oc mice (Scimeca *et al.,* 2000). This raises the possibility that TCIRG1 is the gene responsible for other disorders of BMD that have been mapped to 11q12–q13. It is currently unclear whether OPS and/or the high bone mass syndrome are also caused by mutations in TCIRG1, however, or whether other genes are responsible.

In contrast to the paucity of classical linkage studies, several sib-pair-based linkage studies have been conducted to try and identify loci involved in the regulation of bone mass (Table 1). Devoto *et al.* (Devoto *et al.,* 1998) conducted a genome search in a series of affected sibling pairs recruited from several families with history of osteoporosis. These studies identified loci on chromosomes 1p36, 2p23–p24, and 4q32–34 where the data were suggestive of linkage to spine or hip BMD (lodscores between +2.5 and +3.5). Several other loci with lodscores between +1.5 and +2.5 were also identified, including a broad region of chromosome 11q, which was seemingly distinct from the 11q12–q13 locus identified by Johnson *et al.* (1997). Further studies by the same group subsequently confirmed evidence of linkage to the 1p36 locus in a second set of sib-pairs (Spotila *et al.,* 1998). Nui and colleagues conducted a genome-wide search for loci that regulated forearm BMD in 153 healthy sib-pairs, drawn from a Chinese population (Nui *et al.,* 1999). In this study, evidence suggestive of linkage to BMD was found on chromosome 2p23–p24 (lodscore +2.15) and chromosome

Table I Candidate Loci for Regulation of BMD Identified by Genome Searches

Author	Type of study	Locus	Peak lodscore	Phenotype
Devoto *et al.,* 1998	Sib-pair	1p36	3.51	Hip BMD
		2p23	2.29	
		4q33	2.95	
Nui *et al.,* 1999	Sib-pair	2p21	2.15	Wrist BMD
		13q24	1.67	
Koller *et al.,* 2000	Sib-pair	1q21–q23	3.86	Spine BMD
		5q33–q35	2.23	Hip BMD
		6p11–p12	2.13	Hip BMD
Johnson *et al.,* 1997	Family	11q12–q13	5.74	Spine BMD
Koller *et al.,* 1999	Sib-pair	11q12–q13	3.50	Hip BMD

13*q*34 (lodscore 1.67). Prompted by the observation that high bone mass, OPS, and osteopetrosis all mapped to chromosome 11*q*12–*q*13, Koller and coworkers evaluate this region for evidence of linkage to spine and hip BMD in a study of 835 healthy Caucasian and African-American sib-pairs (Koller *et al.,* 1999). These studies showed evidence of linkage to the region of interest with a peak lodscore of +3.51 for spine BMD. Although positive lodscores were observed in both ethnic groups, the effect was greater among Caucasian women. Koller subsequently conducted a whole genome search in an expanded sample of sib-pairs derived from the same population (Koller *et al.,* 2000). The highest multipoint lodscore attained in this sample was +3.86 at chromosome 1*q*21–*q*23 in relation to lumbar spine BMD. Other lodscores suggestive of linkage were observed on chromosome 5*q*33–*q*35 (lodscore of +2.23 with femoral neck BMD) and chromosome 6*p*11–*p*12 (lodscore of +2.13 with lumbar spine BMD). Interestingly, while the 11*q*12–*q*13 region continued to show evidence of linkage in the whole genome scan, the results were much weaker than in Koller's candidate locus linkage study (Koller *et al.,* 1999). Duncan and colleagues conducted a sib-pair linkage study in 115 probands and 499 of their relatives using polymorphic markers in the vicinity of 23 candidate genes which have implicated in the regulation of bone mass (Duncan *et al.,* 2000). The highest lodscore was observed at the PTH receptor 1 locus in chromosome 3*p* (lodscores = +2.7–3.5, depending on the method of analysis) but positive lodscores (above +1.7–1.8) were also observed at the vitamin D receptor (VDR) locus, the collagen type I alpha 1 (COLIA1) locus, and the epidermal growth factor locus.

Candidate Gene Studies

These have been widely used in studying the genetic basis of osteoporosis, focusing on candidate genes such as cytokines and growth factors, which regulate bone turnover; those that encode components of bone matrix; and those that encode receptors for calciotropic hormones. Individual candidate genes that have been implicated in the regulation of bone mass or osteoporotic fractures are discussed in more detail below.

Vitamin D Receptor

The active metabolites of vitamin D play an important role in regulating bone cell function and maintenance of serum calcium homeostasis by binding to the vitamin D receptor and regulating the expression of a number of response genes. Polymorphisms affecting both the 3′ and 5′ regions of the VDR gene have been studied in relation to BMD and other phenotypes relevant to the pathogenesis of osteoporosis. The first study of VDR genotypes in relation to calcium metabolism were those of Morrison who found an association between polymorphisms affecting the 3′ region of the gene and circulating osteocalcin levels (Morrison *et al.,* 1992). In a subsequent study, the same group reported a significant association between a *BsmI* polymorphisms in intron 8 of VDR and BMD in a twin study and a population based study, but this association was later found to be much weaker than originally reported due to genotyping errors (Morrison *et al.,* 1997). A large number of studies have now been carried out looking at the association between BMD and other aspects of calcium metabolism in relation to *BsmI* and other polymorphisms at the VDR locus. Several studies have supported the original findings whereas others have found no significant association and still others reported an inverse associations to those originally reported (Houston *et al.,* 1996; Uitterlinden *et al.,* 1996). Gong and colleagues recently reviewed the results of 75 articles and abstracts published between 1994 and 1998 which related BMD and associated skeletal phenotypes to VDR polymorphisms. The main conclusions to emerge from this study were that there was a highly significant association between VDR polymorphisms and BMD overall and that positive results were significantly more common in studies that included premenopausal rather than postmenopausal women (Gong *et al.,* 1999). From this the authors concluded that the body of evidence strongly supported an association between VDR alleles and BMD but that the association may have been missed in some studies because of small sample size and other confounding factors. There is evidence to suggest that the relationship between VDR genotype and BMD may be modified by environmental factors such as calcium intake (Ferrari *et al.,* 1995; Krall *et al.,* 1995) and vitamin D intake (Graafmans *et al.,* 1997). In keeping with this view, intestinal calcium absorption has been associated with the *BsmI* VDR polymorphism in some studies (Dawson-Hughes *et al.,* 1995; Gennari *et al.,* 1997). The mechanism by which this occurs is unclear and no association has been found between genotype and mucosal VDR density (Barger-Lux *et al.,* 1995). Four studies have looked at the association between VDR *BsmI* alleles and fracture. In one, a positive association was found but this was significant only in a subgroup of older women age 75 years and above (Feskanich *et al.,* 1998). Three other studies found no association with osteoporotic fracture (Looney *et al.,* 1995; Houston *et al.,* 1996; Ensrud *et al.,* 1999). Studies which have sought to define functional associations of the 3′ VDR polymorphisms have yielded mixed results. Reporter gene constructs prepared from the 3′ region of the VDR gene in different individuals have shown evidence of haplotype-specific differences in gene transcription, raising the possibility that polymorphisms in this region may be involved in regulating affect RNA stability (Morrison *et al.,* 1994). In support of this view, cell lines which were heterozygous for the *TaqI* polymorphism showed differences in allele-specific transcription of the VDR gene (Verbeek *et al.,* 1997). In this study, however, transcripts from the "t" allele were 30% more abundant than those from the "T" which is the opposite from the result expected on the basis of Morrison's results (Morrison *et al.,* 1994). Other *in vitro* studies have shown no differences in allele-specific transcription, mRNA stability, or ligand binding in relation to the *BsmI* polymor-

phism (Mocharla *et al.,* 1997; Gross *et al.,* 1998a; Durrin *et al.,* 1999). Another common polymorphism has been described in exon 2 of the VDR gene, which is a T → C transition, recognized by the *FokI* restriction enzyme (Arai *et al.,* 1997; Gross *et al.,* 1998b). This transition introduces an alternative translational start codon that results in a shorter isoform of the VDR gene. The *FokI* polymorphism has been associated with BMD in some studies (Gross *et al.,* 1997; Arai *et al.,* 1997; Harris *et al.,* 1997) but not in others (Eccleshall *et al.,* 1998; Sowers *et al.,* 1999; Langdahl *et al.,* 2000a). The *FokI* polymorphism has been associated with osteoporotic fractures in Italian women (Gennari *et al.,* 1999), but no association was found in Danish women (Langdahl *et al.,* 2000a). Functional studies of the *FokI* polymorphism have yielded mixed results. A positive association with intestinal calcium absorption was reported in one study (Ames *et al.,* 1999), but two other studies yielded negative results (Zmuda *et al.,* 1999; Cauley *et al.,* 1999a). Studies *in vitro* have shown that different VDR *FokI* alleles differ in their ability to drive reporter gene expression (Arai *et al.,* 1997; Jurutka *et al.,* 2000) and the polymorphic variant lacking three amino acids ("F") has also been found to interact with human basal transcription factor IIB more efficiently than the longer isoform ("f"). Finally, peripheral blood mononuclear cells (PBMC) from "FF" individuals were also been found to be more sensitive to the growth inhibitory effects of calcitriol than PBMC from "Ff" and "ff" individuals (Colin *et al.,* 2000). Contrasting with these results, however, Gross and colleagues found no evidence of functional differences between *FokI* alleles in terms of ligand binding, DNA binding, or transactivation activity (Gross *et al.,* 1998b). In summary, the studies which have been performed to date indicate that allelic variation at the VDR gene locus may have a role to play in the genetic regulation of bone mass. These effects appear to be modified by dietary calcium and vitamin D intake and in some studies have been associated with differences in intestinal calcium absorption. The association between genotype and BMD is weak, however, and no clear association with fracture has emerged. There is some evidence to suggest that different VDR isoforms encoded by the *FokI* variants differ in terms of receptor function although associations between this polymorphism, BMD, and fracture have been inconsistent.

Type I Collagen

Type I collagen is a major structural protein of bone, and the genes encoding this protein (COLIA1 and COLIA2) are strong candidates for the genetic regulation of bone mass. In support of this view, mutations affecting these genes are estimated to be responsible for up to 90% of cases of osteogenesis imperfecta, a hereditary disease characterized by premature osteoporosis and bone fragility (Rowe, 1991). Polymorphisms affecting the coding regions of the collagen type I genes are rare and do not appear to be associated with osteoporosis (Spotila *et al.,* 1994). This led Grant and colleagues to look for evidence of an association between polymorphisms affecting the transcriptional control regions of the COLIA1 gene and osteoporosis (Grant *et al.,* 1996). In these studies, Grant described a G → T polymorphism affecting a binding site for the transcription factor Sp1 in the first intron of COLIA1, which was more prevalent in osteoporotic patients then in controls (Grant *et al.,* 1996). Positive associations between the COLIA1 Sp1 polymorphism and bone mass or osteoporotic fractures were subsequently reported in many other studies (Uitterlinden *et al.,* 1998; Garnero *et al.,* 1998; Langdahl *et al.,* 1998; Roux *et al.,* 1998; Alvarez *et al.,* 1999; McGuigan *et al.,* 2000; Weichetova *et al.,* 2000) (Fig. 5). Several investigators have found that the association between COLIA1 alleles and fracture persists after correction for BMD and/or is stronger than expected on the basis of allele-specific differences in bone mass (Grant *et al.,* 1996; Uitterlinden *et al.,* 1998; Langdahl *et al.,* 1998; Keen *et al.,* 1999b). This has led to speculation that the polymorphism may principally act as a marker for increased bone fragility rather than reduced BMD. Some investigators have found no association between the COLIA1 Sp1 polymorphism and BMD or fracture although these studies have been small, with limited statistical power (Liden *et al.,* 1998; Hustmyer *et al.,* 1999). The relationship between COLIA1 alleles and bone loss has been studied by two groups, following on from the study of Uitterlinden who found that genotype-related differences in BMD increased with age (Uitterlinden *et al.,* 1998). In the study of Harris and colleagues, the COLIA1 "ss" genotype was strongly associated with increased bone loss over a 5-year period in 243 elderly men and women

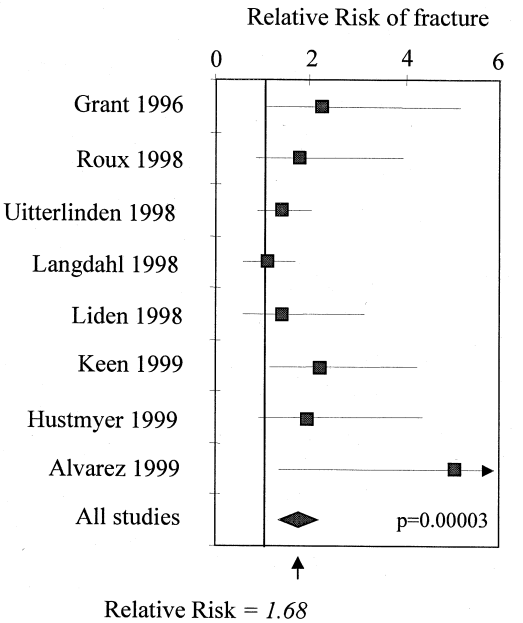

Figure 5 Metaanalysis of COLIA1 polymorphisms and osteoporotic fracture. The odds ratio (± 95% CI) for osteoporotic fracture in relation to carriage of the COLIA1 "s" allele is shown for individual studies performed between 1996 and 1999 and for all studies combined. Overall there was a highly significant 1.68-fold increased risk of fracture in patients who carry the "s" allele when compared to that seen with "SS" homozygotes.

(Harris *et al.,* 2000). In the study of Heegard, however, no association was found between COLIA1 alleles and bone loss in a cohort of 133 women followed over 18 years (Heegaard *et al.,* 2000). The COLIA1 Sp1 polymorphism has been studied in relation to BMD in children but the results have been contradictory; in one study COLIA1 alleles were strongly related to volumetric BMD measured by QCT in Mexican-American girls (Sainz *et al.,* 1999), whereas no association was found in another study of Caucasian girls (Tao *et al.,* 1999). Quite marked ethnic differences have been reported in population prevalence of the COLIA1 "s" allele, which is relatively common in Caucasian populations, but is rare in Africans and Asians (Beavan *et al.,* 1998; Nakajima *et al.,* 1999). This has been suggested as a possible mechanism by which ethnic difference in fracture risk may be determined. The mechanism by which the Sp1 polymorphism predisposes to osteoporosis remains to be fully defined but preliminary data has shown evidence of allele-specific differences in binding of the Sp1 protein to the polymorphic recognition site, differences in allele-specific transcription, differences in collagen protein production, and differences in bone strength in samples derived from patients of different genotype (Mann *et al.,* 2001).

In summary, there is fairly convincing evidence to suggest that the COLIA1 Sp1 polymorphism acts as a marker for osteoporotic fracture. There is also evidence for weak association with BMD, which becomes more marked with increasing age, suggesting that COLIA1 alleles may be associated with age-related bone loss rather than peak bone mass.

Estrogen Receptor Genes

Estrogen, by interacting with its receptors in bone and other tissues plays an important role in regulating skeletal growth and maintenance of bone mass. Knockout mice for ERα and ERβ have reduced BMD compared to that seen in wild-type controls (Korach, 1994; Windahl *et al.,* 1999) and osteoporosis has also been observed in a man with an inactivating mutation of the ERα gene (Smith *et al.,* 1994). These data indicate that the ER genes are strong candidates for genetic regulation of bone mass. Sano *et al.* (1995) reported a positive association between a TA repeat polymorphism in the ERα gene promoter and bone mass in a small study of Japanese subjects. Similar results were reported by groups in the United States and Italy (Sowers *et al.,* 1999; Becherini *et al.,* 2000). Other investigators have reported positive associations between haplotypes defined by *PvuII* and/or *XbaI* polymorphisms in the first intron of the ERα gene and bone mass (Kobayashi *et al.,* 1996; Mizunuma *et al.,* 1997; Ongphiphadhanakul *et al.,* 1998; Sowers *et al.,* 1999) as well as age at menopause (Weel *et al.,* 1999). Other studies in Korean (Han *et al.,* 1997), Belgian (Vandevyer *et al.,* 1999), and Italian (Gennari *et al.,* 1998) women have found no association between *PvuII* polymorphisms and bone mass, however. Polymorphisms of ERα have also been studied in relation to postmenopausal bone loss. In a longitudinal study of Finnish women, early postmenopausal bone loss was associated with the ERα *PvuII* polymorphism (Salmen *et al.,* 2000), whereas a similar study by Willing showed no such association (Willing *et al.,* 1998). No association studies have been performed in which ERα polymorphisms have been related to osteoporotic fractures.

The molecular mechanism by which ERα polymorphisms influence bone mass are as yet unclear. The *PvuII* and *XbaI* polymorphisms lie in an apparently nonfunctional area of the gene but are in linkage disequilibrium with the TA polymorphism in the ERα promoter (Becherini *et al.,* 2000). It is conceivable that the TA repeat could influence gene transcription, but this has not yet been studied. In summary, ERα polymorphisms seem to be associated with BMD in some populations, although the mechanisms by which they do so remain unclear. The ERβ gene has been little researched as a candidate for osteoporosis, apart from one study which showed an association between a microsatellite marker close to the ERβ gene and BMD (Ogawa *et al.,* 2000).

Transforming Growth Factor Beta

Transforming growth factor beta (TGFβ) is a pleiotropic regulatory protein with diverse effects on cell growth and differentiation. Current evidence suggests that TGFβ plays an important role in bone metabolism, where it is thought to act as a coupling factor between resorption and formation and in keeping with this hypothesis, mutations have been described in the signal peptide region of the TGFβ molecule, which is responsible for Camaruti–Engelmann disease, a rare disorder characterized by bone pain and osteosclerosis affecting the diaphysis of long bones (Kinoshita *et al.,* 2000; Janssens *et al.,* 2000). In view of the above, the TGFβ genes represent important candidates for the regulation of bone mass and bone turnover. A rare C-deletion polymorphism in intron 4 of TGFβ-1 has been associated with low BMD, increased bone turnover, and osteoporotic fracture in one study from Denmark (Langdahl *et al.,* 1997), and very similar results were recently reported in another study from Italy (Bertoldo *et al.,* 2000). Although this polymorphism is close to the splice junction, it does not affect the splice acceptor site and the functional effects on TGFβ function (if any) are unknown. Another polymorphism of the TGFβ-1 coding region has been described which causes a Leucine–Proline substitution in the signal peptide region of TGFβ at amino acid 10. The C allele has been associated with high BMD and a reduced frequency of osteoporotic fractures in two Japanese populations (Yamada *et al.,* 1998), with BMD in Japanese adolescents (Yamada *et al.,* 1999), and with reduced rates of bone loss and improved response to treatment with alphacalcidol, an active metabolite of vitamin D (Yamada *et al.,* 2000). This polymorphism is associated with raised circulating levels of TGFβ, suggesting that it may influence protein secretion or stability. Two promoter polymorphisms of TGFβ have also

been described which are also associated with circulating TGFβ levels (Grainger *et al.,* 1999), but the relationship with BMD has not yet been reported.

Interleukin-1 Gene Cluster

Cytokines such as interleukin-1 have important effects on bone cells *in vitro* and have been implicated in the pathogenesis of postmenopausal bone loss. These genes are therefore important candidates for the genetic regulation of bone mass. The interleukin-1 gene cluster on chromosome 2 contains the genes for interleukin 1 (IL-1) alpha and beta genes as well as the gene for the interleukin-1 receptor antagonist (IL-1RA). Polymorphisms of these genes have been widely studied in relation to inflammatory disorders and some alleles have been identified which are associated with production of IL-1 or IL-1RA *in vitro.* This has prompted studies of the IL-1 gene cluster polymorphisms in relation to BMD. Keen and colleagues studied the relationship between BMD and bone loss in 108 postmenopausal women who were genotyped for an 86-base-pair repeat polymorphism in the second intron of the IL-1RA gene. No difference in BMD was observed between genotypes, but the A1/A1 genotype was found to be associated with increased bone loss at the spine. In another study, Langdahl found evidence of an association between IL-1RA polymorphisms and BMD was well as an association with osteoporotic fracture (Langdahl *et al.,* 2000b). The results of this study were in partial agreement with those reported by Keen (Keen *et al.,* 1998) in that the A1/A1 and A1/A3 genotypes were associated with reduced BMD. In contrast, no association was found between IL-1β polymorphisms and BMD. The mechanisms by which these polymorphism associate with BMD remain to be determined. Leukocytes from individuals with the A1/A1 and A1/A3 genotypes have been shown to produce less IL-1RA *in vitro* than the other genotype groups which would be consistent with increased bone loss, but plasma IL-1RA levels have paradoxically found to be higher in those who carry the A1/A1 genotype. These studies are of interest but further work will be required to clarify the relationship between IL-1RA alleles, BMD, and fracture and to identify the mechanisms responsible for the effects that have been observed.

Interleukin-6

Interleukin-6 is another potential candidate gene for the regulation of BMD, in view of its suggested role as a mediator of osteoclast activity and postmenopausal induced bone loss. Murray looked for evidence of an association between association between BMD and osteoporotic fracture in relation to an AT-rich minisatellite repeat in the 3′ flanking region of the IL-6 gene. In this study a positive association was found with lumbar spine BMD but no association was found with osteoporotic fracture (Murray *et al.,* 1997). In a further study a polymorphic AC-rich minisatellite close to the IL-6 gene was also reported to be associated with wrist BMD in Japanese women (Tsukamoto *et al.,* 1998). The most comprehensive study of IL-6 polymorphisms in relation to BMD is that of Takacs, however, who conducted a large-scale linkage and association study of the IL-6 gene in over 800 Caucasian and African-American sib pairs found no evidence to suggest that IL-6 alleles were related to BMD (Takacs *et al.,* 2000). These results do not support an important role for IL-6 in the genetic regulation of BMD.

Apolipoprotein E

The human Apolipoprotein E gene is polymorphic, with three common alleles (ε2, ε3, ε4) coding for three isoforms (E2, E3, E4) which differ from each other by a single amino acid and their binding affinity for the four ApoE receptors. ApoE has been suggested as a candidate for regulation of BMD because it is involved in the transport of vitamin K, which is a necessary cofactor for the hydroxylation of osteocalcin—an osteoblast-specific protein. Shiraki and colleagues reported that BMD values were significantly reduced in Japanese women who carried the ApoE4 allele (Shiraki *et al.,* 1997). While Salamone *et al.* (2000) found that the ApoE4 genotype was associated with accelerated postmenopausal bone loss in women from the United States, Heikkinen *et al.* (2000) found no association between ApoE and BMD or bone loss over a 5-year period in a study of 464 Finnish women. This study was complicated by the fact that half were randomized to HRT and half to calcium and vitamin D supplements, however. In a further study of American Caucasian women Cauley and colleagues (1999b) found a positive association between the ApoE4 allele and hip fracture that was independent of BMD.

In summary, the data assembled so far suggest that ApoE is a promising candidate gene for further study. More work will be required to assess the significance of the association between ApoE and bone phenotypes, however, and to investigate mechanisms by which it affects the skeleton.

Calcitonin Receptor

Calcitonin is a physiological inhibitor of osteoclast function, and its receptor is highly expressed on osteoclasts. A coding polymorphism causing a Proline–Leucine substitution at codon 436 of the calcitonin receptor gene has been described and the relationship between this polymorphism and BMD has been studied in French and Italian populations. Masi *et al.* (1998) reported that individuals homozygous for the Leucine substitution had reduced bone mass when compared with heterozygotes and Proline homozygotes. Taboulet and colleagues (Taboulet *et al.,* 1998) also reported an association between BMD and this polymorphism, but found that heterozygotes had higher BMD and a reduced risk of fracture

when compared with homozygotes. The effects of this polymorphism on calcitonin receptor function has not yet been studied and the absence of an allele–dose effect in the Taboulet study is difficult to explain.

Other Candidate Genes

Several other polymorphisms in candidate genes have been associated with bone mass, ultrasound properties of bone or bone dimensions in isolated studies and these are summarized in Table II. The results of these studies should be regarded as provisional until repeated and confirmed by other investigators.

Gene–Gene Interactions

As stated previously, genetic regulation of bone mass and other determinants of fracture risk appears to be under polygenic control. In view of this several investigators have studied the relationship between combinations of candidate gene polymorphisms and BMD. Willing and colleagues (Willing *et al.,* 1998) looked at the interaction between VDR and ERα polymorphisms in predicting BMD in a series of 171 postmenopausal women and found that individuals with combination of ERα *PvuII* "PP" and VDR "bb" genotypes had very high average BMD values at all skeletal sites examined. Another study by Gennari *et al.* (1998) in a population of postmenopausal Italian women, showed that the combination of VDR and ERα genotypes identified subgroups of individuals with very high and very low BMD. Vandevyer *et al.* (1999), however, found no significant interaction between VDR and ERα genotypes in predicting BMD in Belgian postmenopausal women.

Caution must be exercised in interpreting the results of studies that show an apparent interaction between two genetic polymorphisms in predicting BMD. The studies that have been performed so far have been of limited size with the result that subgroups of patients with different combinations of genotypes have been extremely small. For example, in Willing's study, the high BMD "PP/bb" subgroup consisted of only 11 individuals. Similarly, in Gennari's study, the high BMD "AABBtt-PPXX" subgroup consisted of 9 individuals, compared with 14 individuals for the low BMD "aabbTT-ppxx" subgroup. This raises the possibility of type II (false positive) statistical errors and indicates the need for large sample sizes when assessing gene–gene interactions

Pharmacogenetics and Osteoporosis

Pharmacogenetics is the study of how genetic factors influence the response to drug treatment. This is a subject of increasing interest in clinical medicine since it raises the prospect of being able to predict individual responses to drug treatment on the basis of genetic profiling (Roses, 2000). Several investigators have now looked at associations between candidate gene polymorphisms and the response of BMD to anti-osteoporotic treatments.

Table II Candidate Gene Studies in Osteoporosis

Candidate gene	Function	Type of polymorphism	Association	No. and type of subjects studied	Ethnic group (study)
Androgen receptor	Hormone receptor	Polyglutamine repeat, exon 1	BMD	261 Postmenopausal women	Caucasian (Sowers *et al.,* 1999)
PTH	Regulates calcium homeostasis	Intronic silent	Total body BMD; Bone dimensions	383 Postmenopausal women 91 Healthy women	Japanese (Hosoi *et al.,* 1999) Caucasian (Gong *et al.,* 2000)
PPAR gamma	Regulates adipocyte differentiation	Coding silent H446H	BMD	404 Postmenopausal women	Japanese (Ogawa *et al.,* 1999)
AHSG	Matrix component	Coding transitions T230M and T238S	Heel ultrasound, BMD, height	222 Postmenopausal women	Caucasian (Zmuda *et al.,* 1998, Dickson *et al.,* 1994)
Osteocalcin	Osteoblast product	Promoter polymorphism	BMD	160 Postmenopausal women	Japanese (Dohi *et al.,* 1998)
BMP-4	Osteoblast growth factor	Coding transition V147A	BMD (negative)	154 Postmenopausal women	Italian (Semprini *et al.,* 2000)
Matrix Gla protein	Matrix component	CA repeat	Wrist BMD	460 Postmenopausal women	(Tsukamoto *et al.,* 2000a)
MTHFR	Regulates folate metabolism;	Coding transition A222V	Spine BMD Total body BMD	307 Postmenopausal women	Japanese (Miyao *et al.,* 2000)
TNFR2	Receptor for TNF	Polymorphisms in 3′ UTR:	Spine BMD	34 Men / 125 women	Caucasian; Ashkenasi Jews (Spotila *et al.,* 2000)
CASR	Extracellular calcium receptor	CA repeat	Wrist BMD	247 Women	Japanese (Tsukamoto *et al.,* 2000b)

Calcium and Vitamin D

There is fairly convincing evidence that the VDR *BsmI* polymorphism predicts response of BMD to calcium supplementation. Ferrari and colleagues (Ferrari *et al.,* 1995) were the first to show a link between dietary calcium intake and VDR polymorphisms when they reported an association between bone loss at the lumbar spine in elderly individuals who were heterozygous for the *BsmI* polymorphism. Subsequently Krall and colleagues (Krall *et al.,* 1995) studied the relationship between VDR alleles and bone loss in 229 women who had participated in a controlled trial of calcium supplements in the prevention of postmenopausal bone loss. The calcium supplemented group showed no relationship between VDR genotype and bone loss, whereas in the placebo group, bone loss was significantly greater in the BB group when compared with the other genotype groups. Graafmans *et al.* (1997) studied the response to vitamin D supplementation in a series of 81 postmenopausal Dutch women who had taken part in a placebo-controlled trial of vitamin D supplementation on BMD and fracture incidence. These workers observed that the 2-year change of BMD values in the vitamin D group relative to the placebo group was significantly higher in the "BB" and "Bb" genotypes when compared with the "bb" genotype group. This study is of interest in relation to another study by the same group which showed that the "bb" genotype also had low BMD in a population-based study. Yamada and colleagues (2000) studied the relationship between response of BMD to 1α-hydroxyvitamin D in relation to a signal peptide polymorphism of the TGFβ1 gene. This study comprised 363 postmenopausal women who were treated with 1α-hydroxyvitamin D ($n = 117$); hormone replacement therapy (HRT) ($n = 116$), or who were untreated ($n = 130$). Individuals with the high BMD associated "CC" genotype responded significantly better to vitamin D treatment than the other genotype groups. The same trend was observed in the HRT group but the differences were not significant.

HRT Response

Some information is available on the relationship between candidate gene polymorphisms and response to HRT. Ongphiphadhanakul and colleagues (2000) studied the relationship between ERα polymorphisms and 1-year response to HRT treatment in 124 postmenopausal Thai women. Individuals with the "pp" genotype at the ERα *PvuII* site were found to respond less well to HRT (+2.3% increase in BMD) than the other genotype groups (+6–7% increase in BMD). In a similar but larger study of 248 Korean women, however, Han and colleagues (1999) found no association between *XbaI* or *PvuII* polymorphisms and 1-year response of BMD to HRT. Salmen *et al.* (2000) similarly found no association between ERα genotype and response to HRT in a study of 145 Finnish women. Taken together, these data do not support the view that ERα polymorphisms consistently predict response to HRT. Other candidate genes have been also studied in relation to HRT response. They include TGFβ1 (discussed above) and ApoE, which was analyzed by Heikkinen and colleagues (2000) in a study of 232 women who were treated with HRT and followed up after a 5-year period. No association was observed in this study between ApoE genotype and HRT responsiveness.

Response to Bisphosphonates

This is an area of great clinical relevance but is one that has been little studied. Marc and colleagues (Marc *et al.,* 1999) looked at the relationship between VDR genotype and response to Etidronate therapy in a small series of 24 postmenopausal women undergoing treatment with etidronate. The mean change in BMD over a 2-year period was significantly greater in the BB vs bb group with intermediate values in the heterozygotes. In another study, Qureshi *et al.* (1997) looked at the association between COLIA1 genotype and the response to etidronate treatment in a series of 48 early postmenopausal women who took part in a randomized controlled trial of etidronate in the prevention of postmenopausal bone loss. While no difference was observed in response of spine BMD to etidronate treatment, those with the "s" allele responded significantly less well at the femoral neck when compared with "SS" homozygotes. These preliminary data are of interest in raising the possibility that genetic markers can be used to predict therapeutic response to bisphosphonates, but further work in larger groups of patients will be required to confirm and extend these observations.

Implications for Clinical Practice

Studies on the genetic basis of osteoporosis have important implications for clinical practice. Mapping and identification of genes that regulate BMD offers the prospect of identifying novel molecules that can serve as targets for drug design in the search for new treatments for bone diseases. In addition, genetic markers that are associated with BMD or osteoporotic fracture could be used in the identification of patients at risk of fracture. The most promising candidate genes identified so far in this respect are COLIA1, TGFβ, and ApoE, although it is likely that information from genetic profiling using these and other markers would be combined with other risk factors such as low BMD to identify individuals at risk of fracture. The utility of this approach has recently been demonstrated for the COLIA1 polymorphism which has been found to enhance fracture prediction when combined with BMD measurements in two studies (McGuigan *et al.,* 1998; Weichetova *et al.,* 2000). Another benefit may be in identifying individuals who respond best to anti-osteoporotic treatments. This has already been demonstrated for some polymorphisms in relation to calcium and vitamin D treatment and further

work is certain to be performed in this area to identify genetic markers of response to other drug treatments as well as to identify markers for the development of adverse effects (Roses, 2000).

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CHAPTER 73

Pathophysiology of Osteoporosis

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Introduction

Osteoporosis is a reduction in bone mass and a change in microarchitecture, which increases the susceptibility to fractures. The bone loss is brought about by an imbalance between bone resorption and bone formation, coupled processes that occur continuously throughout the skeleton to carry out its functions. Osteoclastic bone resorption releases calcium, phosphate, and other ions from bone for homeostasis and initiates the structural remodeling, which adapts skeletal architecture to mechanical loads. Bone resorption can also alter the bone marrow space in response to hemopoietic needs (Shinar and Rodan, 1993). As a consequence, the balance between bone resorption and bone formation can be influenced by many factors, which could therefore cause or contribute to osteoporosis. The key to understanding the pathogenesis of osteoporosis is the elucidation of factors that govern osteoclastic bone resorption, osteoblastic bone formation, and the link between the two. Characterization of these processes in the adult should help treat and manage this multifactorial disease. State-of-the-art knowledge of these subjects is covered in several chapters in this volume. Here we shall attempt in summary form to bring this information together in the context of osteoporosis.

Peak Bone Mass vs Rate of Bone Loss

Among the parameters that can be measured today, bone mass, as estimated by bone densitometry, is still the best predictor of osteoporotic fractures (Black *et al.,* 1992; Cummings *et al.,* 1995b; Melton *et al.,* 1993; Wasnich and Miller, 2000; Melton *et al.,* 1998; see also Chapter 74). The amount of bone in all individuals increases during growth, reaches peak in the 20s, starts decreasing in the late 30s, and exhibits an accelerated loss in women during the first 5–10 years after menopause (Melton, 1995), followed by a slower steady decrease thereafter. The latter phase is also seen in men after the age of 40. The amount of bone at a given age, and therefore the risk of osteoporotic fractures, will depend on the peak bone mass of that individual and the rate of bone loss until that age. Bone loss is determined by the balance between the rate of bone resorption and bone formation. The determinants of peak bone mass are primarily genetic (Cooper, 1999; see also Chapter 72), as shown by twin studies (Pocock *et al.,* 1987; Slemenda *et al.,* 1991), with the potential contribution of calcium intake, diet, and exercise during childhood and puberty (Nieves *et al.,* 1995). Family history and the geographic distribution of the incidence of osteoporosis in Europe further point to the importance of genetic background and dietary habits in the pathophysiology of this disease (Johnell *et al.,* 1995, Kanis *et al.,* 1999).

From a therapeutic/medical perspective, the pathogenesis of bone loss is probably most important since the majority of patients are seen many years after reaching peak bone mass. Twin studies (Pocock *et al.,* 1987) suggest that the rate of bone loss is less strongly influenced by genetics than peak bone mass.

It is apparent that multiple genes are responsible for the susceptibility to osteoporosis. Polymorphism in the genes of type I collagen, interleukin-1 (IL-1), and the receptors for estrogen, vitamin D, and IGF1 have been implicated in the pathogenesis of osteoporosis, among others, however, further studies are needed to validate these observations (Cooper, 1999). A couple of "high-bone-density" genes have been fully or partially identified by positional cloning

(Balemans *et al.,* 2001; Brunkow *et al.,* 2001; Johnson *et al.,* 1997; Koller *et al.,* 1998), but their relationship to bone density in the population at large remains to be established.

Most conditions that lead to osteoporosis, including estrogen deficiency, hyperparathyroidism, and hyperthyroidism, are associated with increased osteoclastic bone resorption. Multiple genes are likely to determine the level of these and other resorption stimuli, excessive response to such stimuli, and/or inappropriate "coupling" between resorption and formation. The possibilities are many. Although bone formation increases along with resorption, that increase is often insufficient to maintain bone balance. It should be kept in mind that it takes 3–4 months for osteoblastic bone formation to replace the bone resorbed by osteoclasts in 2–3 weeks (Eriksen, 1986). It is therefore possible that the defect is not in the "coupling," the molecular basis of which requires further study, but in the physiological ability of the bone formation process to keep up with the increased level of resorption. Higher turnover would thus lead to greater bone loss, as it usually does. Following is an analysis of the possible control points in resorption, coupling, and formation and how they might be affected by the etiological factors of osteoporosis.

Bone Resorption

Bone resorption is determined by the number of sites at which resorption occurs (estimated histomorphometrically by activation frequency), by the rate of osteoclast recruitment, and by the intensity and duration of osteoclast activity. These processes are discussed in detail elsewhere in this volume. To briefly summarize, starting with resorption sites, their distribution is probably not random and seems to be related to mechanical function, since the absence of strain (force divided by cross-sectional area), produced for example either by bed rest, lack of gravity, paralysis, or casting of a limb, increases bone resorption in the affected bone(s). Lack of mechanical strain could thus contribute to bone loss.

Extent resorption is influenced by factors that govern osteoclast number and activity. Osteoclasts are derived from hemopoietic precursors, probably granulocyte-macrophage colony-forming units (CFU-GM), branching off the monocyte macrophage lineage and suppression of the macrophage phenotype(Roodman, 1995; Lean *et al.,* 2000). Studies in mice have demonstrated the obligatory requirement of receptor-activating NF-κB (RANK) ligand (RANKL) a tumor necrosis factor (TNF)-related cytokine and its receptor RANK (receptor activating NF-κB) for osteoclast formation and activity (see Chapter 7). RANK-ligand activity is tightly modulated by its soluble neutralizing receptor osteoprotegerin (OPG), the lack of which produces in mice osteopenia, and the excess osteopetrosis (Bucay *et al.,* 1998; Simonet *et al.,* 1997). These factors became obvious candidates for the pathogenesis of osteoporosis, but no change in their abundance in the circulation has been observed in osteoporotic patients so far. On the other hand, agents that stimulate bone resorption *in vivo* and increase osteoclast formation from bone marrow cultures *in vitro* were shown to modulate RANK/RANKL/OPG production and signal transduction in culture (Chapter 7). Many of these agents have previously been implicated in the pathogenesis of osteoporosis including parathyroid hormone (Silverberg *et al.,* 1996), prostaglandin E (PGE) (Kawaguchi *et al.,* 1995), interleukin-1 (Kitazawa *et al.,* 1994), TNF-α (Kitazawa *et al.,* 1994), and, under certain conditions, IL-6 (Jilka *et al.,* 1995). Estrogen administered *in vivo* was shown to suppress *ex vivo* in human macrophages the production of IL-1 (Kitazawa *et al.,* 1994), TNF-α (Kitazawa *et al.,* 1994 Chapter 36), and prostaglandin E_2 (PGE2) (Kawaguchi *et al.,* 1995) and in murine bone marrow stromal cells, IL-6 (Jilka *et al.,* 1995). Estrogen was also shown in experimental systems to modulate RANK abundance and signaling as well as the production of M-CSF (monocyte-macrophage colony-stimulating factor), essential for osteoclastogenesis (see Chapter 36). In estrogen deficiency, hyperparathyroidism, and hyperthyroidism- associated bone loss, there is clear histological and biochemical evidence for increased osteoclastic bone resorption (Delmas, 1993; Parisien *et al.,* 1990) and increased osteoclast recruitment, which could be explained by effects of these agents on osteoclast differentiation from precursor cell. The histological appearance of osteoclast clusters and analogy with the function of monocytes and leukocytes raises the possibility that osteoclasts themselves may release osteoclast recruiting factors during bone resorption.

The next aspect deals with the extent of osteoclast activity once started. Trabecular perforation is a characteristic histological feature of cancellous bone in postmenopausal osteoporosis (Parfitt *et al.,* 1983). It has therefore been suggested that the intensity or duration of osteoclast activity may be increased. However, direct measurement of the depth of bone resorption lacunae, albeit imprecise (Compston and Croucher, 1991), failed to detect estrogen effects on this parameter (Steiniche *et al.,* 1989). It is therefore possible that trabecular perforation reflects the cumulative effect of resorption over time rather than the result of osteoclast activity in a single cycle of remodeling. Trabecular perforation is not a pronounced feature of bone loss in hyperthyroidism, for example, where it was reported that the trabeculae are thinner, but their number is not decreased. The data base for these observations is limited, moreover, they are all based on analysis of the iliac crest, which was shown to have different rates of cancellous bone turnover than vertebrae, and the cancellous bone volume of the two bones was also found to differ, possibly due to differences in mechanical function.

The stimuli responsible for stopping osteoclast activity during remodeling are not known. *In vitro* an osteoclast does not resorb a specific site for more than a couple of hours (Kanehisa and Heersche, 1988) and, when it moves away, it leaves behind nonmineralized, undigested matrix. Mineralized matrix seems to be a requirement for effective osteoclast activity (Chambers *et al.,* 1984). In addition to the systemic hormone calcitonin, local humoral factors, shown

experimentally to inhibit osteoclast formation or activity, include transforming growth factor (TGF)-β (Hattersley and Chambers, 1991), IL-4 interferon-γ. (Lacey *et al.,* 1995), prostaglandin E (Chambers and Ali, 1983) and IL-18, via GM-CSF (granulocyte-macrophage colony-stimulating factor) ((Horwood *et al.,* 1998). There is no conclusive evidence showing that these factors stop osteoclast activity during bone remodeling. It has also been suggested that mechanical strain in the matrix may inhibit osteoclast activity (Hillam and Skerry, 1995); mechanical strain increases prostaglandin production in bone cells (Somjen *et al.,* 1980), but there is no evidence that it plays a role in this case.

Recent evidence suggests that in postmenopausal osteoporosis the rate of bone resorption, reflected in biochemical markers, participates in the susceptibility to fractures not only by determining bone loss, but by increasing bone fragility directly (see Chapter 74), via effects on bone structure. Increased resorption could weaken trabeculae and cortical bone, due to indentations and incomplete mineralization at multiple remodeling sites.

To summarize this section, in most osteoporoses there is increased bone resorption, resulting from the combined effects of increased osteoclast "tonus" caused by sex steroid deficiency, with the potential contribution of secondary hyperparathyroidism due to inadequate calcium intake and vitamin D deficiency, all superimposed on reduced mechanical loads. Quantitatively, osteoclast recruitment, i.e., osteoclast number, which is strongly influenced by sex steroids, seems to be the rate-limiting factor.

Coupling Bone Resorption to Formation

The next point relevant to the pathophysiology of osteoporosis is the coupling of formation to resorption. Histologically, bone formation follows resorption. Calcium kinetics as well as biochemical markers have shown that the two change usually in tandem in the same direction. This led to the postulate that the two processes are mechanistically coupled and to the search of "coupling" factors. It is of interest that many molecular factors that stimulate bone resorption can also stimulate, apparently independently, bone formation. These include prostaglandin E (Jee *et al.,* 1995), parathyroid hormone (Dempster *et al.,* 1993), and possibly others, such as IL-1 (Boyce *et al.,* 1989) and TGF-β (Centrella *et al.,* 1994). Stimulation of bone resorption and formation by these agents would be one form of coupling. Unequal effects of these factors on resorption and formation, respectively, could bring about the inadequate coupling seen in osteoporosis (Martin and Rodan, 2001). It has been reported, for example, that estrogen increases skeletal resistance to parathyroid hormone-induced resorption (Cosman *et al.,* 1994).

Another proposed coupling mechanism is the release of bone- forming growth factors from the matrix during resorption, for example, IGF (Mohan and Baylink, 1991) or TGF-β (Oreffo *et al.,* 1989). Since estrogen may stimulate production of these factors by osteoblasts (Centrella *et al.,* 1994; Schmid and Ernst, 1992), their abundance in bone would decrease in estrogen deficiency.

A third interesting hypothesis proposed by Frost states (paraphrased) that since bone structure and mass are regulated by mechanical load, the set point of that feedback is altered by estrogen deficiency (Frost, 1987). For example, in the absence of estrogen, a hypothetical sensor would perceive the mechanical load to be lower and reduce bone mass accordingly. This idea can be further developed to consider mechanical forces themselves as a coupling factor (Rodan, 1991, 1996) since they stimulate bone formation and may inhibit resorption. The feedback loop would be local at the site of bone remodeling and would work as follows. Bone resorption, initiated, for example, by a decrease in mechanical strain or a need for calcium mobilization, would proceed until dissipation of that stimulus. A thinner trabecula subjected to the prevailing force would experience a higher strain. Bone formation would follow and continue as long as osteoblasts perceive locally increased strain. Such a feedback loop would explain the resorption of mechanically nonfunctional cancellous bone and the well-known adaptation of trabecular architecture to the lines of mechanical force. This is consistent with the observation that in perforated trabeculae, which do not carry load, bone resorption seems to be more extensive and is not followed by formation and the fact that in vertebrae the vertical trabeculae, which play a primary role in weight-bearing, are most conserved during bone loss (Mosekilde, 1990). The adaptation of bone architecture to mechanical loads could not be explained in any other way. In essence, this is biomechanical coupling of bone resorption to formation, since resorption inevitably would increase the degree of mechanical strain in the tissue and formation would take place to relieve that strain. The most likely receptors for mechanical strain, at this time, are the integrins (Hynes, 1992) through which cells bind to the extracellular matrix, where the strain is produced (Rodan, 1991).

Why does this coupling fail in estrogen deficiency? It is possible, as suggested by Frost, that the response of osteoblasts to strain is diminished, possibly via effects on integrin signaling. Another simple reason may be that, in the face of excessive bone resorption, the increase in bone formation cannot keep pace. As mentioned, it takes 3–4 months to replace bone resorbed in 2–3 weeks. Once resorption is abated by estrogen, bisphosphonates, or calcitonin, the remaining trabeculae can increase in size until they optimize their load-bearing capacity. This could take longer than one remodeling cycle, which may explain the continuous increase in bone density observed with bone resorption inhibitors well beyond the 4 months required for the filling of the "remodeling space" (Tonino *et al.,* 2000).

Bone Formation

The next arm in the remodeling cycle to be considered in the pathophysiology of osteoporosis is bone formation. A reduction in bone formation could lead to inadequate bone

replacement during remodeling and to gradual bone loss. This would be consistent with reduced "wall thickness," the amount of bone formed in a remodeling cycle (Compston and Croucher, 1991; Kimmel *et al.,* 1990), and reduced cancellous bone formation (Parfitt *et al.,* 1995), reported in some osteoporotic patients by histomorphometry of the iliac crest. However, using biochemical markers which measure bone turnover in the whole skeleton, several studies have shown on the average a significant increase in bone turnover (resorption and formation) in patients with postmenopausal osteoporosis, regardless of age (Delmas, 1993; and Chapter 74). It is possible that reductions in bone formation observed in iliac crest biopsies, the only accessible site in patients, are not representative of the skeleton as a whole. Nonetheless there is probably a population of patients, possibly among the very old ones, in which decreased bone formation plays a role in their osteoporosis, for example in growth hormone and/or IGF-1 deficiency (Langlois *et al.,* 1998; Kurland *et al.,* 1998). Both growth hormone and IGF-1 injected in humans were shown to increase the level of biochemical markers for bone formation as well as for bone resorption, although the total "bone balance" estimated by bone mineral density did not improve (Marcus, 2000). Our knowledge of the physiological control of bone formation during bone remodeling is more limited than that of bone resorption, which limits the understanding of "coupling" and effective use of bone-forming agents in therapy.

Following this general discussion of pathophysiological mechanisms, focused on the failure of bone remodeling to maintain adequate bone mass, we shall now briefly discuss each of the known etiological factors. This discussion is meant to complement related chapters in this volume.

Local Factors in Osteoporosis

There are many reasons for exploring the roles of local regulators of bone resorption and formation in the pathogenesis of osteoporosis. However, most of the evidence is either based on animal models or indirect data in humans. Direct evidence for an alteration in local factors in osteoporotic patients remains an exciting, but not yet realized, research goal. Among the reasons for exploring local factors are that it has been difficult to demonstrate consistent, relevant differences in the levels of systemic hormones in osteoporotic patients compared with appropriate controls (Lips *et al.,* 1987; Silverberg *et al.,* 1989; Tiegs *et al.,* 1985) and many osteoporotic patients have site-specific changes related to their particular fracture syndrome (Cummings *et al.,* 1995a). This exploration has been reinforced by the discovery of many local factors that influence bone metabolism. These are discussed in detail elsewhere in this volume. This chapter will summarize results that point to their possible pathogenetic role in osteoporosis. The problem has been difficult, not only because there are so many local factors, but also because these factors have complex effects on bone cells, probably related to multiple receptor and signal transduction systems. Moreover, these factors can be produced by nonbone cells adjacent to the skeleton, including hematopoietic and vascular elements.

Estrogen and Local Factors

Osteoporotic women and men are usually estrogen deficient. While estrogen may affect systemic hormones and play a direct role in the activity of differentiated bone cells, there is also evidence that estrogen can affect the production or activity of local factors, including cytokines, prostaglandins, and growth factors. There are many local factors that respond to estrogen, and these factors can modulate each other, so that decreasing or increasing a single factor may not give an accurate picture of its physiologic or pathologic role. Data from animal models and cell and tissue cultures suggest that estrogen deficiency increases and estrogen replacement decreases the activity of IL-1, TNF-α, and IL-6, as well as prostaglandins (Kawaguchi *et al.,* 1995; Kimble *et al.,* 1995; Manolagas and Jilka, 1995). Estrogen can increase TGF-β, leading to osteoclast apoptosis (Hughes and Boyce, 1997), and increase osteoprotegerin which would block both formation and activity of osteoclasts (Hofbauer *et al.,* 1999).

Cytokines and Osteoporosis

The cytokines that affect bone metabolism can be classified in three groups. IL-1 and TNF-α are potent stimulators of bone resorption *in vivo* and *in vitro.* IL-4, IL-13, and interferon-γ (INF-γ) can inhibit bone resorption. IL-6, IL-11, and related cytokines that interact with a receptor system involving the protein GP-130 probably act as stimulators of an early stage of osteoclast formation, thus enhancing bone resorptive responses to other agonists. All of these factors can influence prostaglandin production (see below).

IL-1 and TNF-α probably play a central role in mediating bone loss in the ovariectomized rat (Kimble *et al.,* 1995). Bone loss can be prevented in this model, not only by estrogen, but by a combination of IL-1 receptor antagonist and TNF binding protein. The role of TNF-α is further supported by the finding that bone loss after ovariectomy is blunted in a transgenic animal model in which the TGF binding protein is overexpressed (Ammann *et al.,* 1995). However, bone loss after ovariectomy is also blocked in transgenic mice which lack the IL-1 activating receptor (Lorenzo *et al.,* 1998).

These cytokines may come from marrow hematopoietic cells rather than bone cells. Marrow supernatants from ovariectomized animals stimulate prostaglandin production and bone resorption, and this effect can be blunted by adding the IL-1 receptor antagonist to the supernatants (Kawaguchi *et al.,* 1995). In humans, peripheral macrophages or marrow cells of estrogen-deficient women have been reported to produce more IL-1, TNF-α, and IL-6 (Pacifici *et al.,* 1991a;

Abrahamson *et al.,* 2000; Kassem *et al.,* 1996; Bismar *et al.,* 1995). However, these results are variable and not been confirmed in all studies (Hogasen *et al.,* 1995, McKane *et al.,* 1994, Kassem *et al.,* 1996). Nevertheless, there does appear to be increased bone resorbing activity produced by cultured monocytes of postmenopausal women, compared to premenopausal women (Cohen-Solal *et al.,* 1998).

IL-4 and IL-13 can inhibit bone resorption, in part by decreasing prostaglandin production (Kawaguchi *et al.,* 1996, Miyaura *et al.,* 1995; Nakano *et al.,* 1995). However, IL-4 has also been shown to enhance macrophage colony-stimulating factor production in marrow cells, which could have a positive effect on osteoclastogenesis (Lacey *et al.,* 1994). IL-4 overexpression in transgenic mice produces a picture of osteoporosis with low turnover and decreased osteoblastic function (Lewis *et al.,* 1993). The mechanism is not known and may depend on effects of IL-4 early in development.

A role for IL-6 is supported by two lines of evidence (Manolagas and Jilka, 1995). (1) IL-6 can enhance osteoclastogenesis when added with its soluble receptor. Some of this effect may also be due to stimulation of prostaglandin synthesis (Tai *et al.,* 1997). (2) IL-6 production is regulated by systemic hormones. Its hepatic production is increased by parathyroid hormone (PTH) (Mitnick *et al.,* 2000). An initial study suggested that the bone loss induced by ovariectomy did not occur in animals in which the IL-6 gene had been knocked out, but subsequent analysis of later generations of this line did not confirm this point. IL-6 antibodies can diminish osteoclastogenesis in cell cultures but do not prevent bone loss in ovariectomized animals and do not block the stimulation of prostaglandin production by marrow supernatants (Kawaguchi *et al.,* 1995, Kimble *et al.,* 1995). Circulating IL-6 levels decrease with age in humans, but no difference has been found between osteoporotic patients and age-matched controls (McKane *et al.,* 1994). However, marrow cells from estrogen-deficient humans produce more IL-6 (Bismar *et al.,* 1995). Other cytokines may be involved. IL-11 can enhance osteoclast production (Manolagas and Jilka, 1995), and LIF has biphasic effects on bone resorption (Lorenzo, 1991). Overexpression of LIF also results in osteoporosis in animals (Metcalf and Gearing, 1989).

Macrophage colony-stimulatory factor (M-CSF or CSF-1) is important in osteoclast formation and acts through a pathway involving the c-*fos* gene (Grigoriadis *et al.,* 1994). Animals with deficiency of M-CSF or a knockout of c-*fos* have osteopetrosis. However, overexpression of c-*fos* does not cause osteoporosis, but does result in increased bone and cartilage tumor formation.

Prostaglandins, Leukotrienes, and Nitric Oxide in Osteoporosis

Prostaglandins (PGs), leukotrienes (LTs), and nitric oxide (NO) can be produced by bone cells and adjacent hematopoietic and vascular cells and have potent effects on bone resorption and formation (Gallwitz *et al.,* 1993, Kawaguchi *et al.,* 1995, Ralston *et al.,* 1995). Their production is increased in a number of inflammatory models. These factors may interact with each other. For example, the response of the bone cells to fluid shear stress involves an increase in both NO and PG production, and both can be blocked by blocking NO synthesis (Klein-Nulend *et al.,* 1998). The increase in PG production is attributed to induction of COX-2. Impact loading increases both PG and NO production (Pitsillides *et al.,* 1995; Burger and Klein-Nulend, 1999). LTs can stimulate bone resorption by both prostaglandin-dependent and -independent mechanisms and may activate osteoclasts directly, in contrast to PGs, which increase osteoclastogenesis but have a direct inhibitory effect on the activity of isolated osteoclasts (Flynn *et al.,* 1999).

PGs may mediate some of the resorptive effects of cytokines. PGs have biphasic effects on both formation and resorption of bone. Exogenous PGs and circulating PGs produced by malignant tumors can increase both resorption and formation of bone in humans. There are data supporting a role for endogenous PGs in the bone loss associated with immobilization and ovariectomy in animals, and inhibitors of PG synthesis may decrease bone resorption, although their multiple effects on other organs limits their use (Bell *et al.,* 1994). This problem may be overcome by the use of COX-2 selective inhibitors (Raisz, 2001). On the other hand, the anabolic response to impact loading could be blocked by COX-2 inhibitors (Chow and Chamber, 1994).

Growth Factors and Osteoporosis

Both the IGF and TGF-α families are expressed abundantly in bone. It seems likely that a loss of the activity of these factors could be important in the impairment of bone-formation responses that is characteristic of osteoporosis (Boonen *et al.,* 1999). Studies of these factors are complicated by the presence of multiple binding proteins for IGFs and by the complex activation pathways of TGFβ. There is conflicting evidence concerning the levels of IGFs and their binding proteins (IGFBPs) in osteoporosis. Local production of IGFs and activation by changes in binding protein could play an important role. For example, IGFBP-5 may be a factor that binds and decreases the activity of IGF in bone (Nicolas *et al.,* 1995) as well as a direct stimulation of growth. PTH and PGE_2 can cause the release of IGFBP-5, and this, together with the ability of these hormones to increase IGF-1 production, may be responsible for some of their anabolic effect (Hakeda *et al.,* 1996). Decreased levels of TGF-β, but not IGFs, have been reported in ovariectomized rats (Finkelman *et al.,* 1992). Other growth factors such as fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) could be involved, but these have not yet been studied either in animal models or in human disease.

Systemic Factors in the Pathophysiology of Osteoporosis

In addition to local factors that are ultimately and mechanistically of pivotal importance in the cellular pathophysiology of osteoporosis, systemic regulators of mineral metabolism are believed to play an important role. Clearly, a reduction in circulating concentrations of estrogens is the single most important reason why postmenopausal women are at greater risk for bone loss than premenopausal women (Lindsay, 1995). More recent obseervations from the Study of Osteoporotic Fractures have established further that the low "residual" estrogen levels in postmenopausal women not taking estrogens are determinants in bone mineral density and in fracture risk (Ettinger *et al.,* 1998; Cummings *et al.,* 1998). The importance of estrogen sufficiency in men also has elegantly been elucidated in two male models of estrogen deficiency involving the estrogen receptor (Smith *et al.,* 1994) and the aromatase enzyme that converts androgens to estrogens (Morishima *et al.,* 1995; Carani *et al.,* 1997; Bilezikian *et al.,* 1998; Grumbach and Auchus, 1999). Animal knockout experiments of the alpha-estrogen receptor and of the aromatase gene provide further support for the key role of estrogens in male development (Lubahn *et al.,* 1993; Couse and Korach, 1999; Windahl *et al.,* 1999; Oz *et al.,* 2000).

The importance of estrogen in male skeletal health is amplified further in epidemiological studies correlating declining estrogen levels, but not declining testosterone levels, in age-related bone loss in men (Khosla *et al.,* 2001). Falahati-Nini *et al.* (2000) have shown recently that when men are rendered temporarily but acutely hypogonadal, estrogen is the prime regulator of the subsequent increase in markers of bone resorption.

The importance of androgens in establishing and maintaining bone mass is also well recognized (Orwell, 1996, 1998). In addition to the sex steroids, glucocorticosteroids are key systemic factors in bone metabolism. We recognize the glucocorticosteroids not so much for any major role they may play in normal mineral homeostasis but rather for their devastating impact on bone metabolism when present in excess (Reid, 1999). In Cushing's disease or when glucocorticosteroids are used therapeutically, bone loss is a major complication. Excessive thyroid hormone may also have a deleterious effect on bone metabolism (Stern and Lakatos, 1999). Elsewhere in this book, these points are considered in depth. This section deals primarily with evidence that casts a potential role for the calciotropic hormones in the pathophysiology of osteoporosis.

Vitamin D

1,25-Dihydroxyvitamin D, the active metabolite of vitamin D, enhances the absorption of calcium from the gastrointestinal tract, helps to regulate calcium handling in the kidney, serves to maintain normal bone remodeling, and is an important regulator of parathyroid hormone (Clemens and O'Riordan, 1995). Any abnormality in vitamin D formation, metabolism, or action could lead, therefore, to profound changes in calcium balance and result in bone loss. Many studies report a fall in the circulating concentration of 1,25-dihydroxyvitamin D with advancing age (Eastell *et al.,* 1991; Epstein *et al.,* 1986; Tsai *et al.,* 1984; Fujisawa *et al.,* 1998). More consistently, levels of the substrate for 1,25-dihydroxyvitamin D, namely 25-hydroxyvitamin D, fall as a function of age (Blumsohn and Eastell, 1995). Reduced concentrations of these vitamin D metabolites are thus considered to be one reason why vitamin D deficiency is implicated in age-associated reductions in bone mass.

Inadequate sources of vitamin D from either diet or sunlight are the most obvious possibilities for reduced levels of vitamin D in older individuals. In the skin, ultraviolet B, at 290–315 nm, converts 7-dehydrocholesterol to previtamin D (Clemens and O'Riordan, 1995) 7-Dehydrocholesterol levels in the skin fall by ~50% between 20 and 80 years of age (MacLaughlin and Holick, 1985). The process by which skin produces previtamin D is also a function of sunlight itself. In northern latitudes, sunlight may contribute very little to vitamin D stores during the long winter months (Bouillon *et al.,* 1987; Hodkinson *et al.,* 1973; Webb *et al.,* 1988, 1990). Even in Maine and Massachusetts, no synthesis of previtamin D occurs in the skin during the late fall and winter (Rosen *et al.,* 1994; Webb *et al.,* 1988). Other factors such as avoidance of sunlight and the use of highly protective UV-blocking creams may contribute to the diminishing importance of sunlight as a source of vitamin D.

The other source of vitamin D, the diet, is also a factor contributing to the potential for inadequate stores with age. Among adult Americans, vitamin D intake is rather marginal, 75–150 IU daily, well below the recommended daily allowance of 400–600 IU (Consensus Development Conference, 1994). In addition, mechanisms associated with absorption of vitamin D from the gastrointestinal tract begin to decline with age (Barragry *et al.,* 1978).

Subclinical vitamin D deficiency has been shown in some groups of osteoporotic American women (Villareal *et al.,* 1991). A number of studies indicate subclinical vitamin D deficiency among home-bound subjects or those living in extended care facilities (Chapuy *et al.,* 1992; Gloth *et al.,* 1995; Komar *et al.,* 1993).

Adequate vitamin D depends not only upon sufficient sources and absorption but also upon normal metabolism of the parent vitamin. Alteration of hepatic or renal hydroxylation of vitamin D or 25-hydroxyvitamin D, respectively, can lead to deficiency (Rao, 1993; Siegel and Bilezikian, 1995). The kidney becomes a pivotal organ in this discussion because it is the source of the active metabolite, 1,25-dihydroxyvitamin D. Factors that help to regulate the renal formation of 1,25-dihydroxyvitamin D are parathyroid hormone, phosphorus, calcium, and 1,25-dihydroxyvitamin D itself. A decline in the ability of the kidney to form 1,25-dihydroxyvitamin D develops with age (Gallagher, 1990).

Older individuals respond to the stimulating effects of parathyroid hormone on 1,25-dihydroxyvitamin D production less well than young, normal individuals (Riggs *et al.,* 1981; Tsai *et al.,* 1984). Despite attempts to demonstrate a specific defect in renal hydroxylating capacity in osteoporosis (Slovik *et al.,* 1981; Tsai *et al.,* 1984), most studies have equated the defect with declining renal function *per se* (Halloran *et al.,* 1990, 1996)

Other hypotheses related to the role of vitamin D in the pathogenesis of osteoporosis focus on reduced sensitivity of the small intestine to 1,25-dihydroxyvitamin D. Intestinal resistance to 1,25-dihydroxyvitamin D is a proposed primary alteration (Eastell *et al.,* 1991; Pattanaungkul *et al.,* 2000) due to an acquired alteration in binding of 1,25-dihydroxyvitamin D to its receptors in the small intestine (Francis *et al.,* 1984), or to an age-related reduction in intestinal vitamin D receptor concentration (Ebeling *et al.,* 1992; Gennari *et al.,* 1990; Horst *et al.,* 1990).

Finally, controversial observations implicating genetic polymorphisms of the vitamin D receptor point to possible underlying genetic bases for osteoporosis (Eisman, 1995; Peacock, 1995). Such genetic alterations could have as a functional counterpart altered physiological action of vitamin D on calcium absorption (Dawson-Hughes *et al.,* 1995). This subject, reviewed elsewhere, does not provide the kind of certainty that one requires to establish the vitamin D receptor in mechanisms associated with the pathophysiology of osteoporosis (Grant and Ralston, 1997; Cooper, 1999; Nguyen and Eisman, 2000).

The potential role of vitamin D in the pathophysiology of osteoporosis should be distinguished from overt vitamin D deficiency that is associated with the clinical syndromes of rickets (children) or osteomalacia (adults). This discussion highlights, instead, the concept that subclinical vitamin D inadequacy occurring by any of the mechanisms described above leads to suboptimal peak bone mass or to impaired maintainance of calcium balance. The clinical endpoint, osteoporosis, is not believed to be different from any other presentation of age-related osteoporosis. Thomas *et al.* have shown that subclinical vitamin D deficiency as defined by levels of 25-hydroxyvitamin D below 15 ng/ml occurs rather commonly among free-living subjects in Boston, Massachusetts, during both the winter and the summer months (Thomas *et al.,* 1998).

Parathyroid Function

Parathyroid hormone is a central regulator of calcium homeostasis by virtue of its actions to remodel bone, to reclaim filtered calcium in the kidney, and to facilitate absorption of calcium from the gastrointestinal tract via its action to stimulate the renal production of 1,25-dihydroxyvitamin D (Goltzman and Hendy, 1995). Normal bone remodeling depends upon normal dynamics and actions of parathyroid hormone. One could postulate that excessive or deficient parathyroid hormone could account for bone loss associated with aging. Indeed, two central, but opposing, hypotheses state that either too much or too little parathyroid hormone contributes to age-related bone loss (Bilezikian and Silverberg, 2000; Silverberg and Bilezikian, 2001). These two concepts will be exlored in this section.

There is now general agreement that parathyroid hormone concentrations increase with age. Immunoradiometric (IRMA) and immunochemiluminometric (ICMA) assays, which selectively measure circulating intact parathyroid hormone, have helped to demonstrate that the increase in parathyroid hormone is not due to declining renal function alone (Nussbaum and Potts, 1994). Earlier radioimmunoassays that measured circulating, inactive fragments typically showed increases that could have been due to reduced renal clearance of these fragments by the aging kidney. It is now clear, however, that there is a clear increase in biologically active levels of parathyroid hormone as a regular function of aging, independent of any change in renal function. The increase in parathyroid hormone may be associated with a similar rise in bone turnover as assessed both by bone markers (Duda *et al.,* 1988; Eastell *et al.,* 1988) and by histomorphometric indices of bone metabolism (Vedi *et al.,* 1982).

A key hypothesis implicating parathyroid hormone in the development of osteoporosis relates either to the increase in parathyroid hormone concentrations with age or to enhanced sensitivity at target organs (Kotowicz *et al.,* 1990; Riggs and Melton, 1983). If sensitivity to parathyroid hormone is enhanced in the postmenopausal state, for example, normal circulating concentrations of parathyroid hormone could be deleterious in older individuals. Enhanced sensitivity to parathyroid hormone has been invoked as an explanation for why, in the first decade after the menopause, primary hyperparathyroidism seems to be commonly revealed. However, there are no prospective data showing that in these women, primary hyperparathyroidism existed in a subclinical form before the menopause. Nevertheless, it is generally accepted that when postmenopausal patients with primary hyperparathyroidism are given estrogen, calcium levels decline without any change in the circulating concentration of parathyroid hormone (Marcus *et al.,* 1984; Selby and Peacock, 1986). These observations argue for an inhibitory effect of estrogen on parathyroid hormone action in bone and for enhanced sensitivity to the skeletal effects of parathyroid hormone when women become estrogen deficient. Such a mechanism could be operative in postmenopausal women without primary hyperparathyroidism. Some reports have attempted to show that for each picomole per liter rise in parathyroid hormone, osteoporotic women have a specific and quantitatively higher level of bone turnover and bone loss (Kotowicz *et al.,* 1990). However, enhanced sensitivity to parathyroid hormone has not been universally demonstrated (Ebeling *et al.,* 1992a; Tsai *et al.,* 1989).

The nuances of parathyroid hormone dynamics calls attention to the circadian rhythm of parathyroid hormone secretion. Daily parathyroid hormone secretion follows a biphasic profile with peaks at 1800 and 0200 hr (Calvo

et al., 1991; Markowitz *et al.,* 1988; Tohme *et al.,* 1995). Presumably, the larger nocturnal peak is secondary to mild hypocalcemia induced by nighttime fasting. In older osteoporotic women, whose baseline level of parathyroid hormone was elevated, it was shown that the normal diurnal pattern of parathyroid hormone secretion was not altered but rather that the nocturnal secretion of bone resorption markers was increased. Although Ledger *et al.* (1995) implicated parathyroid hormone as causative in the nocturnal increase in bone markers, other explanations, independent of parathyroid hormone, are equally plausible. Nevertheless, McKane *et al.* (1996) have shown that the age-related increase in parathyroid hormone can be controlled by increasing dietary calcium, albeit to rather high levels, ~2.5 g daily, and that concomitant reductions in elevated urinary markers of bone resorption are seen.

One avenue for investigation of a definitive role for parathyroid hormone in the pathogenesis of osteoporosis has focused upon a possible genetic basis. This possibility has been much less intensively studied than that for the vitamin D receptor but the results are similarly inconclusive. Suggestive, but by no means definitive, data have been provided, linking the parathyroid hormone type 1 receptor to osteoporosis (Duncan *et al.,* 1999). Another recent study found a polymorphism in the parathyroid hormone gene to be a marker for low bone mineral density in a group of postmenopausal Japanese women (Miayo *et al.,* 1999).

In the discussion of parathyroid hormone as a possible causative factor in the pathogenesis of osteoporosis, its interactions with estrogen are of particular interest. Clearly, there is functional interaction between parathyroid hormone and estrogens, best illustrated in the menopausal woman who becomes estrogen deficient. At this time, primary hyperparathyroidism often surfaces as a clinical disorder (Silverberg and Bilezikian, 2000).

In addition, use of estrogen in postmenopausal women with primary hyperparathyroidism is associated with a reduction in serum calcium levels (Marcus *et al.,* 1984a; Selby and Peacock, 1986). The reasonable postulate follows that estrogen is somehow an antagonist to the actions of parathyroid hormone. The logic follows that in the context of estrogen deficiency (i.e., the menopause), the "deleterious" actions of parathyroid hormone are no longer opposed.

Gallagher has shown that estrogen treatment increases calcium absorption with a concomitant increase in parathyroid hormone levels (Gallagher *et al.,* 1980). If this increase in parathyroid hormone is a response to a small decrease in serum calcium levels—assuming a direct effect of estrogen of gastrointestinal calcium absorption (see later)—one would expect to see an increase in PTH levels, but the results have not been consistent (Stock *et al.,* 1985; Marshall *et al.,* 1984; Boucher *et al.,* 1989). Several studies have proposed in fact that estrogen deficiency alters the set point for parathyroid hormone secretion in normal women (Boucher *et al.,* 1989), and that in osteoporotic women, blunted parathyroid gland sensitivity to circulating calcium is also seen (Cosman *et al.,* 1991). In addition, estrogen therapy in postmenopausal women leads to reduced bone resorption, but not bone formation, in response to parathyroid hormone (Cosman, 1993).

The concept of parathyroid hormone as a pathophysiological culprit in postmenopausal osteoporosis has been stated even more boldly by Riggs *et al.* (1998), who have proposed a "unitary model for involutional osteoporosis." In this hypothesis, Riggs *et al.* advance the idea that not only is parathyroid hormone involved in the early postmenopausal mechanisms of estrogen deficiency—a relative suppression—but that increases in parathyroid hormone are important later in the setting of age-related bone loss. They have proposed that in the second, age-related phase of bone loss, the actions of estrogen deficiency are no longer primarily at skeletal sites but rather at the gastrointestinal and renal systems. These extraskeletal actions of estrogen deficiency lead to increased urinary calcium excretion and reduced calcium absorption. The resulting secondary increase in parathyroid hormone then is responsible for the ensuing bone loss that continues as part of the aging process. The hypothesis could be strenghtened by more direct experimental observations that estrogens indeed do have important nonskeletal effects at these sites in the way the hypothesis envisions.

The hypothesis also is problematical from a chronological point of view. The actions of estrogens are seen in two time frames, the early one being a skeletal action and the later one being directed at nonskeletal sites. It is not clear how the actions of estrogen deficiency would affect these tissues differentially in time. Other confounding elements to the hypothesis are covered by Bilezikian (1998).

An important alternative hypothesis is that the age-related increase in parathyroid hormone is adaptive, protecting against bone loss, and that in osteoporosis, heightened parathyroid glandular responsiveness is required to maintain bone mass. In this context, reduced responsiveness of the parathyroid glands is viewed as contributing to the development of osteoporosis.

Altered responsiveness to parathyroid hormone could be due to changes in circadian rhythmicity of parathyroid hormone secretion. In contrast to the studies of Ledger *et al.* (1995) showing normal circadian rhythm of parathyroid hormone, Eastell *et al.* (1992) reported that women exhibited a blunted parathyroid hormone peak compared with men, and subsequently, a less dramatic decline in nighttime urinary calcium excretion. Nighttime urinary calcium excretion declined in men by 34% whereas in women, it decreased by only 17%. Postmenopausal osteoporotic women showed a further blunting of their nocturnal parathyroid hormone peak, with no change in fractional excretion of calcium at night (Eastell *et al.,* 1992). Such inefficient renal calcium conservation could contribute to the osteoporotic process by excessive nocturnal urinary calcium excretion.

In addition to a possible blunted circadian rhythm of parathyroid hormone associated with osteoporosis, Prank *et al.* (1995) have shown that pulsatile secretory dynamics of parathyroid hormone in osteoporotic women are abnormal.

Pulsatile parathyroid hormone secretion is normally superimposed upon the diurnal rhythmicity of the hormone, a phenomenon typical of many hormones besides parathyroid hormone, such as growth hormone, ACTH, and cortisol. Prank and colleagues postulated that such abnormalities in pulsatility could contribute to osteoporosis. The reduction in pulsatility could also be a physiological explanation for why intermittent parathyroid hormone is a promising therapy to restore bone mass in osteoporosis (Dempster *et al.,* 1993; Slovik *et al.,* 1986). The demonstration that intermittent administration of parathyroid hormone in low dosage is anabolic for osteoporotic bone further underscores the strength of the hypothesis implicating relative parathyroid hormone deficiency in osteoporosis while also illustrating the complexity of parathyroid hormone actions on bone. (Lindsay *et al.,* 1997; Kurland *et al.,* 2000; Neer *et al.,* 2000; Roe *et al.,* 1999).

Further evidence for abnormalities in parathyroid hormone secretory dynamics in osteoporosis comes from the work of Silverberg and colleagues (1989), who studied parathyroid hormone glandular responsiveness in osteoporosis. After a hypocalcemic stimulus, osteoporotic women demonstrated a much less exuberant increase in parathyroid hormone than their age-matched nonosteoporotic counterparts. It is of further interest that the normal older women exhibited a much greater response to the same hypocalcemic stimulus than younger subjects (Silverberg *et al.,* 1986), arguing for a physiological need for greater parathyroid hormone responsiveness with aging. The greater parathyroid hormone response was required to maintain 1,25-dihydroxyvitamin D levels in the older, nonosteoporotic subjects. It is not clear whether this age-related increase in parathyroid hormone responsiveness with aging reflects an altered set point for calcium or not (Cosman *et al.,* 1991; Ledger *et al.,* 1994).

The evidence thus seems to suggest that parathyroid hormone has the potential to be both catabolic and anabolic on the skeleton. This point is well illustrated further in primary hyperparathyroidism in which the anabolic potential for cancellous sites (vertebral spine) and the catabolic potential for cortical sites (distal radius) are often seen in the same patient (Silverberg *et al.,* 1989, 1994). How parathyroid glandular responsiveness and parathyroid hormone *per se* are important in the pathogenesis of osteoporosis remain to be clearly elucidated.

Calcitonin

Calcitonin, a natural product of C-cells in the thyroid gland, has intrinsic antiresorptive properties on bone (Becker *et al.,* 1995). Despite this property and several clinical examples of its antiresorptive efficacy in Paget's disease of bone and in hypercalcemia of malignancy, it has been difficult to establish a physiological role for calcitonin in normal calcium metabolism. Nor surprisingly, therefore, attempts to link deficiencies in calcitonin to osteoporosis have been disappointing (Blumsohn and Eastell, 1995).

Basal calcitonin concentrations, metabolic clearance, and production rates are all unchanged in osteoporosis (Reginster *et al.,* 1992). Calcitonin levels do not appear to decline with age (Body and Heath, 1983; Tiegs *et al.,* 1986). Moreover, in the menopause, calcitonin kinetics to provocation do not appear to be consistently altered (Pansini *et al.,* 1988; Stevenson, 1982). It would seem at this point that calcitonin is not an important systemic factor in the development of osteoporosis.

Summary

This chapter has reviewed underlying hypotheses to account for the pathophysiology of osteoporosis and the potential roles of local and systemic factors in its development. In chapters found elsewhere in this volume, many of these concepts are developed more fully in the context of what is currently known about the processes and mechanisms underlying the biological, biochemical, and molecular characteristics of the skeleton.

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CHAPTER 74

Evaluation of Risk for Osteoporosis Fractures

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Introduction

Osteoporosis is a systemic disease characterized by a low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in skeletal fragility and susceptibility to fracture (Anonymous, 1993). This definition implies that the diagnosis can and probably should be performed before any fragility fracture has occurred, which is a real challenge for the clinician. The level of bone mass can be assessed with adequate accuracy by measuring bone mineral density (BMD) using dual X-ray absorptiometry (DXA). However, this measurement does not capture all risk factors for fracture. Bone fragility also depends on the architecture and remodeling of bone, i.e., the quality of bone that cannot be readily assessed. It has been suggested that this component of bone strength may be assessed by ultrasonic measurements of bone and by measuring bone turnover using specific serum and urinary markers of bone formation and resorption. In addition the risk of fracture is also influenced by the propensity to fall and the ability to adapt to such falls, and these extraskeletal risk factors for fracture can be evaluated with simple questionnaires and clinical tests. In this paper we will briefly review the diagnostic values of clinical risk factors, bone mass assessment by DXA and ultrasound, and biochemical markers, and we will discuss the potential utility of combining these various tests for predicting fracture risk.

Clinical Risk Factors for Fractures

Clinical risk factors for osteoporosis can be used in two different ways: (1) to identify those women who are more likely to have osteoporosis and therefore who should have a BMD measurement; (2) to improve the fracture risk prediction in women who have already had a BMD measurement. Several clinical risk factors are associated with a low BMD, but none is powerful enough to predict BMD level in individuals (Aloia *et al.,* 1985; Liel *et al.,* 1988; Eiders *et al.,* 1989; Kleerekoper *et al.,* 1989; Stevenson *et al.,* 1989; Slemenda *et al.,* 1990; Pouilles *et al.,* 1991; Ooms *et al.,* 1993; Dawson-Hughes *et al.,* 1993; Kanis *et al.,* 1997). Some diseases such as anorexia nervosa, intestinal malabsorption, chronic renal failure, severe hyperthyroidism, Cushing's syndrome, and prolonged immobilization are often associated with osteoporosis, but their prevalence in the general population is low, and they have limited value for screening purposes. Conversely, some risk factors such as low calcium and high caffeine intake and alcohol and tobacco use have a high prevalence but a poor predictive value. Thus, models based on the combination of clinical risk factors evaluated by extensive questionnaires have failed to predict bone mass with sufficient accuracy to be useful in the management of the individual patient as they have poor sensitivity and specificity (Kleerekoper *et al.,* 1989; Slemenda *et al.,* 1990; Pouilles *et al.,* 1991).

We looked at the predictive value of clinical risk factors for osteoporosis in a cohort of 666 healthy postmenopausal women belonging to the OFELY cohort who completed an extensive questionnaire on fracture and medical history, diet, and exercise habits. We found that seven variables were independent predictors of femoral neck BMD, i.e., age, years since menopause, weight, history of fragility fracture after age 45, estrogen and corticosteroid therapy, and some rare diseases associated with osteoporosis. These were used to compute a score ranging from 0 to 19, and we found that a score of 4 had a sensitivity of 93% and a specificity of 61%

for detecting osteoporosis defined by a femoral neck BMD *T* score of $\leqslant -2.5$ (Albrand *et al.,* 1998).

More recently, in a population of 6958 elderly women age 75 years and over who participated in the EPIDOS study, it was found that the most predictive factors for very low BMD defined as a *T* score of $\leqslant -3.5$ were low body weight, history of fracture after the age of 50 years, slow gait, balance impairment, low grip strength, and dependence for instrumental activities of daily living. Using a score based on these risk factors has a sensitivity of 80% at the median cutoff (Dargent-Molina *et al.,* 2000). Weight was the strongest determinant of very low BMD and has about the same sensitivity as the complete score. If these findings can be confirmed in other larger cohorts, such simple rapid questionnaires might be useful for detecting those asymptomatic postmenopausal women who would benefit from a BMD measurement.

Clinical risk factors can also be used to improve the prediction of fracture risk in women who already had a bone mass measurement. In elderly women, several clinical features have been shown in prospective cohort studies to be associated with the risk of hip fracture even after adjustment for the level of hip BMD (reviewed in Eddy *et al.,* 1998). Ninety percent of hip fractures in elderly people result from a fall, and factors that influence the risk of falling and/or the protective response during a fall are likely to influence the risk of hip fracture (Grisso *et al.,* 1991). In a large prospective study of 9516 white American women, 65 years of age or older, Cummings *et al.* (1995) identified several fall-related risk factors that predicted hip fracture, including the use of long-acting benzodiazepines and anticonvulsant drugs, low physical activity, the inability to rise from a chair without using one's arms, visual impairment, and some indices of poor health status. Neuromuscular impairment increases the risk of falling but also influences the speed of adaptation, the coordination and the protective response during a fall. In a cohort of 7575 French women age 75 years of older, the risk of subsequent hip fracture was significantly increased, even after adjustment for femoral hip BMD, in women with a slow gait speed, with difficulties in doing a tandem (heel-to-toe) walk—i.e., with neuromuscular impairment—and in women with a visual acuity $<2/10$ (Dargent-Molina *et al.,* 1996). The incidence of hip fractures among women classified at high risk based on both a high fall-risk status and low BMD was found to be 29 per 1000 woman-years, compared with 11 per 1000 in women at risk on the basis of either a high fall-risk status or a low BMD, and with 5.4 per 1000 woman-years in those at low risk on the basis of both criteria (Dargent-Molina *et al.,* 1996).

Even with this combined approach, about one-third of women with hip fracture had not been identified as being at high risk, indicating that other factors are important in the pathogenesis of hip fracture. A maternal history of hip fracture doubles the risk of hip fracture even after adjustment for femoral neck BMD (Cummings *et al.,* 1995), stressing the importance of heredity in determining the geometry and structural properties of bones. One such factor is the length of the femoral neck, which has been shown in prospective studies to be associated with hip fractures, especially of the femoral neck (Faulkner *et al.,* 1993). A history of wrist and vertebral fractures, but also of any fracture, increases the risk of hip fracture after adjustment for BMD.

Although these clinical risk factors can easily be assessed with a rapid questionnaire and physical examination, they are probably of limited value in younger women. A personal history of fractures is still predictive, but their prevalence is much lower in women younger than 65 years of age. Fall-related factors have not been tested but are likely to be poor discriminants in younger and healthier women. On the basis of data generated in the Study of Osteoporotic Fractures, the National Osteoporosis Foundation of the USA recommended four clinical risk factors that are likely to provide information about the risk of osteoporotic fractures independently of the level of BMD: maternal history of hip fracture, personal history of fragility fracture after 45 years of age, smoking, and low body weight (<57 kg) (Eddy *et al.,* 1998).

Bone Mineral Density and Ultrasound Measurements

Several cross-sectional studies have shown that bone mass—measured by a variety of techniques including single and dual photon absorptiometry, DXA, quantitative computed tomography, radiography absorptiometry, and radiogrammetry of the metacarpal—is significantly lower in osteoporotic patients than in controls. The strength of the association has been confirmed by several prospective studies showing that each decrease of 1 standard deviation of BMD measured by DXA is associated with an age-adjusted 50–150% increase in the risk of osteoporotic fractures in postmenopausal women (Marshall *et al.,* 1996; Kanis and Gluer, 2000). The risk prediction has been found across various age ranges (but is weaker after 80 years of age), in ambulatory and long-term care residents (Broe *et al.,* 2000), and in studies with short- and long-term (up to 15 years) follow-up and is stronger when BMD is measured at the site of the future fracture. Measurements of BMD with an adequate technique at the spine, hip, forearm, or heel or total-body BMD/BMC have similar value in predicting the risk of all osteoporotic fractures. If the main concern is to detect the risk of hip fracture, measurement of hip BMD is preferable. Prediction of intertrochanteric fractures is improved by measuring the trochanteric area; prediction of femoral neck fractures is increased by measuring the femoral neck and further enhanced when measurement is limited to the upper half of the femoral neck (Schott *et al.,* 1998; Duboeuf *et al.,* 1997). The WHO definition of osteoporosis and low bone mass provides adequate cutoffs for identifying women at high risk of hip fracture, as shown in Fig. 1, from the EPIDOS study (Schott *et al.,* 1998).

Bone quantitative ultrasonography (QUS) is a promising recent technique for the diagnosis of osteoporosis (reviewed

Figure 1 Hip fracture incidence per 1000 women-years assessed prospectively over 2 years of follow-up according to baseline level of femoral neck BMD in 7598 healthy women 75 years of age and older. The EPIDOS study. Adapted from Schott *et al.* (1998).

in Jergas and Schmidt, 1999; Bauer, 1999). Provided that revised diagnostic criteria are used for QUS (Frost *et al.,* 2000) which, however, may be device specific, it has recently been shown that a similar proportion of postmenopausal women are identified as osteopenic as with spine or hip BMD (Frost *et al.,* 2001). Low calcaneal broadband ultrasound attenuation and speed of sound predict the risk of hip fracture in elderly institutionalized (Porter *et al.,* 1990) and healthy (Hans *et al.,* 1996; Bauer *et al.,* 1997) women with a power similar to BMD. More recently in a small prospective study of younger postmenopausal women, ultrasound transmission velocity measured at the distal radius and patella was predictive of all nonspine fractures, with a higher prediction of wrist fractures by distal forearm ultrasound and hip and ankle fractures by patella ultrasound (Gnudi *et al.,* 2000). It has been suggested that QUS may give information not only about BMD but also about the architecture and elasticity of bone, and that a combination of DXA and calcaneal QUS may be useful for identifying women at very high risk of fracture (Porter *et al.,* 1990; Hans *et al.,* 1996; Bauer *et al.,* 1997; Gnudi *et al.,* 2000). QUS is an attractive technique as it is relatively cheap, does not involve ionizing radiation, and is portable and is therefore suitable for screening strategies. There are, however, some unresolved issues: the accuracy, long-term stability and precision of the technique are unknown, and it is yet unclear whether the prospective data obtained with QUS of the calcaneus can be extrapolated to other sites (tibia, phalanges, patella) measured with devices that may have quite distinct technical features. Prediction of fractures others than hip remains to be further evaluated in large prospective studies. In addition, the various devices have not been cross-calibrated. Once these different issues are resolved, QUS may play an important role in the assessment of fracture risk.

Assessment of Fracture Risk by Biochemical Markers

Hormones

Because the trauma associated with hip fracture produces acute changes in hemodynamics and in a variety of hormones, hormonal measurements performed after fracture are difficult to interpret. Only data from prospective studies in which blood and urine sampling was performed prior to the fracture will be reviewed. Although vitamin D deficiency and age-related secondary hyperparathyroidism have been suggested to play a role in the skeletal fragility of elderly women, measurements of serum 25-hydroxyvitamin D (25-OHD), 1,25-dihydroxyvitamin D (1,25-(OH)2D), and serum parathyroid hormone (PTH) appear to have limited value in healthy women for the prediction of fracture. In the EPIDOS cohort, serum PTH and 25-OHD were not predictive of hip fracture risk (Garnero *et al.,* 1996b). These findings were confirmed in the Study of Osteoporotic Fractures (SOF) in women over 65 years of age showing no predictive value of serum PTH and 25 OHD for either hip or vertebral fractures (Cummings *et al.,* 1998). In the same study women in the lowest quintile for serum 1,25-(OH)2 D (<23 pg/ml) had a 2-fold increase in the risk of hip (but not of vertebral) fracture that was no longer significant after adjustment for

calcaneal BMD (Cummings *et al.,* 1998). In a younger population of healthy postmenopausal women from 50 to 89 years of age (OFELY study, mean age 64 years), we recently found no significant association between serum levels of 25 OH D and the risk fractures (40% vertebral, 60% nonspine fractures), whereas women in the highest quartile of PTH had a 1.8-fold increased risk (Garnero *et al.,* 2000a).

Estrogen deficiency is believed to be one of the few major determinants of postmenopausal bone loss and skeletal fragility. However, circulating 17β-estradiol levels explain only a small proportion of interindividual variability of BMD and bone loss (Slemenda *et al.,* 1996) and most of cross-sectional studies have failed so far to show different serum 17β-estradiol values in fracture cases and in controls. In a recent study, Cummings *et al.* (1998) showed that women 65 years of age and older with undetectable serum estradiol (<5 pg/ml) had a 2.5-fold increase in the risk of subsequent hip and vertebral fractures as compared with women with detectable serum estradiol, an association that remained significant after adjustment for age, weight, serum estrone, sex-hormone-binding globulin (SHBG) and calcaneal BMD. Such a finding was, however, not confirmed in another large prospective study (EPIDOS) in women 75 years of age and older in whom women with low estradiol levels were not at increased risk of hip fracture (Chapurlat *et al.,* 2000). In contrast, we found that elderly women with serum levels in the highest quartile were moderately protected from fracture with a relative risk of 0.66 compared to the other women. However, this protective effect disappeared after adjustment by body weight, suggesting that the association between increased estradiol levels and decreased hip fracture risk is mainly mediated by body weight. The reasons of the discrepancies between the SOF and EPIDOS studies on serum estradiol data are unclear and could be due to differences in body weight (higher in the SOF study), but more probably to difference in age which was on average 8 years lower in the SOF study. It seems indeed reasonable to believe that the role of residual secretion of steroids is more likely to play a role in younger postmenopausal women. This hypothesis is supported by results obtained in the OFELY cohort comprising younger postmenopausal women. In that population, serum estradiol levels in the lowest quartile (<11 pg/ml) had a relative risk of all osteoporotic fractures of 2.2 compared to the other women (Table I), an association that remained significant after adjustment for age and body weight (Garnero *et al.,* 2000a). In the same study we also found that decreased levels of dehydroepiandrosterone sulfate (DHEAs)—which can be metabolized to both active androgens and 17β-estradiol—were also associated with increased fracture risk independently of body weight and estradiol levels (Garnero *et al.,* 2000a). Thus, all together, these recent data suggest that the influence of the residual secretion of estrogen on the risk of fracture may decrease with advancing age. The increased risk associated with low estradiol levels in younger postmenopausal women does not seem to be mediated by increased bone resorption as in the OFELY study markers of bone resorption were predictive of fracture risk independently of serum estradiol levels (Garnero *et al.,* 2000a). A potential mechanism could be that low estradiol levels are associated with increased osteocyte death (Tomkinson *et al.,* 1997), a common finding in elderly women with hip fracture (Dunstan *et al.,* 1990). SHBG binds 17β-estradiol and thereby decreases its bioavailability. In the three above-described studies a high serum SHBG was moderately—and not consistently across all fracture types—associated with an increased risk of osteoporotic fractures (Cummings *et al.,* 1998; Garnero *et al.,* 2000a; Chapurlat *et al.,* 2000). In the SOF study women with both undetectable serum 17β-estradiol and a high SHBG had a marked increase in hip and vertebral fractures with relative risks of 6.9 and 7.9, respectively (Cummings *et al.,* 1998).

Table I Serum Hormone Levels and the Risk of Osteoporotic Fracture In Postmenopausal Women: The OFELY Study

Hormones at baseline	Cutoff of high risk	Relative risk (95% Cl)
Estradiol	Lowest quartile	2.2 (1.2–4.0)
	each 10 pg/ml decrease	1.5 (0.95–2.2)
DHEA sulfate	Lowest quartile	2.1 (1.2–3.8)
Sex-hormone binding globulin	Each additional 1 μg/dl	1.5 (1.1–2.1)
	Highest quartile	1.6 (0.9–2.9)
Estrone	Lowest quartile	1.5 (0.8–2.8)
Testosterone	Lowest quartile	1.4 (0.7–2.8)
PTH	Highest quartile	1.8 (1.01–3.4)
25 (OH) vitamin D	Lowest quartile	1.2 (0.6–2.2)

Note. Osteoporotic fractures (20 vertebral and 35 peripheral) were recorded during an average 5-year follow-up in 435 healthy postmenopausal women (mean age 64 years). Adapted from Garnero *et al.* (2000a).

Biochemical Markers of Bone Turnover and the Rate of Bone Loss

Several cross-sectional studies indicate that bone turnover increases rapidly after the menopause and this increase in both bone formation and bone resorption is sustained long after the menopause, up to 40 years (Garnero *et al.,* 1996a). BMD measured at various skeletal sites correlated negatively with bone turnover assessed by various markers in postmenopausal women. We have shown that the correlation between bone markers and BMD becomes much stronger with advancing age, so that in women more than 30 years after the menopause bone turnover accounts for 40 to 50% of the variance of bone mineral density of the whole skeleton (Garnero *et al.,* 1996a). These cross-sectional data suggest that a sustained increase of bone turnover in postmenopausal women induces a faster bone loss and therefore an increased risk of osteoporosis.

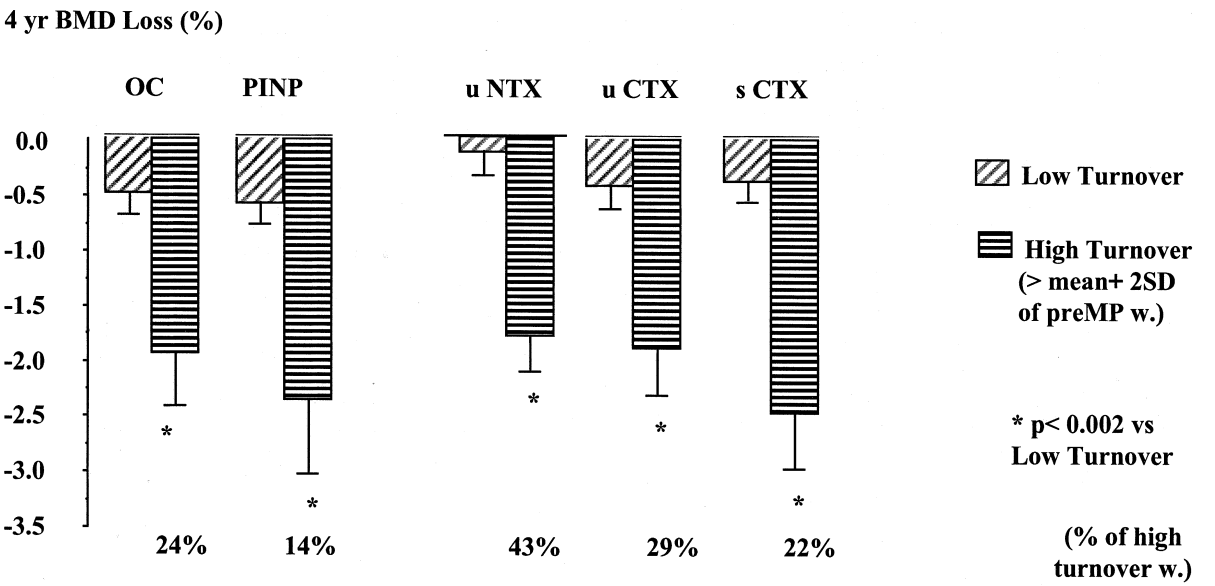

Figure 2 Rate of bone loss in postmenopausal women with high and low bone turnover. Bone loss at the forearm was evaluated by measuring BMD by DXA on four occasions over a 4-year period in 305 postmenopausal women (mean age, 64 years). For each bone marker each women was classified as a low or high bone turnover using as a cutoff the mean value + 2 SDs of 134 premenopausal women. The figure shows the percentage of bone loss from baseline during the 4-year study period in low and high bone turnover groups. Women whose baseline marker values indicated high bone remodeling lost bone two to six times faster over the next 4 years than those whose marker values were normal. Adapted from Garnero *et al.* (1999).

Longitudinal studies, which are necessary to avoid potential confounding factors, suffer, however, from methodological issues: indeed, when bone loss is assessed by annual measurement of BMD at the spine, hip, or radius over 2–4 years, the amount of bone loss is of the same order of magnitude as the precision error of repeated measurements in a single individual, i.e., 3–4%. This technical limitation precludes a valid assessment of the relationship between the rate of bone turnover and the subsequent rate of bone loss in individual postmenopausal women, and probably explains some of the conflicting results that have been published and recently extensively reviewed (Stepan, 2000). Indeed, the association between baseline levels of bone markers and rate of bone loss is more consistent and stronger when bone loss is measured at a very precise site such as radius than when measured at the spine and hip (Stepan, 2000). When the precision error on the rate of bone loss is reduced by performing nine measurements over 24 months, the correlation coefficient between bone markers and bone loss tends to improve from about −0.3 to −0.8 (Johansen *et al.,* 1988). Recently in a cohort of 305 postmenopausal women ages 50–88 years (mean 64 years) who had annual radius BMD measurements over 4 years, we have investigated the predictive value of baseline values of bone turnover markers (Garnero *et al.,* 1999). Serum and urinary C-terminal cross-linking telopeptide of type I collagen (CTX) and urinary N-terminal cross-linking telopeptide of type I collagen (NTX) for bone resorption, and serum osteocalcin and procollagen type I N propeptide (PINP) for bone formation, were found to be highly correlated with the rate of bone loss. In addition, in women within 5 years of menopause who had the highest rate of bone loss, correction of the observed correlation coefficients by errors on bone loss and bone marker measurements resulted in a marked increase in the predictive value of bone markers (Table II). Women with baseline values of bone turnover above the premenopausal range had a rate of bone loss four- to sixfold higher than women with a low turnover (Fig. 2). Ultimately, the weight of evidence will come from long-term studies. A retrospective study (Ross and Knowlton, 1998) performed over 13 years in women (mean age at baseline, 62 years) in whom calcaneal BMD loss was assessed by eight measurements, showed that 1 standard deviation increase in new specific bone markers such as osteocalcin, bone-specific alkaline phosphatase, and free pyridinoline cross-links was associated with a twofold increased risk of rapid bone loss defined as the upper tertile of rate of loss. There is currently only one prospective longitudinal long-term study in which the assessment of bone turnover with nonspecific markers (serum alkaline phosphatase, fasting urinary calcium, and hydroxyproline) at the time of menopause was correlated with the spontaneous rate of bone loss over the next 12 years. This study showed that early postmenopausal women classified as fast losers based on these markers had lost 50% more bone 12 years later than those diagnosed as slow losers (Hansen *et al.,* 1991).

In summary, with the current performance of bone marker and bone loss measurements, it appears that a single measurement of biochemical markers of bone turnover cannot predict the absolute rate of bone loss in an individual

Table II Correlation between Baseline Levels of Biochemical Markers of Bone Turnover and the Forearm Bone Loss over 4 Years in 51 Women within 5 Years of Menopause: The OFELY Study

Marker of bone turnover	Observed *r* value	*R* value after correction for error of the estimation on individual		
		Error on bone loss	Error on bone marker	Error on rate of bone loss and bone marker
Osteocalcin	−0.53	−0.72	−0.68	−0.91
PINP	−0.40	−0.54	−0.48	−0.66
U-NTX	−0.39	−0.53	−0.56	−0.76
U-CTX	−0.40	−0.55	−0.59	−0.81
S-CTX	−0.47	−0.64	−0.58	−0.79

Note. The error on the rate of bone loss was estimated from the mean and standard errors of the estimate for individual regression analyses of BMD against time. The error on the bone turnover marker levels was estimated from published intraindividual coefficients of variation for each marker. Correction of the observed correlation coefficients by errors on bone loss, bone markers, and both parameters were based on the formula proposed by Hassager *et al.* (1991). Adapted from Garnero *et al.* (1999).

woman. However, clearly increased levels of bone markers in postmenopausal women can be regarded as a risk factor for rapid bone loss in the subsequent years.

Markers of Bone Turnover and Fracture Risk

Several retrospective studies have compared bone marker levels in patients with osteoporotic fractures and in controls. However, bone turnover can change after a fracture because of immobilization, because of callus formation and/or because of the frequent regional activation of bone turnover (Ingle *et al.,* 1999a,b). To overcome these limitations, relationships between bone turnover markers and fracture have been investigated when samples were taken within a few hours after the hip fracture or several years after when the primary remodeling period of high activity has passed (reviewed in Garnero, 2000). Although most of these retrospective studies suggested that bone resorption is increased and bone formation is decreased in fracture cases compared to age-matched controls (Akesson *et al.,* 1993, 1995), we cannot exclude that a proportion of these changes of bone turnover may be the result of acute changes in body fluid and hormone levels related to the trauma. Thus, it seems difficult from retrospective studies to determine whether differences in bone turnover levels are related to the underlying rate of bone turnover leading to fracture, or to changes of bone turnover occurring after the fracture.

Relating baseline bone turnover levels with the subsequent risk of osteoporotic fractures is the valid methodology to assess their clinical utility. The previously mentioned study (Hansen *et al.,* 1991) was extended to a 15-year follow-up of 182 women during which 23 women experienced a peripheral fracture and 25 had one or more spinal fractures (Riis *et al.,* 1996). The fracture group had a significantly lower BMD than the group without fracture and a higher initial 3-year rate of bone loss after the menopause. Interestingly, bone mass and the rate of bone loss predisposed to fracture to the same extent, with odds ratios of about 2. Women with both initial low bone mass and an increased rate of bone loss had a threefold increase in the risk of fracture compared with the whole population. This study suggests that initial peak bone mass and postmenopausal rate of bone loss are both important determinants of osteoporosis.

More recently prospective studies have looked at the relationships between levels of biochemical markers of bone formation and bone resorption and the risk of fractures (reviewed in Garnero, 2000). For bone formation markers, conflicting results have been obtained. In the large multicentric cohort of elderly women in France (EPIDOS), no significant relationships were found between levels of serum osteocalcin and bone alkaline phosphatase and the risk of hip fracture occuring during a 2-year follow-up (Garnero *et al.,* 1996b). More recently in two prospective studies performed in younger healthy postmenopausal women [OFELY and Hawai Osteoporosis Study (HOS)], a significant positive association between increased levels of bone alkaline phosphatase and the risk of vertebral and nonvertebral fracture was observed (Garnero *et al.,* 2000a; Ross *et al.,* 2000). The different findings between these studies may be related to the type of fracture but more probably to the duration of follow-up which was of 22 months in the EPIDOS study and of 5 years in the OFELY study. As discussed above, high levels of bone formation markers are associated with a greater bone loss (Garnero *et al.,* 1999). Thus, if the increased risk of fracture is mediated partly through a more rapid bone loss (Hansen *et al.,* 1991), a follow-up of several years may be necessary to detect the association.

In contrast to bone formation markers, very consistent data on the relationship between increased levels of bone resorption markers and fracture risk have been reported in four large prospective studies (Rotterdam, EPIDOS, OFELY, and HOS). Indeed, levels of bone resorption assessed by urinary or serum CTX or urinary free deoxypyridinoline above the premenopausal range were consistently associated with about a twofold higher risk of hip, vertebral, and nonhip and

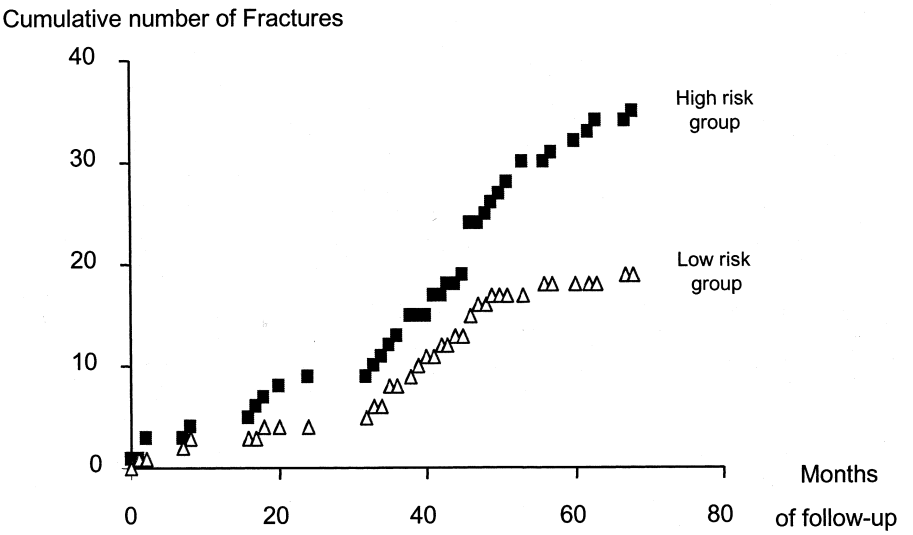

Figure 3 Cumulative number of fractures according to time of follow-up in postmenopausal women with baseline levels of urinary CTX in the highest quartile (high-risk group) and in women with levels in the three lowest quartile (low-risk group). The OFELY study. The number of incident fractures is higher in those women with baseline levels of urinary CTX in the highest quartile compared to women with levels in the three lowest quartiles, irrespective of time of follow-up. For the design of the OFELY study please refer to legend to Table I. Adapted from Garnero *et al.* (2000a).

nonvertebral fractures (Garnero *et al.,* 1996b, 2000a; Ross *et al.,* 2000; van Daele *et al.,* 1996) (Fig. 3). Serum CTX should be measured on fasting morning samples to reduce the variability of the measurement. The odds ratio was not modified after adjusting for potential confounding factors such as mobility status and were only marginally decreased after adjusting for BMD measured by DXA. Thus, the combination of BMD and bone turnover measurement allows the identification of a subgroup of elderly women at much higher risk of hip fracture than those identified by each test alone (Table III). Increased bone resorption was associated with increased fracture risk only for values above the upper limit of the premenopausal range, indicating that bone resorption becomes deleterious for bone strength only when it exceeds a normal physiological threshold, which appears to be the upper range of premenopausal women. These data also suggest that increased resorption could lead to increased skeletal fragility through two independent mechanisms. First, a prolonged increase of bone turnover will lead after several years to a lower BMD, which is a major determinant of reduced bone strength. Second, increased bone resorption above the upper limit of the normal range may induce microarchitectural deterioration of bone tissue such as perforation of trabeculae, a major component of bone strength.

Table III Combination of Bone Mineral Density (BMD) by DXA and Bone Resorption Markers to Predict the Risk of Fractures in Postmenopausal Women: The OFELY Study

	Odds ratio	Probability of fracture (%) (for 5 years)
All women		12.6
Low femoral neck BMD (T score <-2.5)	2.8 (1.4–5.5)	39
High S-CTX (T score $>+2$)	2.1 (1.2–3.8)	25
High urinary free DPD (T score $>+2$)	1.8 (1.0–3.4)	24
Low BMD + high S-CTX	3.8 (1.9–7.3)	54
Low BMD + high free DPD	2.1 (0.7–6.1)	45

Note. S-CTX, serum C-terminal crosslinking telopeptide of type I collagen; Free DPD, free deoxypyridinoline.

For the design of the OFELY study, please refer to legend of Table I. Adapted from Garnero *et al.* (2000a).

Modifications of type I collagen structure, the major organic component of bone tissue, is likely to be involved in decreased bone strength and increased susceptibility of fracture associated with osteoporosis. Indeed, several biochemical studies performed on bone specimens taken from patients with osteoporosis have shown abnormalities in posttranslational modifications of type I collagen molecules. These, which result from enzymatic processes, include an overhydroxylation of lysine residues, an overglycosylation of hydroxylysine, and a reduction in the concentration of nonreduced cross-links that can be associated with reduced bone strength (Bailey *et al.,* 1992; Kowitz *et al.,* 1997; Oxlund *et al.,* 1996). More recently new age-related nonenzymatic posttranslational modifications of type I collagen, so-called racemization and isomerization, have been demonstrated to occur in bone tissue and more specifically involve the aspartic acid residue in position 19

of the C-telopeptide of the alpha 1 chain (Fledelius *et al.,* 1997; Cloos and Fledelius, 2000) (see Chapters 12 and 90). The degree of isomerization and racemization of type I collagen can be detected *in vivo* by the urinary excretion of the different isoforms of CTX: α L CTX, which reflects newly synthesized collagen and three age-related isoforms, the isomerized isoform, β L CTX, the racemized form, α D CTX, and the isomerized and racemized form, β D CTX. In patients with Paget's disease of bone there is a larger increase of urinary α L CTX compared to β L CTX, resulting in a ratio, αL/βL, that was threefold higher than in controls (Garnero *et al.,* 1997). These data suggest that type I collagen within the abnormal woven pagetic bone, which is characterized by decreased strength, is associated with a decreased isomerization as indicated by a higher urinary αL/βL ratio, an hypothesis that was actually confirmed by immunohistochemistry analysis of pagetic bone tissue (Garnero *et al.,* 1997). More recently in the OFELY cohort, we found that the CTX ratios αL/βL and αL/αD were also increased in the 69 women who sustained incident fractures during an average 7-year follow-up compared to the 366 women of the same cohort who did not fracture (Garnero *et al.,* 2000b). In addition, an increased urinary ratio between native and age-related forms of type I collagen CTX was associated with increased fracture risk independently of the level of BMD of the hip and of bone resorption rate with an odds ratio of 2.0 (1.2–3.5). Thus, if confirmed in other prospective studies, a decreased degree of type I collagen isomerization/racemization as reflected by increased urinary αL/βL and αL/αD ratio, respectively, could be associated with alterations in the structure, maturation, or orientation of collagen fibers which may result in reduced bone strength.

Osteocalcin contains three residues of γ-carboxyglutamic acid (GLA), a vitamin K-dependent amino acid. It was postulated that impaired γ-carboxylation of osteocalcin could be an index of both vitamin D and vitamin K deficiency in elderly populations. In two prospective studies performed in a cohort of elderly institutionalized women followed for 3 years (Szulc *et al.,* 1993, 1996) and in a population of healthy elderly women (EPIDOS study) (Vergnaud *et al.,* 1997), levels of serum undercarboxylated osteocalcin over the premenopausal range, but not total osteocalcin, was associated with a two to threefold increase in the risk of hip fracture. More recently in a population of healthy home-dwelling women and men age 70 years and older, the ratio between carboxylated on total osteocalcin — an indirect index of undercarboxylated osteocalcin — was strongly associated with increased fracture risk, with highest predictive value in individuals older than 80 years (Luukinen *et al.,* 2000). Like markers of bone resorption, the prediction of undercarboxylated osteocalcin was still significant after adjusting for hip BMD, and those women with both a low bone mass and high undercarboxylated osteocalcin had a fivefold increased risk in hip fracture compared with the general population (Vergnaud *et al.,* 1997).

Combined Assessment of Fracture Risk

Cummings *et al.* (1995) looked at the value of combining a variety of clinical risk factors obtained by questionnaire and physical examination with BMD of the calcaneus to predict the risk of hip fracture in women over 65 years of age. The incidence of hip fracture ranged from 1.1 per 1000 woman-years among women with no more than two risk factors and calcaneal BMD in the upper tertile, to 27 per 1000 woman-years among those with five or more risk factors and BMD in the lowest tertile.

In the OFELY prospective cohort we have shown that combination of the strongest single clinical risk factor (history of fracture after the age of 45 years), with a low hip BMD and high levels of bone resorption assessed by urinary CTX improve the predictive value of a single test with relative risk of fracture increasing from 1.8–2.8 to 5.8 (Fig. 4) (Garnero *et al.,* 2000a). Similarly in a nested case–control analysis of the EPIDOS study, we have compared the ability of history of fracture after the age of 45 years, hip BMD, heel broadband ultrasound attenuation and urinary CTX to predict the risk of hip fracture and we investigated whether a combination of these parameters could improve the predictive value (Garnero *et al.,* 1998). The outcome of these combinations depends on whether the goal is to improve the sensitivity, or the specificity and therefore depends on the treatment strategy. If the strategy is to increase the sensitivity, i.e., to detect as many at risk patients as possible and to avoid false negatives, combining different tests is no more effective than lowering the diagnostic threshold of a single test. Conversely, combining two tests with adequate cutoffs is useful for increasing the specificity without decreasing the sensitivity obtained with a single test — a strategy to target a subset of high-risk patients. For example, the combination of urinary CTX with either hip BMD or heel BUA increases the specificity by 10% with a sensitivity similar to hip BMD or heel BUA alone. Such a combined diagnostic approach might be more cost-effective than BMD measurement alone, as it results in a lower number of patients to be treated to avoid one hip fracture. If DXA or ultrasound is not available, we found that the combination of a high bone resorption marker and a positive history of any type of fracture gave a predictive value similar to that obtained with BMD or heel BUA alone (Garnero *et al.,* 1998).

Conclusion

Osteoporosis can be identified early during the course of the disease before the occurrence of major fragility fractures such as vertebral and hip fractures using diagnostic tests with appropriate prognostic value. With the emergence of effective — but rather expensive — treatments, it is essential to detect those women at higher risk of fracture. Because the pathogenesis of fragility fractures is multifactorial, involving not only the level of BMD but also bone architecture, bone

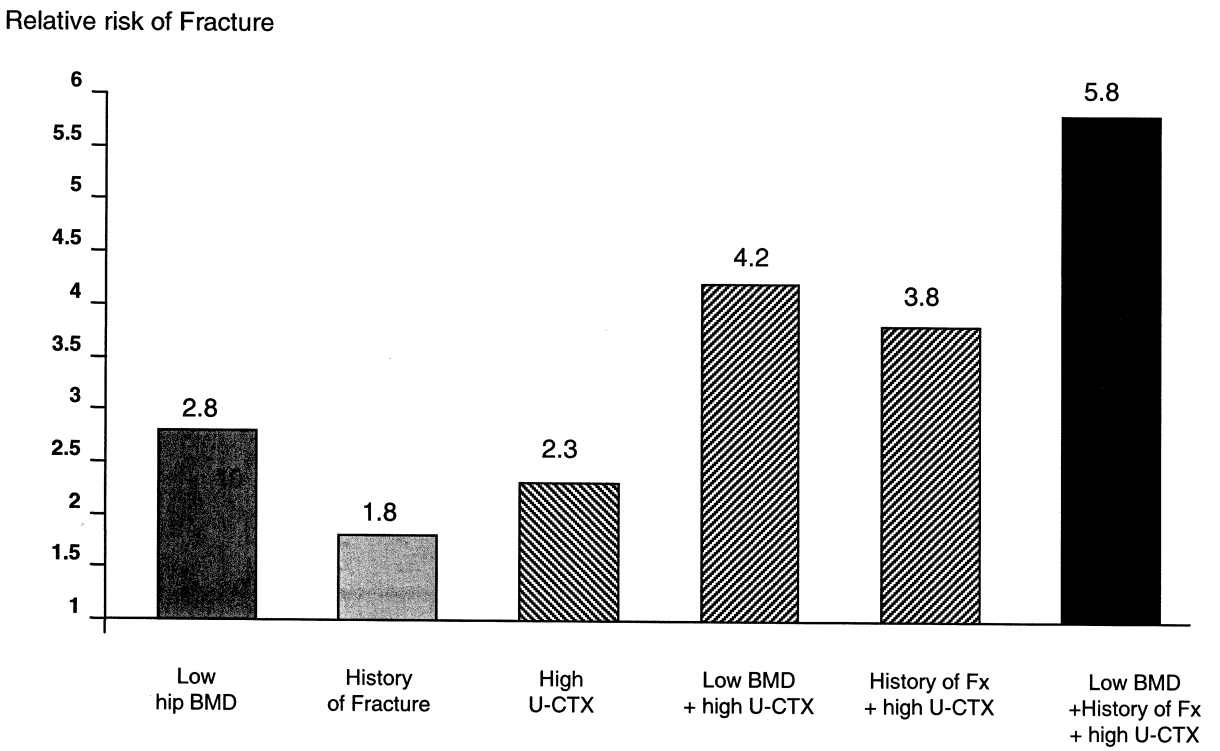

Figure 4 Combination of clinical risk factors, bone mass, and bone turnover measurements to identify women with the highest risk of fracture. Low hip BMD was defined as values at 2.5 SD or below the mean of young adults. High urinary CTX corresponds to values in the highest quartile. Relative risks are adjusted for age and physical activity. For the design of the OFELY study please refer to legend to Table I. Adapted from Garnero *et al.* (2000a).

turnover and fall-related factors, a global diagnostic approach is probably desirable. There is some evidence that such an integrated strategy using major clinical risk factors (e.g., history of fractures, low body weight), measurement of BMD by DXA or heel ultrasound and measurement of bone resorption by urinary or serum biochemical markers could be useful for detecting high-risk patients. These combinations of diagnostic tests should be validated in other prospective studies and operational thresholds should be developed and adapted to treatment strategies.

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PART III

Pharmacological Mechanisms of Therapeutics

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CHAPTER 75

Parathyroid Hormone

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Introduction

In all but the most recent textbooks of physiology, parathyroid hormone (PTH) is thought to increase serum calcium, partly by increasing the rate of bone remodeling. PTH is thought of as a bone-resorbing hormone. Although bone resorption is a prominent histological event seen in bone sections obtained from patients suffering from pathological secretion of PTH, osteoclasts do not present PTH receptors, and the enhanced bone resorption accompanying pathological PTH secretion appears to require the presence of osteoblasts (McSheehy and Chambers, 1986; Parfitt; 1976). Patients with primary hyperparathyroidism may be at increased risk for ongoing bone loss and fractures, together with evidence for increased bone turnover (Christiansen *et al.,* 1999; Grey *et al.,* 1994; Khosla *et al.,* Parisien *et al.,* 1995; Silverberg *et al.,* 1999).

The anabolic properties of exogenously injected PTH (bovine parathyroid gland extracts) were first reported in a series of serendipitous findings dating back to 1931 (Pehue *et al.,* 1931; Selye, 1932), in both human and animal situations. Some 45 years later Reeve and colleagues, citing circumstantial evidence in both clinical hyperparathyroid states and animal data, used a synthetic human PTH(1–34) amino terminal fragment to treat a small group of elderly individuals with osteoporosis. (Reeve *et al.,* 1976, 1980). They reported a dramatic histological improvement in bone turnover and structural trabecular growth.

Solid-state synthesis of peptides is expensive. However, the small number of clinical trials evaluating the potential of exogenous PTH therapy to treat osteoporosis, consistently demonstrated a beneficial anabolic effect. (Bradbeer *et al.,* 1992; Finkelstein *et al.,* 1994; Hesch *et al.,* 1989; Hesp *et al.,* 1981; Hodsman and Fraher, 1990; Hodsman *et al.,* 1991, 1993, 1997; Lindsay *et al.,* 1997; Neer *et al.,* 1987, 1991; Reeve *et al.,* 1981, 1987, 1990, 1991, 1993; Slovik *et al.,* 1981, 1986; Sone *et al.,* 1995). Commercial development of PTH awaited the technologic advances needed for inexpensive large-scale manufacture of peptides by fermentation. Indeed, the first report of a commercial dose-finding 12-month controlled clinical trial evaluating the efficacy of hPTH(1–84) on bone mineral density (BMD) against placebo is available only in abstract format (Lindsay *et al.,* 1998).

Advantages of Anabolic Agents for Reversal of Osteoporosis

There are strong epidemiologic associations between BMD measurements at either central (lumbar spine) or appendicular sites (femoral neck, distal radius, os calcis) and the current or future risk for osteoporotic fractures (Cummings, *et al.,* 1993; Marshall *et al.,* 1996; Ross *et al.,* 1991). There is approximately a twofold increase in the risk of fracture for every 1.0 standard deviation below average age- and sex-adjusted BMD, increasing dramatically if there is a preexisting fragility fracture in an individual with a low BMD (Marshall *et al.,* 1996; Ross *et al.,* 1991). Relatively small BMD increments reported in published placebo-controlled clinical trials, translates into an incident fracture reduction of approximately 50% within 1–4 years of therapy. The evidence for this is best illustrated in the randomized controlled clinical trials of the bisphosphonates (Black *et al.,* 1996; Cummings *et al.,* 1993; Harris *et al.,* 1999; Liberman *et al.,* 1995; McClung *et al.,* 2001; Reginster *et al.,* 2000).

There are currently two therapeutic strategies to treat osteoporosis. These strategies are illustrated in Fig. 1. For the purposes of this figure a fracture threshold has been indicated, but the gradient of risk is continuous (as it is for many biological variables such as blood pressure and cholesterol in ischemic vascular disease). The most important role for an anabolic agent in bone would be the rapid induction of new bone formation in the skeleton to reduce the risk of fragility fractures. It is against this background that the role of PTH therapy is discussed.

Anti-resorptive Agents

This approach relies upon the inhibition of osteoclastic activity during the bone mineralization unit (BMU) remodeling activity, allowing osteoblastic function to continue unimpeded. For example, the randomized, placebo-controlled clinical trials (RCT) of the bisphosphonates, alendronate and risedronate, have defined the benefits of this strategy, resulting in an increased BMD and significant reductions in incident fracture risk (Black *et al.,* 1996; Cummings *et al.,* 1993; Harris *et al.,* 1999; Liberman *et al.,* 1995; McClung *et al.,* 2001; Reginster *et al.,* 2000). Evidence from observational cohort studies suggests that estrogen also reduces osteoporosis-related fractures by up to 50% (Eiken *et al.,* 1996; Kiel *et al.,* 1987; Lofkin *et al.,* 1992; Maxim *et al.,* 1995; Naessen *et al.,* 1990), however, only one short-term RCT supports this observation (Lufkin *et al.,* 1992).

The large-scale RCT with raloxifene (a selective estrogen receptor modulator) demonstrated comparable anti-fracture efficacy for vertebral (but not for peripheral) fractures, after a gain in vertebral BMD of $<3\%$ over 36 months (Ettinger *et al.,* 1999). Another anti-resorption agent, calcitonin, was shown to reduce incident vertebral fractures without any significant increment in BMD (Chestnut *et al.,* 2000). These RCT's of anti-resorptive therapy have called into question that increments in BMD automatically translate into a reduced fracture risk. Nonetheless, overall gains in BMD at an axial measurement site following bisphosphonate therapy (typically 4–8% at the lumbar spine over 3 years) may reduce incident fractures by approximately 40–50%. Detailed reports of anti-resorptive therapies are reviewed in other chapters.

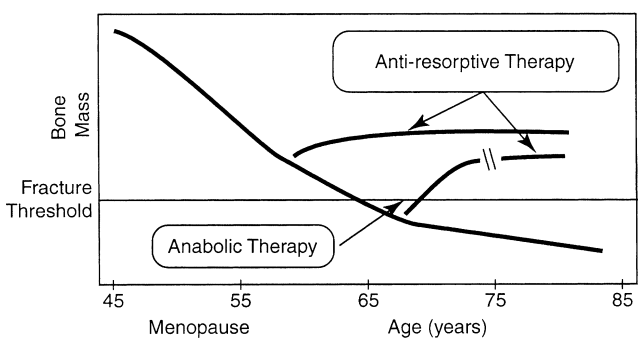

Figure 1 Depicts the relative efficacy of different strategies to maintain bone mass. (Top) Anti-resorptive strategy. Various agents (e.g., estrogens, bisphosphonates, calcitonin) will arrest ongoing age-related and postmenopausal bone loss. Reductions in bone resorption and turnover are observed, and gains in skeletal bone mass may occur due to the reduced bone resorption and perhaps to a reversal of the remodeling deficit. (Bottom) Anabolic therapy. Agents such as PTH and sodium fluoride directly stimulate osteoblastic activity to increase bone mass, despite concurrent increases in bone turnover (i.e., there is an increase in both bone formation and resorption). The rapid increase in bone mass should accelerate the reduction in future fracture risk more effectively than the use of an anti-resorptive agent alone.

Anabolic Agents

There are two principal agents which have been shown to stimulate osteoblast function within bone: sodium fluoride and analogs of PTH. Sodium fluoride and its slow-release formulations are discussed elsewhere in this publication. The landmark publication of Riggs *et al.* (1982) suggested that plain sodium fluoride significantly reduced vertebral fracture rates. A subsequent placebo-controlled trial demonstrated a rapid and linear gain in lumbar spine BMD of 9% per year over 4 years (Kleerekoper *et al.,* 1991). However, this result provided no evidence for a reduction in incident fractures (Kleerekoper *et al.,* 1991). Since the fluoride dose in this trial might have been toxic and adversely affected the matrix quality of newly formed bone, the lower dose, slow-release fluoride formulation studied by Pak *et al.* (1995) may be of interest. In this randomized trial, fluoride-treated patients gained BMD in the lumbar spine of 4% per year and experienced a 64% reduction in incident vertebral fractures.

By whatever outcome measure used, PTH analogs have been shown to be anabolic, resulting in comparable gains in bone mass by comparison with fluoride. These gains are considerably more rapid than those seen with anti-resorptive agents. However, fluoride therapy has not supported the dictate that gains in BMD translate into lower fracture rates. Since there are as yet few controlled trials reporting incident fractures in patients treated with PTH analogs, it is necessary to evaluate the potential mechanisms by which PTH has seemingly paradoxical anabolic effects, focusing on the preclinical data in osteopenic animal models, as well as the available clinical evidence.

Potential Mechanisms of Anabolic Action

The physiological action of PTH is to maintain the ambient concentration of ionized calcium in blood by: (a) influencing calcium reabsorption from the glomerular filtrate of kidney tubule cells and; (b) indirectly by enhancing calcium absorption from the gut through increased activity of renal vitamin D-1-hydroxylase, which produces $1\alpha,25(OH)_2D_3$ (Bilezikian *et al.,* 1994; Brabant *et al.,* 1992). PTH also initi-

ates a series of events which result in release of skeletal calcium by osteoclasts.

In 1931, Pehue *et al.* described the case history of an 8-year-old Parisian child who had succumbed to anemia due to the obliteration of his marrow space by bone; he was subsequently discovered to have had hypertrophic parathyroids (Pehue *et al.,* 1931). Subsequently Selye suggested that, in contrast to the findings in primary hyperparathyroidism, PTH could have a potent anabolic effect (Selye, 1932). Selye was able to mimic this clinical situation in 30-day-old albino male rats with daily injections of 5 IU of Lilly parathyroid extract for 30 days. These low doses of parathyroid extract resulted in the production of osteopetrotic bone with no sign of osteoclastic bone resorption. Numerous investigators have since confirmed the anabolic action of PTH (used as either the 1–84 holohormone or various amino-terminal analogs) when administered as multiple intermittent doses and have coined this effect "the PTH paradox" (Morley *et al.,* 1997).

The amino-terminal third of the PTH molecule appears to contain most of the biological activity of bone. Both the intact PTH(1–84) and the synthetic amino terminal 1–34 PTH peptide act through signaling pathways in target cells. In osteoblasts, the type 1 PTH/PTH-related protein (PTHrP) receptor is coupled to both the adenylate cyclase-activating G protein (Gs) and the phospholipase C-activating (Gq) protein. PTH requires the first two amino acids and some part of the amino acid 25–34 region to activate Gs, but only the 28–32 portion to activate Gq (Whitfield *et al.,* 1998). PTH fragments such as (1-desamino) hPTH (1–34), hPTH (8–84), or hPTH (28–48), which stimulate membrane bound protein kinase C (PKC), but not adenylate cyclase in isolated osteoblasts, do not stimulate bone formation in the oophorectomized rat model of osteoporosis. However, hPTH (1–31) amide, which stimulates the adenylate cyclase pathway as effectively as hPTH (1–34) or hPTH (1–84), but yet does not activate the PKC pathway, is capable of stimulating the growth of both trabecular and cortical bone in the same animal model (Rixon *et al.,* 1994; Whitfield *et al.,* 1996). Administration of analogs or activators of cAMP mimic many of the effects of PTH on osteoblasts in culture, thus strongly suggesting that the bone anabolic properties of PTH are associated with activation of the cAMP–protein kinase A pathway.

The exact cellular mechanisms and principal mediators of PTH action on osteoblasts have not been fully elucidated, but likely results from a combination of growth factor actions available in the immediate bone marrow environment, as well as the recruitment of new preosteoblasts from marrow stromal cells. There is strong evidence that PTH stimulates both the insulin-like growth factor (IGF) and transforming growth factor-β (TGF-β) systems in osteoblasts (Dempster *et al.,* 1993; Johansson and Rosen, 1998). The insulin-like growth factor regulatory system is composed of two growth factors (IGF-I and IGF-II), two receptors, six binding proteins which regulate IGF bioavailability, and IGF-binding protein (IG-FBP)-specific proteases. After PTH binding to the osteoblast PTH receptor and the generation of cAMP, these cells produce more IGF-II, IGFBPs -1, -4, and -5, and the IGFBP-3 and -5 proteases (Johansson and Rosen, 1998). PTH also directly stimulates the synthesis of TGF-β by mature osteoblasts (Canalis, 1996). IGF-I, IGF-II, and TGF-β secreted by osteoblasts can be bound to the collagen matrix of bone where they reside as a sort of growth factor bank. When PTH induces osteoclastic bone resorption during the bone remodeling cycle, growth factors are released into the immediate environment where they can act on resident osteoblasts (Canalis, 1996). The anabolic effect of PTH also results in the appearance of multistacked active osteoblasts (whose origin is uncertain) at sites of bone formation. They may be osteoprogenitor cells from marrow precursors, PTH responsive postmitotic cells which have been recruited to the site by chemotaxis, or bone lining cells which have been induced to re-enter proliferative cycles and pile up (Dobnig and Turner, 1995; Watson *et al.,* 1995).

Recent observations suggest that the receptor for PTH, the type 1 PTHR, may be targeted to the nucleus of receptive cells (Watson *et al.,* 2000a,b). In epithelial-derived cells of rat liver, kidney, uterus, ovary, and small intestine there exists a proportion of cells with PTHR immunoreactivity in the nucleus, an observation borne out by cell fractionation and Western and/or ligand blot analysis (Watson *et al.,* 2000a). A potential nuclear localization signal (NLS) has been identified in the cytoplasmic tail of the PTHR that is conserved across species (Watson *et al.,* 2000a). The PTHR is the first member of the G protein-coupled receptor family identified to have nuclear translocation activity (Burysek and Houstek, 1996; Jans and Hassan, 1998; Radulescu, 1995). It is well-documented that receptors of other growth factor (Jans and Hassan, 1998; Radulescu, 1995) cytokine families (Burysek and Houstek, 1996; Jans and Hassan, 1998) (e.g., the insulin and fibroblast growth factor 1 α receptor) translocate to the nucleus where they (or their cognate ligand) have a direct influence on nuclear function. The physiological implication of this data is, as yet, unclear but there is obviously the potential for the PTHR to have a direct effect on nuclear function independent of its nominal signaling pathways. In studies of bone cells in culture, the localization of the PTHR to the nucleus coincided with the onset of mitosis, perhaps suggesting a role for the nuclear PTHR in PTH anabolism via cell proliferation (Watson *et al.,* 2000b). Clearly, the anabolic effect of PTH is not through a simple mechanism but most likely involves a coordinated effort from all of its signalling abilities.

Animal Models of PTH Effects on Bone Metabolism

Detailed investigation of the anabolic action of PTH requires a suitable animal model. The ovariectomized (OVX) mature rat rapidly develops osteopenia which is characterized by a significant decrease in both cancellous bone mineral and bone mass, and a marked increase in osteoblast and osteoclast activity typical of increased bone turnover (Frost and Jee,

1992; Hock *et al.,* 1988; Hori *et al.,* 1988; Liu and Kalu, 1990; Liu *et al.,* 1991; Wronski *et al.,* 1989, 1991). The OVX rat has been widely accepted as a model for human postmenopausal osteoporosis since it closely parallels the early, rapid phase of bone loss characteristic of postmenopausal humans (reviewed in Kalu, 1991; Liu *et al.,* 1991). The osteopenia of OVX rats shares many characteristics in common with the bone manifestations of perimenopausal bone loss including increases in both bone resorption and activation frequency, with a relative deficiency in bone formation leading to a negative modeling balance (Frost and Jee, 1992; Kimmel *et al.,* 1993; Liu and Kalu, 1990; Liu *et al.,* 1991; Wronski *et al.,* 1989).

The OVX rat has been extensively used as a model for the *in vivo* anabolic action of intermittent treatment with PTH (Hori *et al.,* 1988; Jerome, 1994; Kimmel *et al.,* 1993; Liu and Kalu, 1990; Shen *et al.,* 1992; Tada *et al.,* 1990; Wronski *et al.,* 1993). Studies by the Kalu laboratory (Liu and Kalu, 1990) have shown that hPTH 1–34 treatment of mature, OVX rats prevents the 50% reduction in trabecular bone volume following OVX, and enhances bone volume to a level threefold that of sham-operated controls. PTH also reverses preexisting bone loss induced by OVX. (Kimmel *et al.,* 1993; Liu *et al.,* 1991; Shen *et al.,* 1992; Tada *et al.,* 1990; Wronski *et al.,* 1993). Dempster and colleagues have shown that the increase in bone formation rate after intermittent therapy with PTH is primarily due to an increase in both total mineralization surface and the mineral apposition rate which occurs in the first week after the initiation of treatment (Meng *et al.,* 1996). Measurement of BMD in PTH-treated rats has also shown positive increments in femur (Mosekilde *et al.,* 1994), vertebrae (Mosekilde *et al.,* 1994; Wronski; *et al.,* 1988), and tibia (Gunness-Hey and Hock, 1989; McMurtry *et al.,* 1992; Mosekilde *et al.,* 1994), confirming the histological data. All of these studies show a PTH-induced increase in bone formation chiefly by the induction of osteoblast function. Thus, intermittent PTH therapy increases osteoblastic bone formation via increased activation frequency, resulting in positive bone balance (Wronski *et al.,* 1993).

Another important consideration is the quality of the bone produced by PTH therapy. Data from the rat have shown that PTH works primarily by increasing trabecular thickness, not number (Kimmel *et al.,* 1993; Lane *et al.,* 1995; Li *et al.,* 1995; Shen *et al.,* 1993). Assessment of trabecular connectivity demonstrates that PTH produces well-connected trabecular bone of normal three-dimensional architecture when used in combination with estrogen as an anti-resorptive agent (Shen *et al.,* 1993, 1995; Tada *et al.,* 1990), but not when used on its own (Shen *et al.,* 1993). One last point to bear in mind is the biomechanical strength of PTH-induced bone, since nothing is gained if the newly formed bone does not provide the strength and support to protect against fractures. Numerous studies of load-to-failure rates in vertebral bodies, femoral shafts and femoral necks have shown that bone from PTH-treated OVX rats consistently performs better than that from sham-operated controls (Li *et al.,* 1995; Meng *et al.,* 1996; Mosekilde *et al.,* 1994, 1995; Shen *et al.,* 1995; Sogaard *et al.,* 1994). This pattern held true even in a primarily cortical bone site (femoral shaft) (Meng *et al.,* 1996) and correlated well with observations of increased tabecular thickness and bone mineral density (Li *et al.,* 1995; Meng *et al.,* 1996).

Much effort has been applied to the problem of either augmenting the bone formation induced by PTH therapy or maintaining the gains of bone following cessation of PTH therapy with an anti-resorptive agent. The bisphosphonates are the anti-resorptives of choice for a variety of reasons, but most notably because of their efficacy in achieving gains in bone mineral density in osteoporotic patients. Risedronate has been carefully evaluated to determine if concurrent therapy is additive to the anabolic effect of PTH in the osteopenic rat model. These studies have shown that combination therapy with risedronate and PTH provides no further advantage over PTH alone in the mature ovariectomized rat when vertebral bone mass and strength (Mosekilde *et al.,* 1994), and femoral neck strength (Sogaard *et al.,* 1994) are assessed. Similar results were obtained with aged ovariectomized rats after evaluation of the first lumbar vertebra and proximal tibia by histomorphometry. (Qi *et al.,* 1995). There has been some concern that PTH therapy may adversely affect cortical bone width and strength but studies with PTH alone or risedronate cotherapy show no effects of PTH on cortical bone in the ovariectomized rat and no advantage to risedronate cotherapy (Baumann and Wronski, 1995; Mosekilde *et al.,* 1995; Wronski and Yen, 1994).

However, studies with other bisphosphonates suggest that these compounds can blunt the anabolic effect of PTH on bone formation rates in cancellous bone of the ovariectomized rat. This has been found for pamidronate (Cheng *et al.,* 1995), tiludronate (Delmas *et al.,* 1995), and YM-175 (Mashiba *et al.,* 1997). Thus, *concurrent* therapy of bisphosphonates and PTH does not appear to confer therapeutic advantages. On the other hand, *maintenance* of bone mass following cessation of PTH therapy might be successfully achieved by bisphosphonates (Ritlmaster *et al.,* 2000).

Clinical Studies

There are approximately 32 published papers and numerous recent abstracts describing the outcomes of therapy with PTH (Bradbeer *et al.,* 1992; Finkelstein and Arnold, 1999; Finkelstein *et al.,* 1994, 1998a,b; Fujita *et al.,* 1999a,b; Gallagher, 1999; Hesch *et al.,* 1989b; Hesp *et al.,* 1981; Hodsman and Fraher, 1990; Hodsman *et al.,* 1991, 1993, 1997, 2000; Kurland *et al.,* 2000; Lane *et al.,* 1998a,b, 2000; Lindsay *et al.,* 1997, 1998; Neer *et al.,* 1987, 1993, 2001; Plotkin *et al.,* 1998; Reeve *et al.,* 1981, 1987, 1993, 1994, 1990, 1976a,b, 1980; Rittmaster *et al.,* 2000a,b; Roe *et al.,* 1999; Slovik *et al.,* 1981, 1986; Sone *et al.,* 1995). Treatment protocols have been very heterogeneous, over periods of 6 to 36

months. Most subjects have been women, over 60 years of age with a diagnosis of osteoporosis, but more recently there have been studies involving younger women with acute estrogen deficiency (Finkelstein and Arnold, 1999; Finkelstein *et al.,* 1998b, 1994), men with osteoporosis (Korland *et al.,* 2000) and glucocorticoid-induced osteopenia (Finkelstein and Arnold, 1999; Finkelstein *et al.,* 1998b; Lane *et al.,* 1998b, 2000). Only recently have the studies involved a randomized design in which acceptable control subjects did not receive PTH (Finkelstein and Arnold, 1999; Finkelstein *et al.,* 1998b, 1994; Fujita *et al.,* 1999b; Gallagher, 1999; Kurland *et al.,* 2000; Lane *et al.,* 1998b, 2000; Lindsay *et al.,* 1998; Neer *et al.,* 1999; Ross *et al.,* 1990). Many studies have included concomitant therapy with the specific intention of minimizing the resorptive properties of PTH; "cotherapy" with estrogen has been widely used (Bradbeer *et al.,* 1992; Lane *et al.,* 1998b, 2000; Lindsay *et al.,* 1997; Reeve *et al.,* 1990, 1991, 1993), but other studies have included calcitonin (Hesch *et al.,* 1989b; Hodsman and Fraher, 1990; Hodsman *et al.,* 1991, 1993, 1997, 2000) and calcitriol (Neer *et al.,* 1987, 1991; Reeve *et al.,* 1987; Slovik *et al.,* 1986).

There are many unknown issues raised by the use of PTH peptides as a prospective treatment for osteoporosis, although the evidence to date highlights only the potential benefits. We will discuss these clinical issues under the headings by which regulatory agencies currently examine data for the treatment of osteoporosis.

Fractures

While fracture risk reduction is the gold standard of efficacy for all osteoporosis treatments, there is at present limited information regarding the efficacy of PTH in this regard. Two of the recently published trials mention fracture rates as secondary outcomes (Hodsman *et al.,* 1997; Lindsay *et al.,* 1997), but neither study included true control groups for comparison. Hodsman *et al.* (1997) reported a vertebral fracture incidence of 4.5/100 patient years in a group of elderly patients with preexisting vertebral fractures and receiving eight 28-day cycles of hPTH(1–34) over 2 years (Hodsman *et al.,* 1997), Lindsay *et al.* (1997) reported an incidence of 2.5/100 patient years in a comparable group of patients receiving daily hPTH(1–34) injections over 3 years. Since both studies enrolled high-risk female patients with an average of >2 preexisting vertebral fractures, an average age of over 60 years, and comparable BMD measurements, they document fracture incidences in a high-risk population. A reasonable historical comparison can be made with the placebo (calcium supplemented) arm of the Fracture Intervention Trials, in which new vertebral fracture incidence rates were documented at 10/100 patient years in a cohort of women with only one prevalent osteoporosis-related vertebral fracture who were followed for 3 years (Black *et al.,* 1997).

The anti-fracture efficacy of PTH(1–34) has recently been reported in a placebo-controlled RCT, designed to evaluate the anti-fracture efficacy of this analog in post-menopausal women with prevalent vertebral fractures. (Neer *et al.,* 2001). At present this is the only Phase III trial specifically designed to address the anti-fracture efficacy of PTH. The significant trial outcomes for reduced incident vertebral and nonvertebral fracture rates together with BMD measurements can be compared to placebo-treated patients and are shown in Fig. 2 through 4. In this study, PTH(1–34) treatment in daily doses of 20–40 μg/day over 21 months resulted in a significant reduction in incident vertebral fractures by over 65% (Fig. 2, RR 0.43, CI 0.20–0.52). Even nonvertebral fractures were significantly reduced by up to 45% (Fig. 3), despite the relatively short duration of the trial. Thus it seems likely that PTH therapy has the potential to reduce incident fractures not only in the spine, but also at other sites. Although this is only the first trial of a PTH analog with sufficient power to evaluate fracture prevention, the findings contrast with trials of fluoride therapy, in which it has been difficult to detect non-vertebral fracture benefits, despite large increments in BMD. An evaluation of the absolute fracture reductions reported by Neer *et al.* (2001) after only 21 months of therapy with PTH(1–34) reveals a number needed to treat (NNT) estimate of only 10 and 27 women needing treatment to prevent one vertebral or one nonvertebral fracture, respectively. Recognizing differences in subject recruitment and duration of therapy, this compares favorably with the post hoc analysis of the Fracture Intervention Trials with alendronate reported by Ensrud *et al.* (1997); in women selected for alendronate therapy who are at comparable risk (prevalent vertebral fractures and femoral neck *T* score of <1.6), the NNT calculations to prevent one new vertebral fracture or any new clinical fracture were 16 and 26, respectively, albeit these estimates require up to 60 months of treatment.

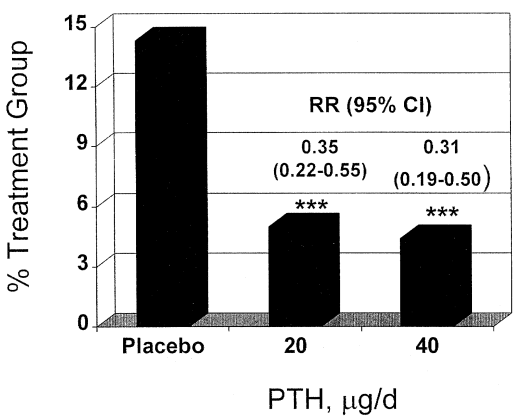

Figure 2 Reduction in vertebral fracture rates during a randomized controlled clinical trial of median duration 21 months. Compared with placebo ($n = 544$), both doses of PTH(1–34) (20 μg/day, $n = 541$; and 40 μg/day, $n = 552$) significantly reduced the risk of one or more vertebral fractures ($^{***}P < 0.001$). Adapted from Neer *at al.* (2001).

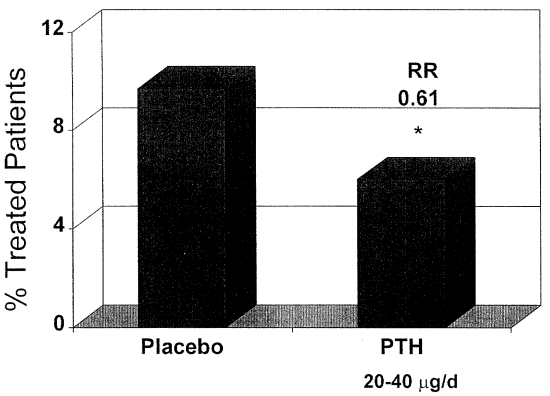

Figure 3 Reductions in all nonvertebral fractures during treatment with PTH(1–34) over 21 months. Adapted from Neer *et al.* (2001). Compared to placebo, new fractures were similarly reduced at both doses of PTH ($^{*}P < 0.04$ to < 0.02).

Bone Mass Measurements

Of all osteoporosis therapies in current use or in late stages of clinical development, PTH has shown the greatest effect on bone mass. Although trials of fluoride may have shown comparable or even greater gains in lumbar spine bone density, major concerns about the quality of new bone stimulated by this treatment remain to be settled. (Riggs *et al.,* 1990). Table I selectively summarizes the results of available studies of PTH therapy in which bone mass was measured. The data provided in the table are the percentage increase over baseline or placebo for the highest dose of PTH analog used in the trial. The method of measurement of bone mass or density (BMD) has varied among these studies and includes both dual-energy X-ray absorptiometry (DEXA) and quantitative computed tomography (QCT) for axial sites, as well as peripheral areal measurements of the forearm. Since these BMD measurements are not entirely comparable, only key points are summarized in this section.

Lumbar Spine

PTH causes a significant increase in BMD in the lumbar spine which exceeds that reported for all other available therapies except sodium fluoride. The studies of PTH as a monotherapy are of relatively short duration (2 years or less) so it is impossible to determine whether this increase is dose- and duration-dependent or if it reaches a plateau in a manner similar to those of antiresorptive therapies (Riggs and Melton, 1992). There are two RCTs of PTH monotherapy: a Phase II study of recombinant hPTH 1–84 (Lindsay *et al.,* 1998) and a Phase III study of recombinant hPTH 1–34 (Neer *et al.,* 2001).

In the first study, daily subcutaneous injections of 50, 75, and 100 μg of hPTH 1–84, were given to women with BMD criteria for osteoporosis (Kanis *et al.,* 1994), in a randomized placebo-controlled 1-year clinical trial sponsored by Allelix Biopharmaceuticals (Mississauga, ON, Canada) (Lindsay *et al.,* 1998). A dose-dependent increase in lumbar spine BMD measured by dual-energy X-ray absorptiometry (DEXA) was seen over the baseline measurement, reaching 6.9% in the 100 μg/d group (Lindsay *et al.,* 1998). Changes in bone mineral content were even greater, reflecting an apparent increase in the area of the vertebral bodies examined by DEXA. However, the total body bone mineral density (TBBM) decreased significantly compared to placebo over the 1 year of treatment by 0.9%. This finding raises the issue that PTH acts to increase trabecular bone mass at the expense of the cortical bone envelope. Alternatively, it seems likely that PTH causes acceleration of cortical remodeling, resulting in an enlarged expansion of the remodeling space. Increments in cortical bone mass would be expected to occur later, as the anabolic action of PTH gradually leads to a net remodeling gain over time. Within this study, the subsequent treatment of approximately one-third of the study cohort with an anti-resorptive agent, alendronate, for a second 12-month interval supports the latter alternative explanation (see below) (Rittmaster *et al.,* 2000b).

The Phase III RCT of PTH(1–34) in the treatment of osteoporosis (sponsored by Eli Lilly, Indianapolis, IN) was reported by Neer *et al.,* (2001). Subjects were recruited with prevalent vertebral fractures due to osteoporosis (Neer *et al.,* 2001). Subjects received daily subcutaneous injections of placebo or 20 or 40 μg of hPTH (1–34). The sponsor terminated this trial after an average of 21 months of therapy, because of a long-term toxicity study in Fisher rats in which a significant incidence of osteosarcoma was found during life-long treatment of these animals with very high doses of PTH(1–34) (Neer *et al.,* 2001). Nevertheless, dramatic effects on BMD and fracture incidence were observed in this clinical trial. In the spine, the increase in BMD was 9.7 and 13.7% in the groups receiving 20 and 40 μg, as opposed to an insignificant increment of 1.1% in the placebo group (Fig. 4). TBBM increased in the PTH-treated groups (Neer *et al.,* 2001) in contrast to the shorter 1-year study of hPTH(1–84) (Lindsay *et al.,* 1998), which reinforces the possibility that the initial loss of BMD reflects the opening of a remodeling space.

The general conclusion from all studies of lumbar spine BMD is that a progressive increase occurs for the duration of therapy (Table I). When PTH is stopped, two studies have shown a continuing gain in bone mass that may reflect the filling in of the increased remodeling space (Finkelstein and Arnold, 1999; Lane *et al.,* 2000). On the other hand, cessation of PTH therapy might be expected to result in reestablishment of the pretreatment pattern of bone losses. One year follow-up of patients in the study by Lindsay *et al.* (1997) suggests that this loss may be attenuated by the continuation of hormone-replacement therapy (HRT). However, a 4% annual loss in lumbar spine BMD was seen during the year after PTH therapy had been discontinued, in spite of continued HRT in another clinical trial combining PTH and HRT (reported by Roe *et al.,* 2000).

Table I Selected Publications Outlining Changes in Bone Mineral Density Measurements during and/or Following PTH Therapy

Author[a] (year)	Age (years)	No. of subjects: %	PTH dose[b]	Study duration (months)	Concomitant therapy	Controls	BMD[c] (% change from baseline)
Postmenopausal women							
Reeve *et al.,* 1990	64	11:1	500 units/day	12	Estrogen or androgen	12 Patients on fluoride	QCT spine +50% Foream ns
Neer *et al.,* 1993	N/A	15:0	400–500 units/day	12–24	Calcitriol	15 on Ca	Spine +12% Forearm −5.7%
Sone *et al.,* 1995	80	14:2	20 units/day	6	None	None	Spine +3.7%
Lindsay *et al.,* 1997	60	17:0	25 μg/day	36	Estrogen	17 on Estrogen	Spine +13% Total hip +2.7% Forearm ns, TBBM +8.0%
Hodsman *et al.,* 1997	67	39:0	65 μg/day, 28-day cycles	24	Calcitonin	None	Spine +10.2% Femoral neck +2.4%
Lindsay *et al.,* 1998, Abstract	65	162:0	PTH (1–84) 50,75,100 μg/day	12	None	54	Spine +6.9% Hip n.s. TBBM −0.9%
Fujita *et al.,* 1999a	70	115:0	15,30,60 μg/week	11	None	None	Spine +8.1% metacarpal cortical thickness ns
Gallagher, *et al.,* 1999, Abstract	Postmenopause	102:0	PTHrP analog (semparatide) 12.5–100 μg/day	6 on 6 off	None 6 months on alendronate	25	Spine +8.8% Total hip +3.2% Forearm −1.9% TBBM −1.9%
Roe *et al.,* 1999, Abstract	Postmenopause	30:0	40 μg/day	24	Estrogen	30	QCT spine +79% Spine +29.2% Femoral neck +11%
Rittmaster *et al.,* 2000b	64	66:0	Maintenance after 12 months PTH (1–84) (see Lindsay 1998)	12 on 12 off	None Alendronate 10 mg/day	66 women: all on alendronate	PTH pretreated patients: Spine +14%, Femoral neck +4.5% TBBM +3.3%

continues

Table I *Continued*

Neer *et al.,* 2001	69	1093:0	20, 40 μg/day	21	None	544 on Ca/D	Spine +13.7% Femoral neck +5.1% Forearm −3.2% TBBM +1.0% (hologic) TBBM +4.5% (lunar)
Other causes of osteoporosis							
Slovik *et al.,* 1986	50	0:8	400–500 units/day	12	Calcitriol	None	QCT spine +98%
Hesch *et al.,* 1989	50	2:6	750 units/day hPTH (1–38),70 day cycles	14	Calcitonin	5 on other treatments	QCT spine +12 to +89% Forearm no change
Finkelstein *et al.,* 1998a[1]	31	21:0	40 μg/day	12	Nafarelin	22 on Nafarelin	Spine, hip, forearm TBBM [**]; ns
Lane *et al.,* 1998a[2]	62	28:0	40 μg/day	12	Prednisone Estrogen	23 on Prednisone + estrogen	QCT Spine +35% Spine +11%, All other sites ns
Finkelstein *et al.,* 1999[1]	34	15:0	None Follow-up Observation post-Nafarelin	12	None	Remained within original cohort	Both groups gained spine BMD after stopping Nafarelin, but prior PTH-treated group gained more
Lane *et al.,* 2000[2]	62	28:0	40 μg/day	12 on +12 off	Prednisone Estrogen	23 on Prednisone + estrogen	QCT spine +45.9% Spine +11% Femoral neck +5.2%, Forearm ns
Kurland *et al.,* 2000	50	0:10	40 μg/day	18	None	13 on Ca/D	Spine +13.5% Femoral neck +2.9% Forearm −1.2% (ns)

Note. TBBM, total body bone mineral (usually excludes skull). Data are significantly different from controls unless stated (ns).

[**] Control group had a significant loss of BMD (i.e., PTH prevented loss).

[a]Superscript by author indicates published data from the same study cohort.

[b]Analog is hPTH(1–34) unless otherwise stated.

[c]BMD measurement made by DEXA or similar areal method unless stated for QCT. (quantitative computed tomography).

Hip

There are differences in the BMD responses to PTH among different regions of the skeleton. One of the major concerns about "anabolic agents" is that the increase in spinal (largely trabecular) bone mass may be at the expense of cortical bone, in particular, at important fracture regions such as hip and forearm. Some studies with sodium fluoride have shown dramatic increases in the spine but loss, or no improvement, at cortical bone sites. If PTH causes a marked increase in cortical bone remodeling, one might expect a decline in BMD in cortical sites during the early stage of PTH therapy, with a later increase if the net effect of PTH is increased bone formation. In reviewing the results of the clinical studies which included BMD measurements at the hip (Table I), a net positive effect of PTH is seen.

Several studies showed no change or a decline in femoral neck or total hip BMD within the first year of treatment (Finkelstein *et al.,* 1998a; Lane *et al.,* 1998a), and this is particularly clear in the 1-year study of hPTH(1–84) (Lindsay *et al.,* 1998), in which there was no change in hip BMD.

The few studies, which have gone beyond 12 months of therapy, have shown a gain in hip BMD (Hodsman *et al.,* 1997; Kurland *et al.,* 2000; Lindsay *et al.,* 1997; Neer *et al.,* 2001; Roe *et al.,* 1999). In the Phase III trial of hPTH(1–34) reported by Neer *et al.* (2001), there were significant BMD gains seen in the hip after just under 2 years of therapy. In the total hip BMD measurements, the placebo group lost 1.6% while gains of 2.6% (20 μg dose) and 3.6% (40 μg dose) were seen in the PTH-treated groups (Fig. 4). Similar dose-dependent improvements in trochanter, femoral neck, and intertrochanteric regions were observed, and the 5.1% increase in femoral neck is greater than that reported for any anti-resorptive therapy after 2 years.

Again, these observations can be explained by postulating the activation of an increased number of bone remodeling units, resulting in an increased remodeling space within the skeleton. During the early stages of PTH therapy, this may cause a decline in BMD in some areas, but as the enlarged remodeling space begins to be filled, increased hip BMD appears as the net result.

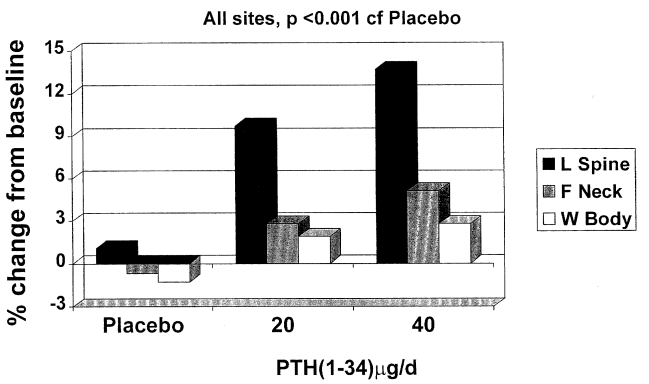

Figure 4 Changes in BMD measurements over 21 months during a randomized placebo-controlled clinical trial of PTH(1–34). Adapted from Neer *et al.* (2001).

Forearm

The earlier studies of PTH have shown inconsistent results in the forearm. In reviewing studies in which PTH was not being used in combination with estrogen, three small studies showed no change (Hesch *et al.,* 1989b; Neer *et al.,* 1987; Reeve *et al.,* 1987), and three showed significant decline (Hodsman *et al.,* 1991; Neer *et al.,* 1997, 2001). One study, using a PTHrP analog (semparatide acetate) reported by Gallagher (1999) also showed a decrease in forearm BMD. The best assessment of hPTH 1–34 effects on BMD of the forearm probably comes from the Phase III trial reported by Neer *et al.,* which averaged 21 months of therapy. It showed a significant, though modest 2–3% decline in BMD of the forearm. This may be a concern, but the decline was not progressive in the second year of the study (Neer *et al.,* 2001).

Total Body Bone Mineral

In the 1-year Phase II randomized placebo-controlled trial of hPTH 1–84, there was a significant decline in TBBM content as assessed by DEXA (Lindsay *et al.,* 1998). In the subjects receiving 75 and 100 μg PTH(1–84), there was a slight but significant decline (0.3 and 0.9%, respectively) in TBBM, reflecting loss in the arms and legs. This loss was thought to represent a generalized increase in cortical bone turnover and would be expected to be transient. Longer term studies with PTH(1–34) have demonstrated small increases in TBBM as shown in Table I and Fig. 4 (Lindsay *et al.,* 1997; Neer *et al.,* 2001).

PTH in Combination with Other Treatments

During the past 20 years, PTH therapy has been used in a heterogeneous fashion: as a single agent, with or without nutritional calcium and vitamin D supplements (Fujita *et al.,* 1999a; Hesp *et al.,* 1981; Kurland *et al.,* 2000; Neer *et al.,* 2001; Reeve *et al.,* 1980a,b, 1981, 1976a,b), with estrogen or androgen as a concurrent anti-resorptive agent (Bradbeer *et al.,* 1992; Lane *et al.,* 1998a; Lindsay *et al.,* 1995, 1997; Reeve *et al.,* 1990, 1991; Roe *et al.,* 1999), with bisphosphonates (Rittmaster *et al.,* 2000b), with calcitriol (Neer *et al.,* 1987, 1991, 1993; Reeve *et al.,* 1987; Slovik *et al.,* 1986); in several cyclical protocols with or without calcitonin (Hesch *et al.,* 1989b; Hodsman and Fraher, 1990; Hodsman *et al.,* 1991, 1993, 1997; Reeve *et al.,* 1987).

Despite the heterogeneity of these protocols, the overall effect of PTH in combination with other therapies has been remarkably consistent: an increase in trabecular bone (as measured by BMD or histomorphometry), with somewhat

smaller changes in bone mass in the appendicular skeleton. Most reported studies had small samples of treated subjects and thus a low power to dissect the interactions of concurrent therapies. Only a minority of studies have evaluated the effects of PTH in a truly randomized trial design in which control subjects received only nutritional supplements of calcium and vitamin D (Fujita *et al.,* 1999a; Gallagher, 1999; Kurland *et al.,* 2000; Lindsay *et al.,* 1998; Neer *et al.,* 2001; Sone *et al.,* 1995). However, some guarded conclusions can be made.

Estrogen

The concurrent use of anti-resorptive agents, specifically estrogen is attractive. If estrogen can selectively blunt the resorptive action of PTH on bone without deleterious inhibition of its anabolic effects, this combination would be ideal. The experimental literature is confusing. Cosman *et al.* infused PTH over 20 hr (approximately 800 units to each of 17 estrogen-treated and 15 estrogen-deficient postmenopausal women with osteoporosis) (Cosman *et al.,* 1993). The estrogen-deficient women had a significantly higher excretion of bone resorption markers (urinary OH–proline and deoxypyridinoline) suggesting a protective effect of estrogen on resorption. However, in a similar experiment Tsai *et al.* (1989) infused 400 units/day for 3 days to three groups of subjects, premenopausal, and postmenopausal women with and without osteoporosis. They found no differences in serum calcium or urinary OH-Pro excretion and concluded that estrogen did not have such a protective effect. Using an alternative approach of calcium deprivation to induce endogenous 2° hyperparathyroidism, the same group reached similar conclusions (Ebeling *et al.,* 1992). Marcus *et al.* (1992) gave acute (20 min.) infusions of graded doses of PTH to 15 postmenopausal women before and after starting estrogen replacement, and no differences were found in serum $1,25(OH)_2D$ increments or urinary cAMP excretion as a result of estrogen therapy. They concluded that the renal–endocrine axis was not affected by estrogen deficiency. The flaw in these arguments is the fact that clinical responses to PTH infusion favor bone catabolism, while intermittent injections favor anabolism (Hodsman *et al.,* 1993).

In the study by Reeve *et al.* (1991), daily PTH injections and concurrent estrogen therapy were combined in nine women. By comparison with historical controls (women treated with PTH alone) (Reeve *et al.,* 1980b), calcium balance studies were significantly improved; cotreatment with estrogen actually led to a 12% decrease in urinary calcium (Reeve *et al.,* 1991), rather than the 14% increase seen historically (Reeve *et al.,* 1980b). To date there are no controlled factorial studies to test the estrogen effect independently of PTH.

However, many clinical studies of PTH therapy have incorporated concurrent therapy with estrogen, or estrogen/-progesterone therapy (HRT; see Table 1). The intent of these combinations has been to limit the ongoing bone resorption induced by PTH. In this context Lindsay *et al.* (1997) reported a progressive total 3-year net gain over baseline lumbar spine BMD of 13%. In a study of glucocorticoid-treated women, who were all on stable HRT, Lane *et al.* (1998a) reported similar increments of 11% in the spine (35% by QCT) over 1 year. In a more recent RCT, employing the same dose of PTH(1–34) (40 μg daily), in postmenopausal women receiving HRT, Roe *et al.* reported average gains of BMD in the lumbar spine of 29% over 2 years (79% increments at this site as measured by QCT) (Roe *et al.,* 1999, 2000). It is difficult to reconcile the dramatic differences in BMD increments on the one hand between the two studies reported by Lindsay *et al.* (1997) and Lane *et al.* (1998a) and on the other hand by Roe *et al.* (1999, 2000), despite the reported dose of PTH being similar for all three studies. Possibly the absolute BMD of patients reported by Roe *et al.* who were approximately 8 years older was much lower than in the other studies, leading to a much higher relative gain. Alternatively the specific activity of the preparation used by Roe *et al.* may have been much higher, leading to a larger functional dose.

Bisphosphonates

No clinical studies combining bisphosphonates with PTH have been reported to date. As discussed in section 4, the data for bisphosphonate interactions with PTH in animal models are also unclear, with contradictory evidence that the *concurrent* use of bisphosphonates may blunt the anabolic agents of PTH. This is an important issue. Given the increase in skeletal bone remodeling induced by an activation drug such as PTH (at the BMU level), it is likely that the remodelling space within all skeletal bone envelopes will be enlarged as a transient state (Parfitt, 1980). The enlarged remodeling space could be exploited by anti-resorptive drugs as *maintenance* therapy after discontinuing PTH. This hypothesis has been tested in only one published study.

Approximately one-third of the randomized Phase II cohort of patients treated with PTH (1–84) for 1 year (Lindsay *et al.,* 1998) were subsequently treated with open-label alendronate, 10 mg daily. As reported by Rittmaster *et al.* (2000), there was a further rapid increment in BMD at all sites, such that the aggregate gain over 2 years in BMD at the highest dose of PTH (1–84) was 14.1% at the spine, 4.5% at the femoral neck, and 3.3% for the whole body. Since there had been significant negative changes in whole body BMD during the 12 months of PTH treatment, the subsequent rapid reduction of bone turnover induced by alendronate was consistent with closing down the skeletal remodeling pace.

Calcitriol

The studies by Neer and co-workers combined concurrent calcitriol with daily PTH injection therapy (Neer *et al.,* 1987, 1991; Slovik *et al.,* 1981, 1986). This strategy was

adopted because the earlier dietary calcium balance studies of Reeve and co-workers demonstrated minimal adaptations of dietary calcium absorption despite obvious histomorphometric restoration of trabecular bone and increased urinary Ca excretion. Since exogenous PTH does increase serum $1,25(OH)_2D$ levels in the short term (Hodsman and Fraher, 1990; Lindsay *et al.,* 1993) and is associated with increased fractional ^{45}Ca absorption, the need for the addition of calcitriol therapy remains unproven. The changes in lumbar BMD reported by Neer and co-workers are comparable to other studies but theirs was the first group to report significant losses of BMD at the radius in a controlled clinical trial (15 patients on calcium, 15 patients on PTH + calcitriol 0.25 μg/day, over 2 years) (Neer *et al.,* 1991). The control group lost 1.7% and the experimental group lost 5.7% in radial BMD—all in the first year of treatment. The greater and abrupt loss of appendicular bone mass in the experimental group might indicate a permanent loss to cortical bone but an alternative explanation includes the possibility of an effect consistent with increased intracortical modeling.

Cyclical Therapy with PTH and Calcitonin

Cyclical therapy with PTH and an antiresorptive agent, calcitonin, is of interest insofar as it might provide insights as to how the hormone might ultimately be given in the most economical fashion. The concept for the cyclical use of PTH should be distinguished from its direct anabolic effects on the skeleton. Since PTH also activates bone remodeling, cyclical protocols have attempted to exploit the ADFR hypothesis (A, activate remodeling; D, depress resorption in the activated bone modelling units; F, a treatment-free period of bone formation; R, repeat the treatment cycle). In fact, none of the cyclical protocols can be considered as true ADFR protocols and should be regarded as hybrid anabolic protocols.

Hesch and Hodsman have combined cyclical PTH(1–34) and 1–38 with concomitant or sequential cycles of calcitonin (Hesch *et al.,* 1989b; Hodsman and Fraher, 1990; Hodsman *et al.,* 1991, 1993, 1997, 2000). There is little evidence that the anti-resorptive action of calcitonin provides any additional clinical benefit. Short cycles of PTH (400–500 units/day, approximately 40 μg) for less than 14 days provide little evidence of skeletal activation of bone turnover or anabolism (Hodsman and Fraher, 1990; Hodsman *et al.,* 1991; Reeve *et al.,* 1987), although biochemical responses are detectable within this time frame. However, 28-day cycles of high-dose PTH (800 units, or 65 μg daily) appear to provide a strong stimulus for activation of bone turnover (Hodsman *et al.,* 1993) and anabolic effects on bone mass (Hodsman *et al.,* 1993, 1997). The reported changes in lumbar spine BMD, with small increments in femoral neck BMD with 28-day cycles of high-dose PTH may provide an alternative approach to harnessing the anabolic effects of PTH, but there are no controlled trials comparing cycles of PTH therapy with daily injections.

Use of PTH in Conditions Other Than Postmenopausal Osteoporosis

As noted previously, PTH has been used successfully to improve BMD in estrogen-treated women with glucocorticoid-induced osteoporosis (Lane *et al.,* 1998a). Of note in that study, BMD continued to increase at the spine and hip measurement site during the 12 months of observation following 12 months of PTH therapy, suggesting that treatment with continued HRT as an anti-resorptive therapy effected similar reductions in bone turnover to those seen previously with alendronate (Table I) (Lane *et al.,* 2000).

PTH(1–34) has also been shown to prevent the large and rapid loss of bone mass following "medical oophorectomy" induced by the gonadotrophin antagonist, nafarelin (Finkelstein *et al.,* 1994). Following cessation of nafarelin and PTH therapy, bone mass continued to increase during 1 year of follow-up, such that in the subjects previously treated with PTH, BMD in the spine was actually higher than it had been at baseline 2 years earlier (Table I).

Finally, PTH appears to have equivalent anabolic effects in men with osteoporosis (Kurland *et al.,* 2000; Slovik *et al.,* 1986), although the numbers of men so far studied are very small (see Table 1).

Histology

As described in earlier sections, changes in bone histology can be detected in animal experiments very early on in the course of PTH therapy. Similar changes have been recorded in clinical studies (Hodsman *et al.,* 1993).

After only 28 days of hPTH (1–34) injections, Hodsman *et al.* (1993) reported that changes in both formation and resorption surfaces were increased 2 to 5 times those measured in a control panel of osteoporotic bone biopsies. Since tetracycline bone markers were given on days 14 and 28, the increased bone formation surfaces were likely the result of osteoid formation on quiescent bone surfaces, since there had not been sufficient time for a *de novo* resorption/reversal cycle to be completed. Dynamic histomorphometric measurements confirmed greatly increased bone formation rates and a two- to threefold increase in the activation frequency of bone remodeling. There was no evidence for impaired mineralization as measured by mineral apposition rates and trabecular architecture was apparently normal (Hodsman *et al.,* 1993). This study involved 28 day cycles of PTH(1–34) injections which were repeated at three-monthly intervals. After 2 years, repeated biopsies demonstrated that bone resorption and formation surfaces remained two- to threefold higher than untreated controls, with comparable increases in activation frequency of bone remodeling (Hodsman *et al.,* 2000). The mean wall thickness of completed trabecular osteons was significantly increased after 2 years of cyclical PTH therapy, suggesting that therapy resulted in a sustained positive remodeling balance. Over 2 years, the cortical thickness in these

iliac crest biopsies remained significantly higher than in controls, with no change in cortical porosity (Hodsman *et al.,* 2000). Although the trial was small ($n = 29$), this study provides histological support for the positive benefits of PTH on trabecular bone without detriment to the cortical bone envelope (Hodsman *et al.,* 2000).

Reeve and co-workers have reported changes in bone histomorphometry after 6 to 12 months of daily PTH therapy (Bradbeer *et al.,* 1992; Reeve *et al.,* 1980b, 1987, 1991, 1993). Using each patient as an internal control, significant increases in trabecular area, trabecular width, and mean trabecular osteon wall thickness of between 5 and 25% over baseline measurements were documented (Bradbeer *et al.,* 1992; Reeve *et al.,* 1991). Although the earlier study by Reeve *et al.,* demonstrated increased trabecular osteoid surfaces (from 23.3 to 35.2%) and resorption surfaces (from 3.9 to 6.2%) (Reeve *et al.,* 1980b), their later study demonstrated that if anything, these same parameters tended to decrease with time (Reeve *et al.,* 1993). Although little kinetic data (based on *in vivo* tetracycline labels) are available from Reeve and co-workers, the available evidence suggests that long-term PTH therapy (i.e., of more than 6 to 12 months duration) favors a positive osteon remodeling balance, and normal bone architecture, but that the dramatic short-term histological evidence for increased bone turnover is not sustained over time.

Biochemical Markers of Bone Metabolism

Biochemical markers of bone turnover provide noninvasive indicators of the balance between bone resorption and bone turnover (Delmas *et al.,* 1991; Garnero *et al.,* 1994; Riis *et al.,* 1995). Because PTH is regarded as a hormone with potent bone-resorbing properties, leading to concomitant increases in urinary calcium excretion, other markers of bone resorption are of great importance. In this section, discussion of biochemical markers of bone resorption will be restricted to urinary hydroxy-proline (OH-Pro), deoxypryidinoline (DPD), and the collagen cross-linked N-telopeptides (NTx). Markers of bone formation will focus on bone-specific alkaline phosphatase (BSAP) and/or osteocalcin. However, a complete interpretation of the data is founded by heterogeneous study designs and duration of therapy.

Reeve *et al.* (1980b) reported a 26% increase in OH-Pro excretion in patients treated with daily PTH, but either no change or a 100% fall in urinary OH-Pro in a small group of 12 patients who were treated with estrogen or androgen as concurrent anti-resorptive therapy (Reeve *et al.,* 1987, 1991). When using a dose of PTH (800 units/day), Hodsman *et al.* reported a 53% increase in urine OH-Pro after 1 month of treatment with no significant changes in urine DPD excretion (Hodsman *et al.,* 1993). However, other investigators have variously reported increments in either urinary OH-Pro, DPD, or NTx excretion that are much higher (150–350% over baseline) (Finkelstein *et al.,* 1998a; Kurland *et al.,* 2000; Lane *et al.,* 2000; Lindsay *et al.,* 1997; Sone *et al.,* 1995). These markers of bone resorption all tend to be maximal within the first 6–12 months of PTH treatment and fall back toward baseline values over longer periods of continuing therapy.

Increments in both BSAP and osteocalcin are consistently seen and vary between approximately 115 and 250% (Finkelstein *et al.,* 1998a; Fujita *et al.,* 1999a; Hodsman *et al.,* 1997; Kurland *et al.,* 2000; Lane *et al.,* 2000; Lindsay *et al.,* 1997). These increments in biomarkers of bone formation precede evidence of bone resorption, but again are maximal within 6 to 12 months and fall toward baseline values in the longer studies.

In summary, the reported measurements of biochemical markers for bone resorption and formation support the rapid onset of skeletal activation and bone turnover in response to PTH as reported histologically by Hodsman *et al.* (1993, 2000). Increments in bone formation markers are consistent and of considerable magnitude, in keeping with direct osteoblastic stimulation and bone anabolism (Hodsman *et al.,* 1997; Lindsay *et al.,* 1997). Increments in bone resorption markers are less consistent, but still of considerable magnitude. Since declines in biochemical markers of bone turnover after 2–3 years of therapy appear to be consistent, these markers may substantiate the premise that skeletal resistance to the anabolic effects of PTH peptides might occur during the first three years of therapy.

Calcium Balance Studies

Prior to 1980, there were few methods to document changes in bone mass in response to any agent. Bone biopsies yield important information, but the measured parameters are not precise (typical CV $\pm$ 20%) (Chavassieux *et al.,* 1985). Therefore, early studies relied on classical dietary calcium balance studies and radio-isotopic techniques of assessing skeletal calcium accretion to determine the extent to which new therapies for osteoporosis improved skeletal bone mass. The methodology of the reports discussed below is not easy to follow, but the cited references provide a source for such documentation. Moreover, the reader should appreciate that the cited clinical protocols vary widely with respect to dose of PTH, duration of therapy and concurrent medication. Given these limitations, the results indicate enough consistency to describe the results in general terms.

In the initial report by Reeve *et al.,* (1976a), four patients treated with PTH (500 units/day), demonstrated an improved dietary calcium balance averaging 7.3 mmol/day. Net diet absorption of ^{47}Ca averaged 3.4 mmol/day. Measured accretion of ^{47}Ca by the skeleton averaged 6.3 mmol/day. These data seemed to confirm that the observed improvements in bone histology were indeed due to an anabolic effect of the injected hPTH(1–34). A subsequent report on the short-term changes (less than 1 month) measured in the same subjects defined dose dependent effects, with calcium

balance improving over doses of 500 to 1000 units hPTH (1–34)/day, but deteriorating at doses of 1500 units/day (Reeve *et al.,* 1976b). In a small group of four patients treated with 450–750 units hPTH(1–34) over 1 month reported by Slovik *et al.,* a similar dose dependency was observed, with positive dietary calcium balance (2.2 mmol/day) seen only at the lower doses of PTH (Slovik *et al.,* 1981).

In summary, kinetic calcium data demonstrate trends toward positive dietary calcium balances during a variety of therapeutic PTH protocols. These techniques have not been as sensitive to changes in bone mass as dual-energy absorptiometric measurements, but they comprise the only existing evidence that prolonged PTH treatment does not result in consistent total body calcium depletion. However, two reports suggest that higher doses of PTH (>1000 units/day) may be deleterious to total body calcium balance (Reeve *et al.,* 1976b; Slovik *et al.,* 1981).

Effects on Serum and Urinary Calcium

The biological effects of PTH when given by continuous intravenous infusion, intravenous bolus or subcutaneous injection are not the same. For example, continuous infusion of hPTH(1–34) caused marked bone resorption in the rat (Tam *et al.,* 1981). When infused into osteoporotic subjects, Hodsman *et al.* reported that a given dose of hPTH(1–34) caused significantly larger increments in total serum calcium over 24 hr than the same dose given by a single subcutaneous injection (Hodsman *et al.,* 1993). The PTH infusion led to a significant fall in biochemical markers of bone formation, suggesting, if anything, that continuous intravenous infusion of PTH is anti-anabolic (Hodsman *et al.,* 1993). Similar findings in osteoporotic subjects were reported by Cosman *et al.* (1993), albeit that concurrent estrogen therapy might have mitigated the catabolic effects of PTH infusion.

Increments in serum calcium following subcutaneous PTH injection are delayed and do not peak until 4–8 hr postdosing (Hodsman *et al.,* 1993; Lindsay *et al.,* 1993), but they are generally within the normal physiological range. Since the clearance of injected PTH is so rapid, no obvious safety concern has arisen after over 20 years of PTH therapy. However, persistent hypercalcemia may occur during daily PTH injections. In the only published RCT evaluating PTH(1–34) against placebo, serum calcium measurements 24 hr after daily injections of PTH in a dose of 20 μg/day (approximately 250 units) were mildly elevated (<11.5 mg/dl) in about 10% of patients, and not sustained in a third of these women (Neer *et al.,* 2001). Dose reduction because of sustained hypercalcemia within the dose ranges chosen in most published trials is seldom necessary, and cessation of nutritional calcium supplementation is often sufficient to manage increments in serum calcium above the normal physiological range. Short-term increments in serum $1,25(OH)_2D_3$ have been reported after subcutaneous hPTH(1–34) (Fujita *et al.,* 1999a; Hodsman and Fraher, 1990; Lindsay *et al.,* 1993), together with increased fractional absorption of dietary calcium (Hodsman and Fraher, 1990) and hypercalciuria (Hodsman *et al.,* 1993; Lindsay *et al.,* 1993). However, of the three studies reporting long-term biochemical changes in response to hPTH(1–34) injections, sustained increments in serum $1,25(OH)_2D_3$, and hypercalciuria were not of clinical importance (Hodsman *et al.,* 1997; Lindsay *et al.,* 1997; Neer *et al.,* 2001).

Pharmacokinetics of PTH Administration

Since most therapeutic protocols have reported the response of the skeleton to daily subcutaneous injections, this section focuses on the pharmacodynamic responses to PTH when given by this route.

In a small study involving four healthy young subjects, Kent *et al.* (1995) reported that after subcutaneous administration of 1250 units hPTH(1–34) (100 μg, 20 nmol) the time to reach maximum plasma concentration T_{max} was approximately 15 min, with immunoreactive PTH concentrations decaying to baseline after 180–240 min. Two additional reports, in estrogen-treated postmenopausal osteoporotic subjects and in both young and untreated postmenopausal subjects (Fraher *et al.,* 1993; Lindsay *et al.,* 1993), provided very similar data, i.e., peak immunoreactive increments in serum hPTH(1–34) levels at 20–30 min postinjection, increasing 10- to 15-fold over baseline (depending upon injected dose), and decaying within a $T_{\frac{1}{2}}$ of about 75 min (Fig. 5).

The only detailed pharmacodynamic study published for other PTH peptides is a dose-finding single subcutaneous injection report on hPTH(1–84). Schwietert *et al.* (1997) reported minimal changes in total serum calcium in response to single injections of up to 5 μg hPTH(1–84)/kg (approximately 0.5 nmol/kg). However, with this peptide, absorption appeared to be associated with a double peak in C_{max} with an

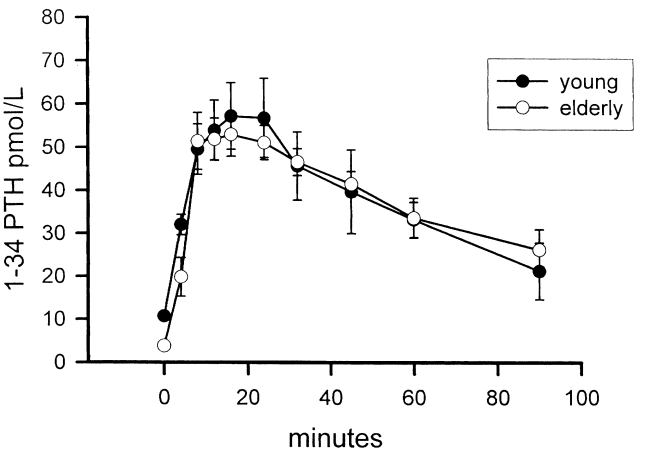

Figure 5 Pharmacokinetics of PTH(1–34) given by subcutaneous injection. Serum concentrations of hPTH(1–34) after subcutaneous injection of 800 IU PTH peptide in 10 young adults ages 25 ± 9 (SD) years and 9 elderly women with osteoporosis, ages 67 ± 11 years. Adapted from Fraher *et al.* (1993).

early peak occurring <20 min postinjection and a second peak appearing $1\frac{1}{2}-2$ hr later. This pharmacokinetic profile occurred at all doses of hPTH(1–84) and appears to be different from that observed after hPTH(1–34) dosing.

It is therefore quite possible that different PTH peptides have different biological properties on bone metabolism together with different safety profiles.

Immunological Responses to Exogenous PTH

Of the available reports in which antibody formation was deliberately sought, 67 of 1168 patients apparently developed anti-hPTH(1–34) antibodies in low titers. (Hesch *et al.,* 1989b; Hodsman *et al.,* 1997; Neer *et al.,* 2001; Reeve *et al.,* 1976a, 1987, 1991) Four patients discontinued hPTH(1–34) therapy because of generalized urticarial reactions or local irritation at the injection site (Reeve *et al.,* 1976a, 1980b). Although the 6% incidence of anti-PTH(1–34) antibodies may seem high, it is likely that the earlier reports reflect impurities in the hPTH(1–34) formulations that were used; native hPTH(1–34) is a naturally occurring peptide and should not be immunogenic. Whether the newer PTH analogs possess this drawback awaits further clinical testing.

Other Analogs and Delivery Systems

The principal analog used in the reported clinical literature was the presumed bioactive amino-terminal fragment of PTH, namely hPTH(1–34). Several have used hPTH(1–38) because it might be more potent than hPTH(1–34) (Hesch *et al.,* 1984, 1989a,b; Hodsman and Fraher, 1990; Hodsman *et al.,* 1991), but there is no compelling reason to accept this. The natural hPTH(1–84) holohormone is currently under development for clinical trials and appears to be anabolically equipotent to PTH(1–34). Other analogs include hPTH-(1–31) amide and its lactam. These analogs stimulate adenyl cyclase with equivalent potency to the holohormone PTH-(1–84), but seem to have no effect on the phospholipase C messenger system; in animal models of anabolic bone formation they are equipotent to PTH(1–34) (Whitfield *et al.,* 1997). There is a single report of continuous intravenous infusion of PTH(1–31) amide in young human subjects (Fraher *et al.,* 1999). When compared to an equimolar dose of infused PTH(1–34), comparable urinary and plasma cyclic AMP responses were seen, but the PTH(1–31) amide resulted in no increase of serum and urinary calcium or in urinary NTx. This suggests that analogs can be designed with more selective potency for bone formation over bone resorption. Other PTH analogs, published in abstract, appear to be potent anabolic agents in animal experiments (Henry *et al.,* 1996; Yee *et al.,* 1996a,b). So far only the PTHrP(1–34) analog, semparatide, has been evaluated in a small Phase II randomized clinical trial. (Gallagher, 1999). Over 6 months, significant dose-dependent increments in BMD measurements of the lumbar spine and total hip were seen, with slight reductions of the radius and TBBM (Table I). Half of the study cohort received a second 6-month period of alendronate treatment and experienced a further acceleration of BMD gain at all sites, similar to that reported by Rittmaster *et al.* (2000). So far this study has been reported only in abstract form, making it difficult to compare safety and efficacy to PTH(1–34).

While all PTH peptides are currently under clinical development using the subcutaneous injection route, alternative delivery systems include:

(a) automated injection or transcutaneous delivery
(b) intranasal delivery
(c) transpulmonary delivery of aerosolised peptide (Deftos *et al.,* 1996).

Alternative delivery systems are in the early stages of clinical evaluation. Intranasal delivery devices have been demonstrated for both PTH and calcitonin peptides, using lyophilized, microparticulate delivery systems (Deftos *et al.,* 1996; Nomura *et al.,* 1996). Transpulmonary systems have been developed in which aerosol delivery to the bronchoalveolar tree leads to 30 to 40% bioavailability of PTH peptide compared with that seen through intravenous routes of administration, with profiles of peptide absorption that are very similar to those delivered by subcutaneous injection (Patton *et al.,* 1994). While noninjectable routes of PTH peptide delivery are still in the realm of experimental medicine, there is no reason to believe that this compliance barrier cannot be crossed in the near future. In the short term, research using injectable PTH peptides has not been a significant barrier to patient compliance.

Adverse Effects during PTH Therapy

Over 2800 patients have received PTH in the published literature, but most studies have been small (fewer than 50 participants) and of short duration. In only four studies were there more than 100 subjects receiving active PTH peptide (Fujita *et al.,* 1999a; Gallagher, 1999; Lindsay *et al.,* 1998; Neer *et al.,* 1999), representing close to 2250 patient years. No serious adverse reactions have surfaced beyond the obvious need to monitor serum and urine calcium concentrations, the majority of which can be handled by appropriate dose reductions during the initial weeks of treatment (Neer *et al.,* 2001). Nausea, headache, dizziness, arthralgia, and leg cramps following injection occur in 5–15% of women, in a dose-dependent fashion, but are likely to be rare in doses chosen for commercial applications (Finkelstein *et al.,* 1994; Neer *et al.,* 2001). Hodsman *et al.* (1997) have raised an issue about the long-term safety of PTH on renal function. In this report, 39 patients were treated with cyclical high dose PTH (800 units per day for four 28-day cycles/year over 2 years). This patient group experienced a significant 10% increase in serum creatinine (albeit within the normal range of age-related serum creatinine). Thus, PTH-induced hypercalciuria might conceivably affect renal function.

The study of PTH(1–34) reported by Neer *et al.* (2001) was terminated prematurely after the consistent dose-dependent appearance of osteosarcomas in a concurrent study in Fischer 344 rats undergoing a standard carcinogenicity bioassay. In that study, animals were treated with near-lifetime daily injections of PTH(1–34), and developed osteosarcoma after 18 months of dosing, by which time osteosclerosis and extramedullary hematopoesis had occurred. PTH did not increase the incidence of nonskeletal tumours, and osteosarcoma has not been reported in other species after similar dosing protocols (Neer *et al.,* 2001). The mitogenic potential of PTH peptides (as given therapeutically for osteoporosis) has not surfaced as an issue in published clinical trials, and since the study reported by Neer *et al.* is the largest trial to date, it is encouraging that the incidence of all neoplasms in the treated patients was actually lower than that observed in the placebo arm (Neer *et al.,* 2001). To date osteosarcoma has not been reported after therapeutic treatment with a PTH peptide in human subjects. In the 2 naturally occurring clinical conditions in which long-term increments in circulating PTH are well documented (primary and secondary hyperparathyroidism associated with chronic renal failure) there is no reported incidence of osteosarcoma (Kaplan *et al.,* 1971; Koppler and Massry, 1988; Maissonneure *et al.,* 1999; Vamvakas *et al.,* 1998). Although the mitogenic potential for PTH analogs has not been confirmed outside of a single laboratory experiment, PTH does appear to act as a growth factor within skeletal tissue and its receptor is widely distributed in other tissues. Therefore the oncogenic potential of these peptides should not be dismissed.

Conclusion

To date, the 25-year clinical experience of PTH therapy indicates that this is an important anabolic agent with the potential to reverse osteoporosis. Very few safety concerns have been raised over treatment periods of 2–3 years. The introduction of PTH peptides/analogs holds promise for a new class of agents which might reduce fractures in osteoporotic individuals. However, there are several outstanding questions raised by the published literature; the order in which we have listed them in no way reflects their order of importance.

(1) The safety prolife of PTH peptides remains to be established. Although ongoing long-term placebo-controlled trials will establish this, a small minority of patients develop sustained hypercalcemia and hypercalciuria, and there is at least one report of declining renal function over time (Hodsman *et al.,* 1997).

(2) While PTH holds the promise of rapid increments in skeletal bone mass in osteoporotic subjects, anti-fracture efficacy has been clearly demonstrated in one completed Phase III clinical trial (Neer *et al.,* 2001). Nonetheless, are the anabolic benefits to the skeleton restricted to the trabecular envelope? Do patients lose cortical bone as suggested by the radial bone mass data reported by Neer *et al.* (1991)? This important question is crucial but other data regarding the radius, total body calcium balance (Lindsay *et al.,* 1997; Neer *et al.,* 2001), and total body bone mineral density (Lindsay *et al.,* 1997) suggest that the effect of PTH treatment is most potent at trabecular sites but not detrimental to cortical bone.

(3) Given that PTH therapy induces rapid gains in bone mass (assessed by BMD measurement), can these gains be translated into sustainable bone mass. Animal data suggest that concomitant or sequential bisphosphonate therapy either blunts the skeletal response to PTH or blunts subsequent therapy with the peptide (Cheng *et al.,* 1995; Delmas *et al.,* 1995; Mashiba *et al.,* 1995), although this is not an invariable finding (Mosekilde *et al.,* 1995). To date, clinical studies suggest that concurrent estrogen therapy and daily PTH injections result in significant increments in total body BMD as well as increments at axial sites (Lindsay *et al.,* 1997). Sequential therapy with alendronate following 1 year of therapy with daily hPTH(1–84) injections resulted in marked gains in both axial and appendicular bone mass (Rittmaster *et al.,* 1998). This approach may suggest a role for anti-resorptive therapy as a sequential regimen in the overall management of osteoporosis, rather than concomitant therapy.

(4) Is concurrent medication necessary during PTH therapy? Most studies have utilized a variety of agents (including calcium, vitamin D or calcitriol, calcitonin, and estrogen). While the published data employing such strategies are not convincing, factorially designed clinical trials have not been done.

(5) Will cycles of PTH injections prove to be as effective as continued daily dosing? This question has a significant impact on cost and compliance.

(6) Does the skeleton develop resistance to continued PTH therapy? If so, will interval rechallenge be effective? Perhaps most importantly, will PTH therapy be as effective in patients previously treated with bisphosphonates but who have had less than adequate responses to this class of therapeutic agents?

(7) Will analogs of PTH be better anabolic agents than hPTH(1–34) or hPTH(1–84)?

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CHAPTER 76

Calcium

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Background

Calcium in the Environment in Which Life Arose

Calcium is the fifth most abundant element in the biosphere, after iron, silicon, oxygen, and aluminum. Unlike silicon and aluminum, whose compounds are quite insoluble, calcium salts exhibit an intermediate solubility that both permits them to be present in solution in the waters in which life evolved and to support development of various hard, solid organs for use by evolving life forms. Calcium is dissolved in seawater, for example, at concentrations up to 10 mM, ~10 times that which is found in the extracellular fluid of mammals. Even fresh water, if it is to support an abundant biota, must have substantial dissolved calcium (~1–2 mM, roughly on the order of mammalian extracellular fluid concentrations). Dissolved calcium is needed to keep carbon dioxide in solution for the photosynthetic activity of algae and aquatic plants, thereby establishing the base of the aquatic food pyramid.

The radius of the Ca^{2+} ion is just right to fit naturally into the folds of many proteins, coordinating with up to eight oxygen atoms in the peptide chain and its side groups. Calcium thus stabilizes critical tertiary structures of both catalytic and structural proteins (Carafoli and Penniston, 1985). This binding to key peptides is true, of course, for many metal cations, but most tend to be highly specific for one or two configurations (e.g., Fe, Mn, Cu, Zn, etc.). Magnesium, calcium's closest relative in the cell, and like calcium a divalent alkaline earth element, has a smaller ionic radius and does not bind as strongly as does calcium. Calcium, by contrast, binds avidly to a profusion of proteins, ranging from actin to calmodulin to the various *gla* proteins. Calcium's functionality is so broad, in fact, that essentially all cells have found it necessary to restrict calcium concentration in the cell sap to something like four to five orders of magnitude below that in the extracellular fluid (ECF) surrounding the cell. These low cytosolic concentrations are the context that permits cells to use calcium as a nearly universal second messenger. They do so by admitting controlled quantities into critical cellular compartments when specific functions are to be activated, and promptly pumping it out, either into the extracellular space or into intracellular vesicles, when the cell action is to be shut off. Intracellular storage of calcium is the rule, rather than the exception. Examples include the sarcoplasmic reticulum of muscle and calcium phosphate crystals in mitochondria. Calcium is so critical to intracellular function that most cells have developed means of maintaining their own supply. In general their dependence on extracellular calcium is limited to initiating the cascade by opening calcium channels in the cell membrane. Movement of ECF calcium into the cytosol through these channels then activates a much larger release of intracellular calcium stores, as in muscle contraction.

However, life at a higher, multicellular level requires integrative functions and, therefore, the maintenance of critical concentrations of many factors in the ECF of complex organisms. Here a higher calcium concentration (in the range of 1.25 mM) is essential for a variety of functions, ranging from neuronal synaptic transmission to blood clotting. While ECF $[Ca^{2+}]$ is essentially constant across the higher vertebrates, the means whereby it is stabilized vary. Fish and amphibia have access to the calcium in the surrounding water and buffer the concentration of calcium in their extracellular fluids by controlling fluxes across the gill membranes. However, terrestrial vertebrates, dependent upon periodic food ingestion for their calcium, need an internal source and sink of calcium for homeostasis. It is partly in this context that bone enters the scene.

Calcium and Bone in Evolutionary Perspective

While the most obvious feature of bone in terrestrial vertebrates, and particularly in humans, is its structural property,

it is likely that bone served somewhat different purposes in the early marine vertebrates (Urist, 1964). The fossil record indicates that bone evolved independently several times over the millennia of evolution — as dermal armor, as teeth, and as internal stiffening, often dropping out again in more modern descendants of ancestral bony members of the same classes. The internal stiffening and mechanical strength provided by bone, so obviously important on dry land, is of less significance in a buoyant medium, and there is a trend for fish to become less bony over the course of evolution. The modern sturgeon, a true bony fish by evolutionary origin, is essentially boneless today (except for the gill covers), but the fossil record shows that early sturgeons were quite as bony as most other modern fish. The *chondrichthyes* (sharks, skates, and rays), to cite another example, have only a cartilaginous skeleton, and yet do not lack for mechanical engineering efficiency.

Evolutionary biologists consider that the skeleton probably served an important homeostatic function, helping the organism maintain the constancy of critical elements in its internal milieu (Urist, 1962). That function is clearly evident in modern mammals, where the ion most prominently buffered by bone is calcium. There is reason to believe, however, that calcium may not have been the critical ion in marine vertebrates, since, as already noted, the surrounding sea provided a relatively large surplus of calcium relative to the ECF in most higher vertebrates. However, phosphorus is a trace element in seawater, and the phosphorus essential for intermediary metabolism, for DNA and RNA structure, and for so many catalytic functions, is acquired by marine vertebrates by ingesting the tissues of other organisms lower in the food chain. Hence, phosphorus needs to be rigidly conserved. Bone meets that need.

While the mechanisms by which bone serves a homeostatic function in terrestrial vertebrates are well worked out, it is less clear precisely how bone functions in maintaining internal homeostasis in fish. Fish produce many of the hormones involved in mammalian calcium and phosphorus homeostasis (calcitonin is an obvious example), but these agents do not seem to have the same effect on ECF [Ca^{2+}] in fish as in mammals. Furthermore it is not certain in fish how minerals are either added to the bony reserve or withdrawn from it.

Bone as the Body's Calcium Sink and Reserve

Without a constant supply of calcium in the fluid bathing the gill membranes, land-living vertebrates were confronted with the problem of maintaining constancy of extracellular fluid calcium levels in the intervals between feedings. This required a place to put calcium acquired from food in the absorptive phase and a place from which to draw calcium during fasting. Bone is that place. However, bone is not simply a passive reservoir. As noted elsewhere in this volume, calcium is stored in bone only in the process of mineralizing newly deposited bone matrix, that is, by adding new volumes of bone *tissue;* and it is withdrawn from bone only by resorption of old bone *tissue.* Thus the reservoir functions of bone are mediated by modulating, on a moment-to-moment basis, the balance between new bone formation and old bone resorption.

A good example of this modulation is afforded by what happens during calcium absorption from milk in infant mammals. The quantity of calcium ingested in a short period of time, coupled with the efficiency of absorption in infants and the small volume of the extracellular fluid water into which that calcium is dumped, are such that near fatal hypercalcemia would ensue if there were not some way to damp the absorptive rise in ECF calcium. This is accomplished by calcitonin-mediated suppression of osteoclastic bone resorption. Since bone formation is continuing, the mineral demands of recently deposited bone matrix effectively soak up the absorbed calcium. Then, as absorptive input decreases, bony resorption resumes. The result is a nearly steady input of calcium into the ECF, with the relative contributions of bone and gut varying inversely.

It is virtually certain that most of the skeletal control of extracellular calcium levels throughout life is exerted on the resorptive side of the remodeling apparatus, for the simple reason that osteoclastic activity is susceptible to very rapid response to humoral mediators, while mineralization is not. Once suitable crystal nuclei are formed (see Calcium in Bone, below), mineralization is mainly passive. The mineralization of newly formed bony units actually constitutes the principal systemic drain to which the control systems must respond (rather than functioning as a response mechanism itself).

An example of the reservoir function of bone is seen during annual spring antler formation in deer (Banks *et al.,* 1968). Rapid bone growth in the antler buds creates a greater calcium demand than the nutrient-poor, late winter and early spring foliage can satisfy. Since intestinal input cannot meet the demand, parathyroid hormone secretion increases and a burst of bone remodeling occurs throughout the entire skeleton. The first phase of bone remodeling is resorptive; hence a great deal of calcium is thereby released into the ECF, countering the drain of antler bone mineralization. Several weeks later, the skeletal remodeling loci reach their own formative stage, more nutrient-rich summer foliage has become available, and antler growth has slowed. The deer are then able to pay back the skeletal calcium loan from food sources. Something similar is seen in the deposition of medullary "egg bone"' early in the egg-laying cycle in birds. Such bone is then resorbed during later phases of egg formation, when its calcium both supports shell formation and helps solubilize protein being transported to the oviduct. The same type of phenomenon occurs in preparation for lactation in most mammals, with pregnancy commonly being a time of more positive calcium balance than can be accounted for by fetal mineralization, and then with substantial loss of bone during the hypoestrin state of lactation (Heaney, 1996a). For example, the nursing rat, across lactation of a full litter of pups, loses on the order of one-third of the skeletal mass she had at delivery (Brommage and DeLuca, 1985).

Thus, it is clear from the comparative biology of the skeleton that bidirectional remodeling imbalances (decreasing and then augmenting local bone mass) are capable of both removing and replacing calcium from bone by tiny minute-to-minute alterations of bone mass. Except for the accumulation of remodeling errors, this in-and-out process would not be expected to exert a deleterious effect on bone; in fact, it probably has a net beneficial effect on bone quality, inasmuch as evoked remodeling focuses preferentially on old, fatigue-damaged tissue, replacing it with fresh, new bone. However, when withdrawals are protracted or payback is incomplete or absent, structural elements may be lost (Fig. 1). When this occurs, the loss becomes irreversible, inasmuch as the scaffolding for osteoblastic replacement vanishes. Nutrient repletion can be expected only to fatten up existing structures, not replace lost ones. In this sense, calcium differs from a nutrient such as iron for which full repletion of functional iron mass is possible even after loss of a major fraction of total body iron stores.

There is an important asymmetry about this bidirectional flux of calcium in and out of bone through the remodeling cycle. While bone can be resorbed essentially without limit, there is a practical ceiling that governs how much calcium can be stored. Given an environmental abundance of calcium, bone mass is regulated not by nutrient availability but by a mechanical feedback loop that works to maintain bony strain under load at a constant level in all skeletal parts and regions. The regulation of this setpoint is complex and poorly understood, but it is influenced, at least in part, by estrogen [which explains why bone tissue is added to the skeleton at puberty (Gilsanz *et al.,* 1988) and during pregnancy (Heaney and Skillman, 1971), when estrogen levels rise, and withdrawn from it at menopause and during lactation (Brommage and DeLuca, 1985), when estrogen levels fall]. However, under a hormonal steady state, bone balance will become zero when the optimal level of strain is reached. Additional dietary calcium cannot be stored (see Defining the Calcium Requirement, below). The upshot is that, while the organism can withdraw calcium virtually without limit, storage is limited by the extent to which the skeleton continues to be subject to normal loading stresses, contingent upon preservation of structural elements.

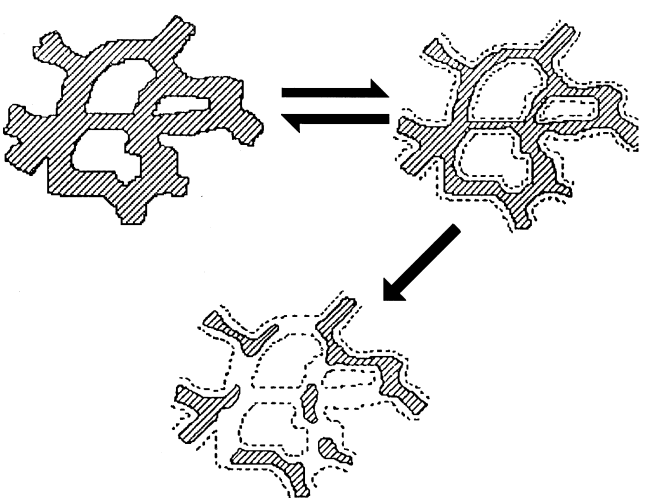

Figure 1 Schematic representation of the bidirectional flux of calcium in and out of the skeleton in a small volume of cancellous bone. Calcium is mobilized by resorbing bone tissue. So long as the basic structure is preserved, lost bone can be fully replaced. However, when whole structures are resorbed (i.e., trabecular plates penetrated and trabecular beams severed), replacement of lost bone becomes difficult or impossible. (Copyright Robert P. Heaney, 1995. Reproduced with permission.)

Calcium in Bone

The adult human body contains, on average, slightly more than 1 kg of calcium, better than 99% of which is in the form of bone and teeth. Calcium is the principal cation of bone, comprising slightly less than 40% of the mass of the bone mineral and slightly less than 20% of the dry weight of bone. Calcium exists in bone in a mineral form that is usually characterized as hydroxyapatite, i.e., $Ca_{10}(PO_4)_6(OH)_2$. This is only approximately correct, and it is more accurate to say, after Posner (1987), that bone mineral is a "structurally imperfect analogue of hydroxyapatite." Bone mineral contains, for example, carbonate, citrate, potassium, and magnesium, among other ionic species. Carbonate content is especially high. It varies fairly substantially from species to species, in humans comprising in the neighborhood of 6–9% of the mass of the mineral. Because the carbonate ion does not fit well into the hydroxyapatite crystal lattice, it is generally presumed to be located in superficial positions on the mineral crystals. The lability of bone carbonate, as reflected in changes in carbonate content with metabolic acidosis, suggests also that its location may be mostly on anatomic surfaces.

The crystals of bone exist in intimate association with the collagen fibers of the bone matrix. They are long and needle-shaped, are ~70 Å in diameter and from 200 to 3000 Å long, and are constrained in size and orientation by the dense, orderly packing of the matrix collagen fibrils, which are laid down before mineralization begins. Matrix, as deposited, consists of about half protein and half water. As mineralization proceeds, mineral crystals displace the water, aligning themselves with the collagen fibrils. Ultimately, at full mineralization, the extracellular bony material contains virtually no free water. One consequence is the fact that mineral ions deep to anatomical surfaces are frozen in place, neither exchanging with nor supporting the level of the corresponding ionic species in solution in the ECF.

The calcification of matrix presents an interesting illustration of the importance of specificity of crystal habitus and takes advantage of the marginal solubility characteristics of calcium phosphate. Both calcium and phosphate (the latter in the form of $H_2PO_4^-$ and HPO_4^{2-} circulate in the body fluids in solution and do not precipitate either in the bloodstream or in healthy body tissues. However, the same minerals, in exactly the same concentrations,

support mineralization when the blood flows past a bone-forming site. The reason, very simply, is that the concentrations of calcium and phosphate in ECF are such that body fluids are approximately half saturated with respect to $CaHPO_4$ (the most likely crystal form at physiological pH and pCO_2). However, bone mineralization is not simple precipitation. Rather, it involves the creation of a template for formation of a crystal more like hydroxyapatite or tricalcium phosphate [$Ca_9(PO_4)_6$]. Such minerals would not form spontaneously below pH 8; they are much less soluble, and body fluid concentrations of calcium and phosphate are approximately twice their K_{sp}. Local creation of such crystal nuclei is the means by which vertebrates have been able to control deposition of mineral, placing it only in loci specifically prepared to receive it, e.g., where it is needed to provide the necessary rigidity for the structural role of bone.

Noncollagenous matrix proteins are believed to play the critical role in configuring Ca^{2+} and PO_4^{3-} ions in space so as to create the hydroxyapatite template. Alkaline phosphatase, produced by the osteoblast late in the matrix deposition process, is believed to function by hydrolyzing pyrophosphate and organic phosphate esters present in the medium, which both makes extra phosphate available and removes components that otherwise function as crystal poisons, inhibiting crystal growth (Whyte, 1989).

The chemistry of formation of the apatite mineral can be summarized as

$$10\ Ca^{2+} + 3.5\ H_2PO_4^- + 2.5\ HPO_4^{2-} + 2H_2O \rightleftarrows Ca_{10}(PO_4)_6(OH)_2 + 13.5\ H^+.$$

The proportion of $H_2PO_4^-$ and HPO_4^{2-} in extracellular fluid at body pH is such that this reaction produces ~13 protons for each unit cell[1] of hydroxyapatite formed. These must, of course, be removed from the mineralizing environment, or the reaction would cease or tend to run in reverse. By the same token, ~13 protons must be produced by osteoclasts to solubilize one unit cell during bone resorption.

The Calcium Requirement

What the Requirement Ensures

The strength of bone, as with all material structures, resides in four features: the instrinsic strength of the mineral–matrix composite, the massiveness of the structure, the geometric arrangement of the bony elements in space, and the loading history of a structural element, expressed in accumulated fatigue damage. As I have noted above, calcium availability may influence the repair of fatigue damage, inasmuch as temporary episodes of calcium deficiency will evoke bone remodeling. But the principal mechanism by which calcium is recognized to influence bone strength is through its effect on bone mass. Since bone functions as the calcium nutrient reserve, it follows inexorably that any depletion of that reserve will carry with it a corresponding decrease in bone strength.

As already noted, the calcium needed for critical cell-metabolic functions is, in most cells, derived from intracellular stores of the mineral. Bone constitutes such a large reserve of calcium that cellular functions could virtually never deplete it, no matter how low the oral intake of the nutrient. The same is true for the maintenance of ECF [Ca^{2+}]. A mere 5 g of bone contains as much calcium as the entire extracellular fluid space of an adult human. The requirement for calcium relates thus, not to the metabolic role of the nutrient, but to building and maintaining the size of the calcium reserve, i.e., to its secondary function, bone strength.

Calcium in the Environment in Which *Homo sapiens* Evolved

The abundance of calcium in the biosphere, previously noted, is expressed in the relatively high calcium content of the foliage, tubers, nuts, and other plant foods available to herbivorous and omnivorous mammals. The massive skeletons of extinct herbivores, the exuberant racks of antlers of deer and elk, both prehistoric and modern, and the large volumes of calcium-rich milk produced by dairy cattle today are all testimony to the abundance of calcium in a plant-based diet. Omnivorous animals, in addition to the calcium from plants, had access to the calcium in bones of animal prey, calcium in the chitinous exoskeletons of insects, and calcium in insect larvae and grubs.

Analysis of the foods consumed on a year-round basis by chimpanzees, our closest primate relatives, indicates a calcium nutrient density in their diets of 2–2.5 mmol (80–100 mg) Ca/100 kCal (Eaton and Nelson, 1991). The !Kung people of South Africa have been found to have diets very nearly as high [1.75–2.0 mmol (70–80 mg) Ca/100 kCal]. Studies of other hunter–gatherer peoples indicate that their diets, too, have calcium nutrient densities in the range of those of the high primates. The energy expenditure of a hunter–gatherer or chimpanzee, scaled up to the body size of contemporary Western peoples, translates these nutrient density figures to a total calcium intake in the range of 50–75 mmol (2000–3000 mg)/day from plant sources. Riverine peoples, with bony fish in their diets, had still higher calcium intakes. By contrast with these pretechnological kinds of intakes, the diet calcium density of urbanized or industrialized nations is commonly under 0.6 mmol (24 mg)/100 kCal (Carroll *et al.,* 1983).

The reason for the difference between primitive and contemporary diets is expressed, graphically, in Fig. 2, which depicts schematically the effect on calcium nutrient density produced by the agricultural and pastoral revolutions (which can be dated to roughly 10,000 years ago, at least in the fertile crescent, and much more recently in the Western hemisphere and northern Europe). Domestication of animals and

[1]A unit cell, in this case containing 10 calcium ions, is the smallest, symmetrical repeating unit of the crystal lattice.

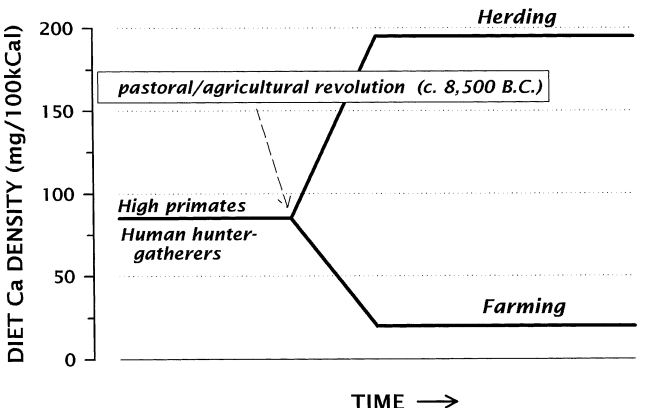

Figure 2 Schematic representation of the effect of the pastoral and agricultural revolutions on calcium nutrient densities of the diets of paleolithic humans. (Copyright Robert P. Heaney, 1995. Reproduced with permission.)

the development of flocks led to a nomadic lifestyle heavily dependent upon the milk produced by the pastoralists' herds. Farming, on the other hand, exploited natural hybrid forms of cereal grasses, which required humans for their propagation (mainly because the excess starch of the seed kernels made them too heavy for efficient dispersal by the wind). At the same time the extra starch of the hybrids made it possible to feed a great many more people. The hunter–gatherer lifestyle makes very inefficient use of the land, and population pressures were one reason for the shift to farming or herding.

With this divergence, the pastoralists thus had a substantial increase in the calcium density of their food supply, while the agriculturists had a substantial decrease. By contrast with milks [at calcium densities in the range of 27.5–50 mmol (110–200 mg)/100 kCal], cereals typically have calcium densities under 0.25 mmol (10 mg)/100 kcal. A contemporary example of a dominantly pastoral economy is provided by the Masai of East Africa, whose energy needs are largely met by fluid bovine milk, and whose calcium intakes, accordingly, are in the range of 150–175 mmol (6000–7000 mg)/day. It is likely that the actual calcium intake of the agriculturists was substantially higher than provided by the staple cereal grains, both because of other vegetables in the diet and because of adventitious calcium entering the food supply in the process of flour milling (Molleson, 1994). Grain was typically dehulled in limestone mortars, and the dehulled kernels then ground in limestone querns. Limestone is, of course, a calcium carbonate mineral; it is relatively soft and workable; and it undoubtedly added a great deal of calcium to the flour produced for bread making. Hence it is likely that only with the development of a more advanced technology, which allowed large-scale milling and the construction of mill stones from harder minerals (silicon based rather than calcium based), that the calcium intake of technologically advanced, urban peoples fell to present levels.

Physiological Adaptations to a High Calcium Intake

The abundance of calcium in the environment in which hominids evolved undoubtedly influenced the physiological mechanisms for extraction of calcium from the food supply and for conservation of absorbed calcium. Mammals typically have low intestinal calcium absorption efficiency, unregulated dermal losses, and poor renal conservation. The significance of these features is perhaps seen most clearly by way of contrast with the physiological adaptations to another nutrient, one which was scarce in the early hominid environment, i.e., sodium, for which absorption efficiency is complete and both dermal and urinary losses can be shut down to near zero. There is a cost to such conservation, of course, and it is likely that analogous mechanisms did not evolve for calcium because, given an environmental surfeit, they would have produced no selective advantage for their bearer. Further, efficient extraction of calcium from food sources would not only have been unnecessary; it would have been dangerous, for the excess would have had to be excreted, lest it lead to hypercalcemia and extraosseous calcification.

Exposure to chronic dietary calcium scarcity is a relatively modern phenomenon, as has been already noted, and fewer than 100 generations have elapsed for most of our lineages between the time of calcium surfeit and the present. This is far too few for the evolution of calcium-conserving mechanisms, particularly when the selective advantage of health at the end of life is much less strong than health during the reproductive years.

Studies Manipulating Calcium Intake

The importance of calcium intake on bone mass has been extensively documented in a number of species. As far back as 1929, Bauer, Aub, and Albright showed that cats developed severe bone loss when placed on low-calcium diets. This work has been replicated in rats and dogs (e.g., Bodansky and Duff, 1939) and has been repeated several times in cats (e.g., Jowsey and Gershon-Cohen, 1964). Figure 3, from one such study, depicts a classical nutritional experiment, in which a nutrient is first removed, the morbid result observed, and then the nutrient restored and recovery noted. The bone loss occurring under these circumstances is dependent upon intact parathyroid function (Jowsey and Raisz, 1968); in the absence of parathyroid hormone, the bone loss is prevented, although at a cost of severe hypocalcemia. These experiments, incidentally, highlight both the central role of bone as the calcium reserve for ECF calcium levels and the importance of parathyroid hormone in releasing calcium from bone to sustain ECF [Ca^{2+}].

Over 40 randomized controlled trials in humans have been published in recent years, establishing that elevating calcium intake enhances bone acquisition during growth, slows bone loss in postmenopausal women, and reduces fracture rates at spine, hip, and other extremity sites. These studies are reviewed in detail elsewhere (Heaney, 2000).

Figure 3 Effect of calcium deprivation and repletion on bone mass in cats, as revealed by micro X-rays of thin slices of undecalcified cat femora. The bone structure is shown (left) of a cat fed a normal, high-calcium diet, a cat (middle) fed a low-calcium diet for just 20 weeks, and a cat (right) depleted for 10 weeks and repleted for 10 weeks. Bone loss in the middle panel is evident. The right-hand panel shows partial healing. (Redrawn from Jowsey and Gershon-Cohen, 1964.)

One clear example of these effects is seen in the report by Chapuy *et al.* (1992) involving nearly 1800 French women, average age 84 (Fig. 4). Elevating calcium intake from ~13 mmol (520 mg)/day to ~42.5 mmol (1700 mg)/day reduced fracture rate at both hip and other extremity sites by 20–40% within 18 months of starting supplementation. The effect on bone mineral density (BMD) was fully as striking as the effect on fracture rate. Control subjects lost ~4.6% of the bone from the hip site over the 18 months of observation (an annual rate of ~3%/year), while the supplemented individuals gained 2.7%. The result was >7% difference in hip bone mass by 18 months. Clearly, 13 mmol Ca/day was not sufficient to prevent erosion of bone mass, while 42.5 mmol Ca/day was.

It may be that some of the benefit obtained in this trial was due to vitamin D repletion, and some also to the added phosphorus contained in the supplement (e.g., on muscle function and coordination); however, the bone mass effect observed in this study was large enough to account for much or all of the fracture difference in its own right. Furthermore, in two further trials in which the control and the calcium-supplemented individuals were both given vitamin D supplements, a similar bone-sparing and fracture-reducing effect was observed in the calcium-supplemented

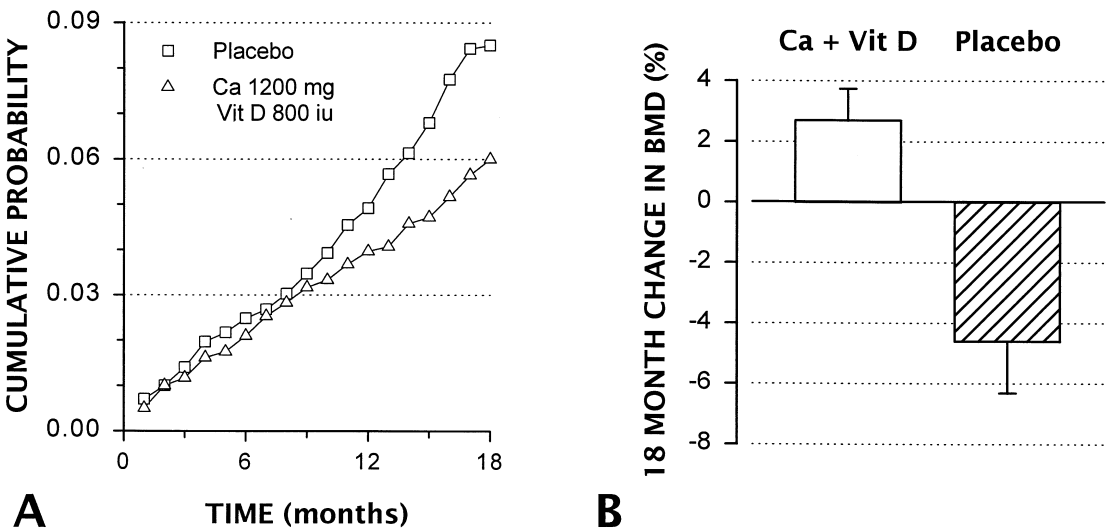

Figure 4 Effects of supplementation with calcium phosphate and vitamin D on hip fracture rate and hip BMD. The cumulative probability of fracture for the supplemented and unsupplemented groups is shown (A), as is the change from baseline in hip BMD for the two groups (B). (Redrawn from the data of Chapuy *et al.,* 1992.)

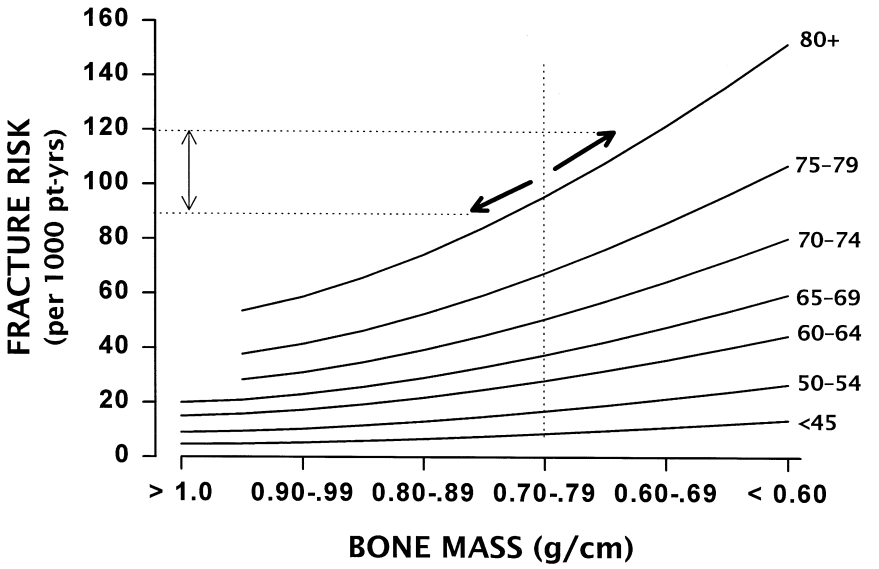

Figure 5 Age-specific fracture risk gradients for forearm BMD. Note the rise in risk with age, even holding BMD constant. Note also the increasing steepness of the gradients with age. A relatively small difference in BMD thus produces a greater fracture effect in the elderly than in younger individuals. (Redrawn from the data of Hui *et al.,* 1988.)

individuals (Chevalley *et al.,* 1994; Recker *et al.,* 1996). In a more recent study, contrasting calcium and vitamin D, Peacock *et al.* (2000) showed that, while calcium alone was able to prevent bone loss entirely, vitamin D alone was not. [It should be noted that blood 25(OH)D levels in Peacock's study were substantially higher than in the study by Chapuy *et al.,* and thus some beneficial effect of vitamin D in the French women cannot be excluded.]

While there is, obviously, a limit to how much fracture reduction can be achieved when one waits to ingest an adequate intake until old age, it is noteworthy that the elderly seem to be both more sensitive to calcium deprivation and more responsive to calcium supplementation than are younger individuals. This is illustrated, for example, in risk gradients for various ages reported by Hui *et al.* (1988) (Fig. 5). While the risk of fracture rises with declining bone mass at all ages, risk rises also with age, even holding bone mass constant. In fact, as Fig. 5 makes plain, the age effect is larger than the bone density effect. The gradient of risk on bone density also becomes steeper with age. Thus, while reducing bone loss in a 60-year-old woman would be expected to produce a modest fracture benefit, the same reduction in bone loss in an 85-year-old produces a much greater reduction in fracture risk. It is not certain why this should be so, but it is likely that the age effect itself is due to increased falling, poor reflex adjustment during the fall, decreased soft tissue padding over bony prominences, and increased intrinsic skeletal fragility from nonmass causes [trabecular disconnection and accumulated fatigue damage (Heaney, 1993a)]. Interaction between these factors may explain the disproportionately large benefit of altering just one of them (i.e., bone mass).

Defining the Calcium Requirement

The calcium requirement can be defined as the intake needed to support genetically programmed acquisition of bone during growth and to sustain acquired bone during maturity and the declining years of life. Because of differing absorption and retention efficiencies, individuals will inevitably have differing requirements. A recommended dietary allowance (RDA) is a figure designed to be at about the 95th percentile of individual requirements. Hence if everyone in a population were ingesting at least as much as the RDA, that intake would ensure that 95% would be getting what they need.

Calcium intake recommendations have undergone substantial revision over the past 10 years and the formats for presenting them have been altered as well. Table I sets forth both the earlier and the current recommendations, using a standardized format to facilitate their comparison. Perhaps the most important of the recent changes is the recognition that the requirement rises substantially in the elderly. This elevated need reflects the age-related decline in ability to adapt to low intakes, and thus in a sense uncovers the optimal intake for all ages, i.e., the intake for which no adaptation would be needed. It is worth recalling, in this connection, that even though the recommended intakes may seem high by contemporary practice, they are still only a fraction of what the primitive human intake would have been for individuals of modern body size.

As previously noted, calcium is a threshold nutrient. This means that, at subthreshold intakes, calcium retention is less than optimal. Bone gain during growth is then a direct function of intake (i.e., it is less than is genetically

Table I Various Estimates of the Calcium Requirement in Women[a]

Age	1989 RDA[b]	NIH[c]	1997 DRI[d]
1–5	800	800	600/1000
6–10	800	800–1200	1000/1600
11–24	1200	1200–1500	1600/1200
Pregnancy/lactation	1200	1200–1500	1200
24–50/65	800	1000	1200/1450
65–	800	1500	1450

[a]All values are given in mg, as this is how the respective bodies reported their recommendations. To convert to SI units, divide the values in the table by 40.

[b]"Recommended Dietary Allowances," Ed. 10. National Academic Press, Washington, DC, 1989.

[c]NIH Consensus Conference: Optimal Calcium Intake (1994). *J. Am. Med. Assoc.* **272,** 1942–1948.

[d]"Dietary Reference Intakes for Calcium, Magnesium, Phosphorus, Vitamin D, and Fluoride. Food and Nutrition Board, Institute of Medicine." National Academy Press, Washington, DC, 1997. The figures given are calculated as RDAs, to facilitate comparison with earlier estimates. The published values were termed "AIs," which in this instance represent estimated *average* requirements (EARs). The RDA figures shown here are 20% higher than the corresponding EAR. The presence of two values in this column reflects the fact that the age categories for the DRIs overlapped those of the NIH.

programmed), while in the mature organisms, subthreshold intakes lead to actual bone loss. On the other hand, the threshold character of the relationship means that intakes above the threshold produce no additional bone effect (see also Bone as the Body's Calcium Sink and Reserve, above). These relationships are depicted schematically in Fig. 6. Because the approach to the threshold is asymptotic, it is difficult to define the precise point at which retention plateaus. For this reason current recommendations are

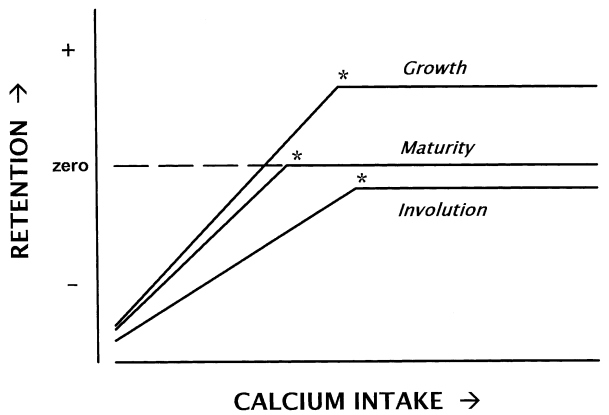

Figure 6 Schematic depiction of the relationship between calcium intake and skeletal retention at three life stages. Given sufficient intake, retention is positive during growth, zero at maturity, and variably negative during involution. Intakes above the threshold do not affect retention, but subthreshold intakes limit bone acquisition or lead to bone loss. The location of the minimum requirement is indicated by the asterisk above each line. (Copyright Robert P. Heaney, 1998. Reproduced with permission.)

actually somewhat conservative. Some hint that the optimal intake may be substantially higher than even the newer recommendations is provided by several lines of evidence. One comes from the balance studies of Heaney *et al.* (1978), in which the *mean* requirement to maintain zero balance in estrogen-deprived women was ~37.5 mmol (1500 mg). An RDA would, inevitably, be higher still. Another pointer in that direction is found in a randomized controlled trial in elderly women supplemented with calcium for 3 years (McKane *et al.,* 1996). Unsupplemented individuals, with intakes averaging close to 20 mmol (800 mg)/day (the then applicable RDA for the age), exhibited the high 24-hr integrated parathyroid hormone (PTH) levels, the enhanced PTH secretory reserve, and the elevated bone resorption which have all been taken to characterize the calcium and skeletal economies of the aged (Epstein *et al.,* 1986). By contrast, women supplemented to 60 mmol (2400 mg)/day decreased both their bone remodeling and parathyroid functional indices, restoring them to young adult normal values. The inference is that the increases in parathyroid cell mass and function represent not so much characteristics of old age as adaptive responses to long-standing calcium deprivation.

Contributing causes for this increased parathyroid activity in the elderly, aggravating the effect of low calcium intakes, include a decline in vitamin D status with age (reflecting both decreased solar exposure and decreased efficiency of skin conversion of 7-dehydrocholesterol to previtamin D) and declining absorption and conservation efficiency for dietary calcium. Holding calcium intake constant at about 20 mmol (800 mg)/day, gross absorption efficiency declines from a mean at age 40 of ~0.24, at a rate of 0.002/year through at least age 65, with a one-time additional drop of 0.02 at menopause (Heaney *et al.,* 1989). Over that period, the decline thus amounts to 0.07, or a fall of ~30% in absorptive efficiency. Nordin and others have shown also that renal conservation of calcium deteriorates across menopause (e.g., Nordin *et al.,* 1991). The net result is that extraction of ingested calcium from food falls with age and conservation of that which is absorbed falls as well. These and similar findings from a number of investigators are the basis for the jump to 36–38 mmol (1450–1500 mg)/day in the 1994 and 1997 recommendations of Table I.

It must be noted that the absorption values cited above are *gross* absorption, such as would be measured by calcium tracer flux from the intestinal lumen into the blood stream. They do not account for the contrary movement of calcium into the lumen in the digestive secretions and sloughed off mucosa (which turns over at a rate of ~20%/day). In adult women, this calcium from endogenous sources entering the intestinal stream amounts to ~3.5–3.75 mmol (140–150 mg)/day (Heaney and Recker, 1994). Most of this calcium is subject to the same absorptive probability as is ingested calcium, but given the low efficiency of calcium absorption in general, inevitably most endogenous calcium will be lost into the feces. Thus *net* absorption from intakes in the range of 20 mmol (800 mg)/day is on the order of only 15%

of ingested calcium. Not all of even this small amount of net calcium gain from the intestine can be retained, since the tiny elevation of blood calcium produced when calcium is absorbed elevates the filtered load at the kidney and leads to an increase in urinary calcium loss.[2] The net result of the low net absorption and poor renal conservation is that, even during times of calcium need, only a small fraction of an ingested load is retained. Further, at the higher intakes achieved by calcium supplements, *fractional* retention is smaller still, since absorption fraction declines with the logarithm of the intake load size (Heaney *et al.,* 1990; Heaney *et al.,* 2000).

Nutritional Factors Influencing the Calcium Requirement

It is a truism of nutritional science that nutrients interact with one another and thereby alter their mutual requirements. Thus, requirements of certain of the B vitamins and of ascorbic acid vary with total energy intake (and expenditure). The lack of evolutionary development of efficient conservation of calcium has rendered this nutrient unusually sensitive to such nutrient–nutrient interactions. Most of this interaction is expressed in urinary calcium excretion, which, for example, is strongly influenced by the ingested load of sodium and protein and by the acid/alkaline residue of the diet (Heaney, 1993b). The protein effect is mainly mediated by the sulfate load produced in the metabolism of sulfur-containing amino acids and amounts to ~1 mg/day of urinary calcium loss for every gram of ingested high-quality protein. The sodium effect is due to the fact that sodium and calcium share a common transport mechanism in the proximal tubule, and thus an increased filtered load of either ion will lead to increased excretion of the other. The magnitude of the sodium effect on the calcium economy is an increase of 0.5–1.5 mM of urine calcium for every 100 mM of sodium ingested (Devine *et al.,* 1995; Nordin *et al.,* 1993; Itoh *et al.,* 1996).

These effects are linear across the full range of intakes of both protein and sodium, from low to high, and are not, thus, consequences only of excessive intakes. With diets characteristic of typical North American and European women, sodium and protein account for ~100 mg/day of obligatory calcium loss through the kidneys. Given the extremely poor net absorption efficiency for calcium, these nutrients can thus easily account for up to 1000 mg/day of the calcium requirement. It has been noted, for example, that individuals with low, but nutritionally adequate, intakes of sodium and protein may have calcium requirements as low as 500 mg/day, whereas those with intakes more typical of contemporary patterns, may have intake requirements closer to 2000 mg/day. This quite extraordinary sensitivity of calcium to the intakes of other nutrients is but one more reflection of the lack of evolutionary acquisition of efficient mechanisms for calcium absorption and retention.

The well-documented effect of the acid/alkaline residue of the diet (Berkelhammer *et al.,* 1988; Sebastian *et al.,* 1994) is probably a function of the "hard" anions (sulfate, chloride), either ingested with food or produced in metabolism, and may not, therefore, be a fully additive effect to what has already been described for protein and salt. In any event, substitution of acetate or bicarbonate for chloride produces substantial reductions in urinary calcium loss and therefore in the ability to maintain calcium equilibrium on low calcium intakes.

It is important to stress this latter point, that these nutrient interactions negatively affect calcium balance mainly at low calcium intakes. This is because they limit an individual's ability to adapt to a low intake. At the higher intakes typical of those that prevailed during hominid evolution, adaptation is easily possible, and high protein intakes, for example, do not then have a negative effect on the skeleton. [Indeed, such evidence as is available indicates that the net effect of protein on the skeleton is distinctly positive, overall.]

Toxicity

It is possible to consume too much of any nutrient, and calcium is no exception. However, in healthy individuals, available evidence indicates that the toxic threshold is quite high. Many young adult males in the United States regularly consume >75 mmol (3000 mg)/day (Carroll *et al.,* 1983), and in pastoralist societies, such as the Masai of East Africa, calcium intakes regularly average >150 mmol (6000 mg)/day. In both instances, there are no known ill effects associated with such intakes. However, there are special circumstances in which high calcium intakes can definitely be harmful. Even at the low absorption fractions typical of high intakes, substantial quantities of calcium will enter the body and will need to be excreted in the urine (unless active bone building is occurring). Under conditions of dehydration or hypovolemia, perfusion of both bone and kidney can be sufficiently compromised so that absorbed calcium produces significant hypercalcemia. Likewise, under conditions of systemic alkalosis, large calcium intakes can produce renal calcinosis and severe impairment of kidney function. Furthermore, under these somewhat unusual and extreme circumstances, elements of positive feedback can cause the situation to deteriorate rapidly. Hypercalcemia impairs the ability of the kidney to retain water and thus aggravates a preexisting dehydration, with consequently worsening hypercalcemia. A fatal downward spiral can easily ensue. Published reports of such toxicity have generally involved intakes of >150 mmol (6000 mg)/day in individuals with preexisting impairments of water and electrolyte metabolism. Whatever the underlying cause, such

[2]This sensitivity of urine calcium to variations in the filtered load is a reflection of the previously mentioned lack of evolutionary development of efficient calcium conservation mechanisms.

problems can be easily handled by rehydration and by correction of the underlying abnormality, so long as these situations are recognized in time.

Kidney stones are perhaps the complication most often thought to be associated with high calcium intakes. However, this association has been largely speculative and is not based on clinical evidence. Most multiple regression analyses of risk factors for renal calculi have found either a very weak or no association at all for calcium intake. Two very large observational studies, in fact, found the converse (Curhan *et al.,* 1993, 1997). In the first, a prospective study of >45,000 men, there was an inverse correlation between calcium intake and stone formation; those with the highest calcium intakes had the lowest risk of kidney stones. This seeming paradox is explained by the fact that renal oxalate excretion in the urine is a more significant risk factor for calculi than is urine calcium. High calcium intakes bind oxalate both of dietary origin and that produced by bacterial fermentation in the gut before it can be absorbed; dietary calcium thereby lowers the renal oxalate burden. In fact, very large calcium supplements have long been standard therapy for the kidney stones occurring with intestinal hyperoxalosis, sometimes found in patients with short bowel syndromes.

In summary, the levels of calcium intake discussed in the foregoing sections of this chapter, as well as those needed to support osteoporosis pharmacotherapy (see Calcium and Osteoporosis Treatment, below), are well within the safety limits for the ingestion of this nutrient.

Interpretation of Published Reports

The importance of a high calcium intake for bone health has often been questioned (e.g., Kanis and Passmore, 1989) and has even more often been termed "controversial." This is because some studies showed strong effects of calcium intake on bone status, while others showed no benefit at all. "Confusing" would have been a more accurate term. There are several reasons for these apparent discrepancies, now sufficiently understood to remove any confusion that may remain.

First, as already noted, osteoporotic fracture is a distinctly multifactorial affair. Nutrition, and particularly calcium nutrition, is only one of many contributing factors. While calcium intake is, as the above discussion indicates, of critical importance in many individuals, it plays little or no role in others, just as iron intake is limiting in only certain forms of anemia. One example of this distinction is seen in menopausal bone loss, which is dominantly due to estrogen deficiency and cannot be substantially influenced by calcium intake (although estrogen-related loss may be exaggerated if calcium intakes are very low) (Heaney, 1990). This is seen, for example, in the fact that even intakes >75 mmol (3000 mg)/day, in the study by Elders *et al.,* (1991), were able only to slow menopausal loss, not prevent it.

However, menopausal loss is self-limited and is mostly confined to the 5-year period surrounding cessation of ovarian function. Every controlled trial of calcium supplementation in women >5 years postmenopausal has, without exception, shown a clear benefit on either bone loss or fracture rate or both. By contrast, roughly one out of five observational studies performed on women in this same age group have failed to show a calcium effect (Heaney, 1993b, 2000); this is now understood to be due to the weak ability to estimate calcium intake from food records, or food frequency questionnaires, no matter how carefully these methods are applied, resulting in substantial classification bias (Heaney, 1991, 1996b).[3] Usually in clinical science, one encounters positive results from observational studies, which are then sometimes not borne out in studies employing the more rigorous design of randomized controlled trials. With calcium, we have the paradoxical situation that the randomized controlled trials are essentially all positive, whereas some of the observational studies are not, and even those that are positive often show what appear to be weak effects. As the controlled trials show, it is not the effects that are weak, but the methods that must be employed in observational studies.

A final consideration relates to the uncritical use of transnational comparisons (e.g., Hegsted, 1986). Differences in fracture rate between ethnic and national populations have many bases, only some of them nutritional. An example is the lower hip fracture rates of contemporary Chinese and Blacks, despite mean calcium intakes for both groups that are lower than those of North American or European Caucasians. Behind this difference there is, first, the large effect of the other nutrients in the diet, previously described. Thus the predominantly vegetable-based and low-protein intakes of many Third World populations would be expected to lower their calcium requirements.

Similarly, there are major racial differences in ability to conserve calcium. American Blacks, for example, both absorb at higher efficiency for any given ingested intake and conserve better at the kidney than do Caucasians (Abrams *et al.,* 1995; Bell *et al.,* 1985, Aloia *et al.,* 1998, Cosman *et al.,* 1997). Thus they have lower calcium requirements. Finally, there are important racial differences in bone geometry that have their own effect on fracture rate. Hip axis length, for example, explains most or all of the difference between the hip fracture rates of Orientals and Caucasians, after adjusting for bone mass (Faulkner *et al.,* 1993; Nakamura *et al.,* 1994).

Instead of making cross-cultural comparisons, the correct approach is to assess the effect of nutrient intakes *within* a cultural and ethnic group. When this has been done, for example in both American Blacks and in Chinese,

[3]There are many sources of this estimation error. One of them, often ignored, is the fact that actual calcium contents of various foods vary over a roughly threefold range (from low to high) around the nominal values in the standard food tables.

bone mass and hip fracture rate are found to be inversely correlated with calcium intake, just as has been found for Caucasian populations (Anderson *et al.,* 1995; Hu *et al.,* 1993; Lau *et al.,* 1988).

Calcium and Osteoporosis Treatment

Effective osteoporosis treatments involve a combination of pharmacotherapy designed to reverse the negative remodeling balance prevailing in the skeleton and physical therapy designed both to increase function and to increase mechanical loading on the skeleton. Currently recommended calcium intakes are presumably adequate to sustain bone mass, but they may not be sufficient to permit bone gain in most individuals for reasons just noted (i.e., net absorption from such intakes is just sufficient to offset obligatory losses). Furthermore, there is essentially no evidence that calcium alone, in any quantity, will lead to substantial gain in bone in individuals who already have osteoporosis. The small increase noted in the study by Chapuy *et al.,* (1992) described above (Fig. 4) probably reflects a remodeling transient (Heaney, 1994) and not a new steady-state rate of change. However, effective *pharmacotherapy,* including fluoride (Pak *et al.,* 1995; Riggs *et al.,* 1990), the bisphosphonates (Liberman *et al.,* 1995; Black *et al.,* 1996; Cummings *et al.,* 1998; Harris *et al.,* 1999), the SERMS (Ettinger *et al.,* 1999), and PTH (Kurland *et al.,* 2000), does seem to be capable of increasing bone mass, for some of these agents at a rate of up to 10% per year. To realize this potential gain, and particularly to optimize it, calcium intakes must be above the maintenance level, i.e., for most osteoporotic individuals that means above 37.5 mmol (1500 mg)/day. Few controlled trials at such intakes have been done, so this conclusion is to some extent an inference from the data summarized in the foregoing.

There is, however, some evidence. With respect to fluoride, Dure-Smith and associates (1996) showed, during effective fluoride therapy, an extraordinary degree of bone hunger, suggesting the need for calcium intakes during fluoride therapy of perhaps as much as 62.5 mmol (2500 mg)/day. Other clear examples of the importance of ensuring adequate calcium intake when using other bone-active agents are provided by a series of recent papers. First is a metaanalysis of 31 controlled trials of estrogen replacement therapy (Nieves *et al.,* 1998), which showed that estrogen in trials using supplemental calcium (at an average intake of 1187 mg/day) produced bone responses more than twice as great as those that did not (at an average intake of 583 mg/day). Additionally, Recker *et al.,* (1999) showed that a reduced dose of estrogen (0.3 mg conjugated equine estrogens) produced a 5% increase in bone mass in women given supplemental calcium and vitamin D. By contrast, in at least three earlier trials without supplemental calcium, this estrogen dose was essentially ineffective.

Summary

Calcium is the principal cation of bone, making up ~20% of its dry weight. Bone constitutes a very large nutrient reserve for calcium in terrestrial vertebrates, a reserve that has acquired a major mechanical function. The requirement for calcium is related to the protection of this mechanical function, not to the metabolic functions of calcium, which could be adequately protected by a reserve several orders of magnitude smaller. Calcium was abundant in the environment in which hominids evolved and in the foods they ate. Probably as a consequence, efficient calcium conservation mechanisms did not develop. Contemporary diets are low in calcium by comparison, and our paleolithic physiologies are poorly adapted to them. Yet other features of the modern diet increase obligatory calcium loss and thereby reduce the ability to adapt to lowered intakes. The evidence is compelling that inadequate calcium intake weakens bone and contributes to the growing osteoporosis problem. A fully adequate calcium diet for persons 65 and older, consuming otherwise typical Western diets, would contain >37.5 mmol (1500 mg)Ca/day and perhaps as much as 60 mmol (2400 mg)/day.

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CHAPTER 77

Calcium Receptors as Novel Drug Targets

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Introduction

Bone and mineral-related diseases comprise a diverse class of disorders affecting nearly every major organ system in the body. By far the most common disorder is osteoporosis, a disease characterized by reduced bone density and an increased susceptibility to fractures. Less common, but also associated with altered bone metabolism, are primary and secondary hyperparathyroidism. All these disorders are associated with aging, especially in women, and, as the world population ages, these diseases are becoming more prevalent. All these disorders might be treated with drugs that alter the circulating levels of parathyroid hormone (PTH).

PTH is the key endocrine factor in systemic Ca^{2+} homeostasis and many of its actions in this capacity involve moving Ca^{2+} between bone and blood. PTH can stimulate bone resorption or new bone formation depending on the levels of circulating hormone. Chronically elevated levels of plasma PTH stimulate osteoclastic bone resorption, resulting in net bone loss and osteopenia. In contrast, PTH stimulates new bone formation when plasma levels of PTH are elevated only briefly (see Chapter 75). The administration of exogenous PTH to cause a brief rise in plasma levels of PTH might be effective in treating osteoporosis. For treating primary and secondary hyperparathyroidism, however, there are no pharmaceuticals that effectively lower the chronically elevated levels of PTH that characterize both these disorders of parathyroid hormone. It is conceivable that drugs that either increased or decreased the secretion of PTH from the parathyroid glands could be useful in treating the various forms of hyperparathyroidism and/or osteoporosis.

Until recently, attempts to regulate secretion of PTH have either failed or achieved only limited success. The significant advance that enables the pharmacological manipulation of several different parathyroid cell functions, including PTH secretion, is understanding how plasma Ca^{2+} acts on parathyroid cells. The key element is the Ca^{2+} receptor, a G protein-coupled receptor whose primary physiological ligand is extracellular Ca^{2+} (see Chapter 23). This cell surface receptor enables parathyroid cells to detect and to respond to small changes in the concentration of extracellular Ca^{2+}. In its structural and functional properties, the Ca^{2+} receptor is akin to other cell surface receptors that initially transduce an extracellular signal into a cellular response. The major difference is that the ligand for the Ca^{2+} receptor is an inorganic ion rather than an organic molecule.

This chapter reviews the progress made in the discovery and development of small molecules that activate or inhibit the parathyroid Ca^{2+} receptor and thereby inhibit or stimulate secretion of PTH. In the first edition of *Principles,* Ca^{2+} receptors on C cells of the thyroid gland and on certain cells of bone were also reviewed in depth but they receive less attention in the present review. The enthusiasm for treating osteoporosis by altering secretion of calcitonin has diminished and, as discussed later, the expression of Ca^{2+} receptors on the various cells of bone is still controversial. In contrast, considerable progress has been made in developing drug candidates that either increase or decrease secretion of PTH. The drug candidate for treating hyperparathyroidism continues to advance toward pivotal Phase III trials and that for treating osteoporosis has entered the clinic. Manipulating the activity of the Ca^{2+} receptor pharmacologically is proving

to be a very effective way of altering at will the circulating level of PTH.

The Calcium Receptor

The Ca^{2+} receptor is a G protein-coupled receptor that shares structural homologies with other members of subfamily C receptors (see Chapter 23). This subfamily includes the metabotropic glutamate receptors (mGluRs) and γ-aminobutyric acid type B receptors ($GABA_BRs$). All these receptors are large by G protein-coupled receptor standards, particularly their extracellular domain, which is believed to bind the cognate physiological ligand glutamate, γ-aminobutyric acid, or extracellular Ca^{2+} (Takahasi *et al.,* 1993; Brauner-Osborn *et al.,* 1999; Hammerland *et al.,* 1999). The human parathyroid Ca^{2+} receptor encodes a protein of 1078 amino acids and it is 92 and 93% identical with the rat kidney and C cell and the bovine parathyroid cell Ca^{2+} receptors, respectively (Brown *et al.,* 1993). At present, only one gene coding for the Ca^{2+} receptor has been identified; no receptor subtypes have been found; splice variants of the Ca^{2+} receptor are few and of low abundance (Garrett *et al.,* 1995a).

Ca^{2+} receptors are expressed on many of the tissues involved in systemic Ca^{2+} homeostasis. In addition to parathyroid cells, parafollicular cells of the thyroid (C-cells) and many epithelial cells along the nephron express high levels of Ca^{2+} receptor messenger mRNA and protein (Brown *et al.,* 1995). In adults, the only other tissues expressing comparably high densities of the Ca^{2+} receptor are the pancreatic islets and the subfornical organ in the brain. At all these sites, the Ca^{2+} receptor messenger RNA transcripts appear to be identical and, as assessed by Western blotting, the final protein products expressed at the cell surface are similar. The major differences between Ca^{2+} receptors expressed in different tissues are posttranslational and seemingly involve different glycosylation patterns (Ray *et al.,* 1998; Bai, 1999).

The Ca^{2+} receptor is expressed at lower levels in other sites throughout the body, as recently summarized in extensive reviews (Brown *et al.,* 1999; Brown and MacLeod, 2001). There is no death of speculation regarding the functions of the Ca^{2+} receptor at these diverse sites but the only functions supported by sufficient evidence are those in parathyroid cells, C cells, and renal epithelial cells.

The Ca^{2+} receptor mediates the initial events of stimulus–secretion coupling in parathyroid cells. It is the key element in the primary physiological mechanism regulating PTH secretion. There are three separate lines of evidence supporting this conclusion. The first derives from molecular genetic studies of patients with the inherited disorders familial benign hypercalcemic hypercalciuria (FBHH) or autosomal dominant hypocalcemia (ADH). These disorders result from point mutations in the Ca^{2+} receptor gene, which result in receptor proteins having altered sensitivity to extracellular Ca^{2+} (Bassett and Thakker, 1995; Brown *et al.,* 1999). The mutated Ca^{2+} receptors in FBHH patients are less sensitive (or completely insensitive) to activation by extracellular Ca^{2+} and this molecular defect is manifest in the phenotype: these patients are hypercalcemic yet plasma PTH levels are normal (Pearce *et al.,* 1995; Pollack *et al.,* 1993). Conversely, the mutant Ca^{2+} receptors in ADH are more sensitive to activation by extracellular Ca^{2+} and these patients are hypocalcemic, but again plasma PTH levels are normal (Pollack *et al.,* 1993; Mancilla *et al.,* 1998). The second line of evidence is based on *in vitro* studies showing that the loss of responsivity to extracellular Ca^{2+} of cultured bovine parathyroid cells correlates with loss of mRNA for the Ca^{2+} receptor (Mithal *et al.,* 1995). Finally, and the focus of the present review, selective pharmacological manipulation of the Ca^{2+} receptor promptly alters PTH secretion *in vitro* or plasma levels *in vivo.*

C cells, like parathyroid cells, have long been known to respond to changes in plasma levels of Ca^{2+}. It seems likely that the Ca^{2+} receptor expressed in C cells functions similarly to that in parathyroid cells and is the essential molecule coupling changes in the levels of extracellular Ca^{2+} to hormone secretion. However, parathyroid cells and C cells use quite different transmembrane and intracellular signaling mechanisms to link the Ca^{2+} receptor to hormone secretion (Nemeth, 1996). In parathyroid cells, increasing the concentration of extracellular Ca^{2+} inhibits PTH secretion. Activation of the parathyroid Ca^{2+} receptor by extracellular Ca^{2+} leads to the rapid formation of inositol 1,4,5-trisphosphate and the mobilization of Ca^{2+} from cellular stores in addition to permitting influx of extracellular Ca^{2+}. The resulting increase in the concentration of cytoplasmic Ca^{2+} ($[Ca^{2+}]_I$) is thus associated with an inhibition of exocytotic hormone secretion, a peculiar relationship that runs counter to the general rule of stimulus–secretion coupling where cytoplasmic Ca^{2+} activates exocytosis. The Ca^{2+} receptor additionally couples through a pertussis toxin-sensitive G_i protein to inhibit adenylate cyclase activation by D_1 dopamine receptors (Chen *et al.,* 1989).

In C cells, increasing the concentration of extracellular Ca^{2+} stimulates calcitonin secretion. Again, activation of the Ca^{2+} receptor by extracellular Ca^{2+} results in a rapid increase in $[Ca^{2+}]_i$ and in this respect, secretion of calcitonin conforms to the general Ca^{2+} hypothesis of stimulus-secretion coupling. In the C cell, the Ca^{2+} receptor couples to voltage-sensitive Ca^{2+} channels and permits the influx of extracellular Ca^{2+} (Raue and Scherübl, 1995). The transmembrane mechanisms involved in this receptor–channel coupling remain unknown. Nearly all the studies on Ca^{2+} receptor coupling to signal transduction pathways in C-cells have used medullary thyroid carcinoma (MTC) cells. These mechanisms might not be representative of those used by the Ca^{2+} receptor in normal parafollicular cells. In rat and human MTC cells, increases in $[Ca^{2+}]_i$ elicited by increases in the concentration of extracellular Ca^{2+} result mostly from the influx of extracellular Ca^{2+} through voltage-sensitive Ca^{2+} channels. Increases in $[Ca^{2+}]_i$ mediated by these channels are important for the regulation of calcitonin secretion because dihydropyridines that activate or block voltage-sensitive Ca^{2+} channels have corresponding effects on secretion of

calcitonin in MTC cells (Hishikawa *et al.,* 1985; Muff *et al.,* 1988; Raue *et al.,* 1993; Scherübl *et al.,* 1993) and in authentic C cells *in vitro* (Cooper *et al.,* 1986). Increasing the concentration of extracellular Ca^{2+} does not evoke rapid increases in inositol phosphates in MTC cells (Fried and Tashjian, 1987) or the mobilization of intracellular Ca^{2+} that is detectable using fluorimetric indicators for cytoplasmic Ca^{2+} (Nemeth, 1987; Fajtova *et al.,* 1991). However, under some conditions (Yamashita and Hagiwara, 1990) and in some primary cultures of human MTC cells (Ridefelt *et al.,* 1994), it has been possible to demonstrate the mobilization of intracellular Ca^{2+} in response to the addition of external inorganic cations. These apparently discrepant observations might reflect the heterogeneous phenotype of different MTC preparations.

Regardless of the details of signal transduction, there is evidence that the Ca^{2+} receptor on C-cells, like the parathyroid Ca^{2+} receptor, is the pivotal molecule enabling these cells to alter secretion in response to changes in the plasma level of Ca^{2+}. Extracellular Ca^{2+} does not affect calcitonin secretion from the human MTC cell line TT (Haller-Brem *et al.,* 1987) and these cells do not express the Ca^{2+} receptor (Garrett *et al.,* 1995b). Authentic C cells and MTC cell lines that do respond to extracellular Ca^{2+} express the Ca^{2+} receptor (Lavigne *et al.,* 1998). There is, then, a tight correlation between the ability of extracellular Ca^{2+} to regulate calcitonin secretion and expression of the Ca^{2+} receptor in C cells. Perhaps the most compelling evidence is that detailed below showing that compounds which selectively activate or inhibit the Ca^{2+} receptor stimulate or inhibit calcitonin secretion *in vitro* and *in vivo.*

Pharmacology of the Calcium Receptor

The pharmacology of the Ca^{2+} receptor can be divided into *calcimimetics* and *calcilytics.* Calcimimetic ligands are those that mimic or potentiate the action of extracellular Ca^{2+} at the Ca^{2+} receptor; these ligands are agonists or allosteric activators of the receptor. Calcilytic ligands are Ca^{2+} receptor antagonists. Calcimimetics can be subdivided into *type I* and *type II* ligands. The former activate the Ca^{2+} receptor in the absence of extracellular Ca^{2+} and behave as true agonists, whereas type II calcimimetics act as allosteric activators. These are operational definitions based on the ability of the ligand to activate the Ca^{2+} receptor in the presence or absence of extracellular Ca^{2+}. The definition is based on functional effects and does not necessarily impart knowledge about the site on the receptor that binds the ligand.

Calcimimetics

From the outset, it was appreciated that the Ca^{2+} receptor is rather indiscriminate with respect to inorganic cations or molecules possessing net positive charges. A variety of inorganic di- and trivalent cations activate the Ca^{2+} receptor and include, in rank order of potency, $La^{3+} \cong Gd^{3+} > Be^{2+} > Ca^{2+} \cong Ba^{2+} > Sr^{2+} > Mg^{2+}$ (Nemeth, 1990). Likewise, many organic polycations activate the Ca^{2+} receptor and include simple polyamines (spermine), aminoglycoside antibiotics (neomycin), and polyamino acids (polylysine) or proteins (protamine) (Brown *et al.,* 1991a,b; Nemeth and Fox, 1999). All these charged ligands behave as type I calcimimetics; they are full agonists and activate the Ca^{2+} receptor in the absence of extracellular Ca^{2+}. While these calcimimetic ligands have been useful in some early studies of Ca^{2+} receptor function, they are far from ideal pharmacological ligands because they lack specificity and cannot be used effectively in tissues or *in vivo.*

The first class of compounds to be developed as selective ligands of the Ca^{2+} receptor are phenylalkylamines typified by NPS R-568 (Fig. 1). This compound does not affect the activity of mGluRs or various other G protein-coupled receptors at concentrations that maximally activate the Ca^{2+} receptor. Most of the phenylalkylamine compounds have at least one chiral carbon and exist as R- or S-enantiomers. Depending on the response measured, the R-enantiomer is 10- to 100-fold more potent than the corresponding S-enantiomer. For example, NPS R-568 increases $[Ca^{2+}]_i$ and inhibits PTH secretion from bovine parathyroid cells *in vitro* at concentrations between 3 and 100 nM, whereas NPS S-568 is without effect on either response at concentrations less than 3 μM (Nemeth *et al.,* 1998). These effects are dependent on the concentration of extracellular Ca^{2+} and NPS R-568 fails to alter parathyroid cell function when extracellular Ca^{2+} levels are reduced to levels below 0.1 mM. Type II calcimimetic compounds like NPS R-568 shift the concentration–response curve for extracellular Ca^{2+} to the left without affecting the maximal or minimal responses (Hammerland *et al.,* 1998; Nemeth *et al.,* 1998a). This effect of type II calcimimetic compounds

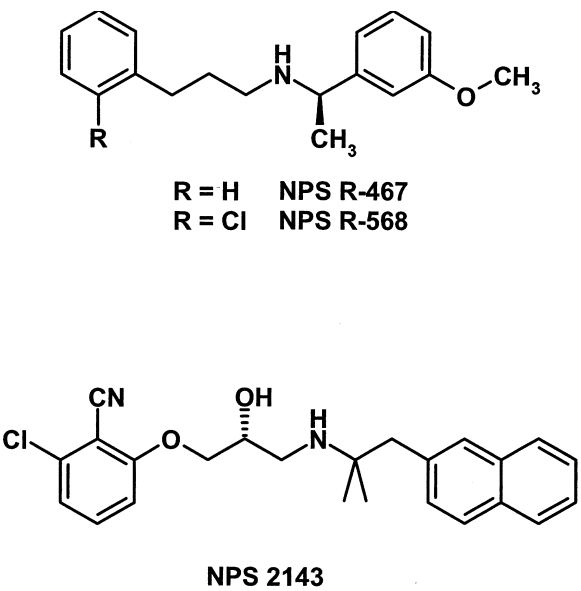

Figure 1 Structure of the calcimimetic compounds NPS R-467 and NPS R-568 and the calcilytic compound NPS 2143.

can be observed in heterologous expression systems and in authentic bovine parathyroid cells. These compounds therefore differ from the inorganic and organic polycations which act as agonists and can elicit responses in the nominal absence of extracellular Ca^{2+}.

Additional studies show that the type I and type II calcimimetic ligands act in different regions of the Ca^{2+} receptor to alter its activity. Using native and chimeric receptor constructs containing domains of the Ca^{2+} receptor and certain mGluRs, it has been shown that type I calcimimetic ligands act predominantly in the extracellular domain of the Ca^{2+} receptor, whereas type II calcimimetic compounds act within the transmembrane domain to cause receptor activation (Hammerland *et al.,* 1996; Hauache *et al.,* 2000). Thus, type I and type II calcimimetic ligands differ in both their presumed binding sites and their functional affects on Ca^{2+} receptor activity. The type II calcimimetic ligands act as positive allosteric modulators to increase the sensitivity of the Ca^{2+} receptor to activation by extracellular Ca^{2+}.

In normal rats, the oral administration of NPS R-568 causes a rapid fall in plasma levels of PTH, which is accompanied by hypocalcemia (Fig. 2). The fall in plasma levels of PTH precedes that of Ca^{2+} and the longer that PTH levels are depressed, the greater is the magnitude and duration of the hypocalcemia that is achieved by a given dose of compound (Fox *et al.,* 1999a). At higher doses, there is a transient increase in plasma levels of calcitonin although these type II calcimimetic compounds are about 50-fold more potent in decreasing plasma levels of PTH than in increasing those of calcitonin (Lavigne *et al.,* 1998; Fox *et al.,* 1999b).

The site of action of type II calcimimetic compounds *in vivo* has been determined by examining the effects of NPS R-568 or NPS R-467 on plasma levels of Ca^{2+} following systematic elimination of tissues expressing the Ca^{2+} receptor and which contribute to systemic Ca^{2+} homeostasis. The participation of renal Ca^{2+} receptors in the response to NPS R-568 was eliminated by an acute, total nephrectomy; this did not affect the hypocalcemic response elicited by NPS R-568 (Fox *et al.,* 1999a). Thus, type II calcimimetic compounds do not cause hypocalcemia by acting on Ca^{2+} receptors in the kidney. Selective surgical ablation of the thyroid, to eliminate C cells but leave the parathyroid glands intact, slowed the rate of onset but did not effect the magnitude of the hypocalcemia following administration of NPS R-568. In contrast, NPS R-568 failed to lower plasma levels of Ca^{2+} in thyroparathyroidectomized animals which were maintained at normocalcemia by continual infusion of PTH (Fox *et al.,* 1999b). Thus, hypocalcemia caused by administering type II calcimimetics results mostly from the ability of these compounds to inhibit secretion of PTH. In the rat, increased levels of calcitonin increase the rate of onset, but not the magnitude of hypocalcemia. These preclinical studies underscore the selectivity of these type II calcimimetic compounds and additionally highlight the pivotal role of the parathyroid Ca^{2+} receptor in regulating PTH secretion and systemic Ca^{2+} homeostasis.

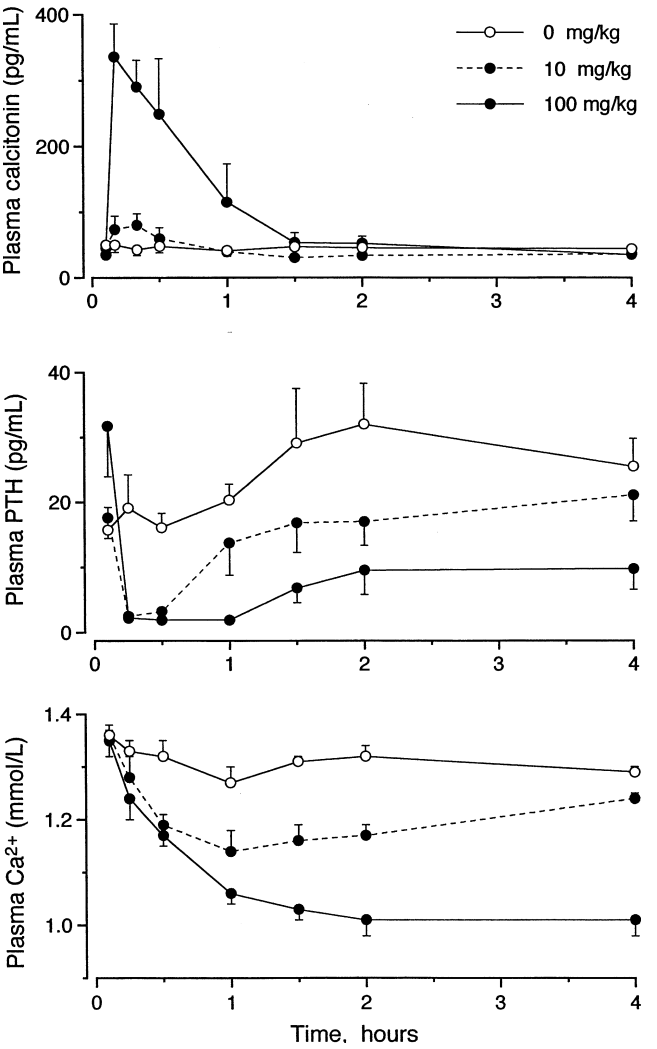

Figure 2 NPS R-568 decreases plasma levels of PTH and Ca^{2+} and increases those of calcitonin. NPS R-568 (or vehicle) was administered orally at the indicated dose to normal male rats. Note that the 10 mg/kg dose, which is close to the ED_{50} for depressing plasma PTH levels, is without effect on plasma levels of calcitonin (Fig. 2 in Nemeth *et al.,* 1996).

Calcilytics

A novel series of compounds acting as Ca^{2+} receptor antagonists has been reported (Fig. 1; Nemeth *et al.,* 2001). The prototype compound, NPS 2143, is the first *substance,* either atomic or molecular, shown to block Ca^{2+} receptor activity. The inhibitory effects of NPS 2143 are not dependent on the properties of the ligand used to activate the receptor in either heterologous expression systems or authentic cells. The potency and the efficacy of NPS 2143 is similar whether blocking cytoplasmic Ca^{2+} responses evoked by type I (Ca^{2+}) or type II (NPS R-467) calcimimetic ligands. NPS 2143 is without effect on the cytoplasmic Ca^{2+} responses elicited by activation of various native or chimeric mGluRs nor does it affect those in cells coexpressing the $GABA_BR1$ and R2. Calcilytic compounds like NPS 2143, when used under appropriate conditions, are potent and selective pharmacological tools

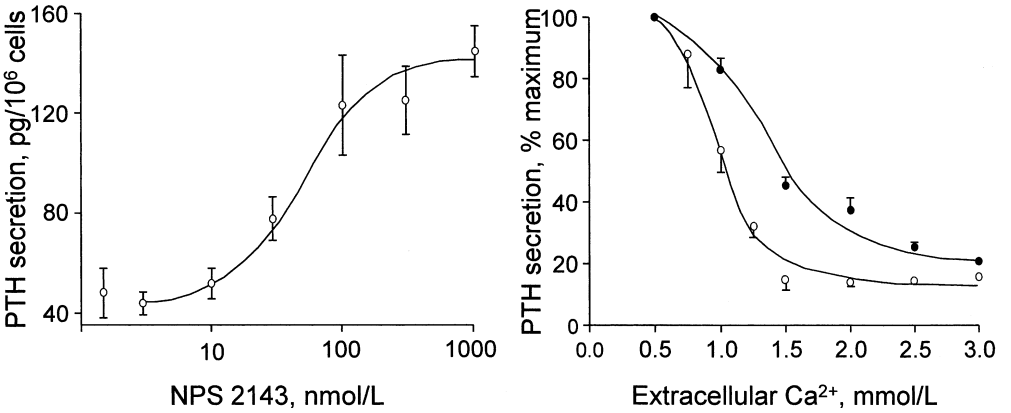

Figure 3 NPS 2143 stimulates PTH secretion from bovine parathyroid cells *in vitro.* (Right) Parathyroid cells were incubated in the presence (closed symbols) or absence of 300 nM NPS 2143 (Fig. 7 in Nemeth *et al.,* 2001).

to study the function of Ca^{2+} receptors expressed in normal and pathological tissues.

The effects of NPS 2143 on PTH secretion from bovine parathyroid cells mirror those of type II calcimimetic compounds. As noted above, these calcimimetics are positive allosteric modulators that shift the concentration–response curves of extracellular Ca^{2+} to the left without changing the maximum or minimum responses. NPS 2143 affects the agonist concentration–response curve in a converse manner: there is a shift to the right which is unaccompanied by changes in either the maximal or the minimal response (Fig. 3). These changes in the agonist concentration–response curves suggest that NPS 2143 decreases, whereas type II calcimimetic compounds increase the sensitivity of the Ca^{2+} receptor to activation by extracellular Ca^{2+}. Although the rightward shift of the concentration–response curve could indicate competitive inhibition by NPS 2143, the shifts in the concentration–response curve shown in Fig. 3 would also be produced by a noncompetitive antagonist acting on a tissue with a large receptor reserve (Zhu, 1993). As discussed below, there might be a large reserve of Ca^{2+} receptors on parathyroid cells.

NPS 2143 stimulates secretion of PTH from bovine parathyroid cells *in vitro* at concentrations similar to those that inhibit evoked increases in $[Ca^{2+}]_i$. When administered intravenously or by oral gavage to normal rats, the compound causes a rapid, three- to fivefold increase in plasma levels of PTH (Fig. 4; Nemeth *et al.,* 2001). These findings were comforting because it was never a certainty that a calcilytic compound would in fact stimulate PTH secretion. While intuitively appealing, this assumption rests solely on the finding that lowering the level of extracellular Ca^{2+} stimulates PTH secretion. This is not, however, equivalent to blocking the activity of the receptor in a normocalcemic setting. The

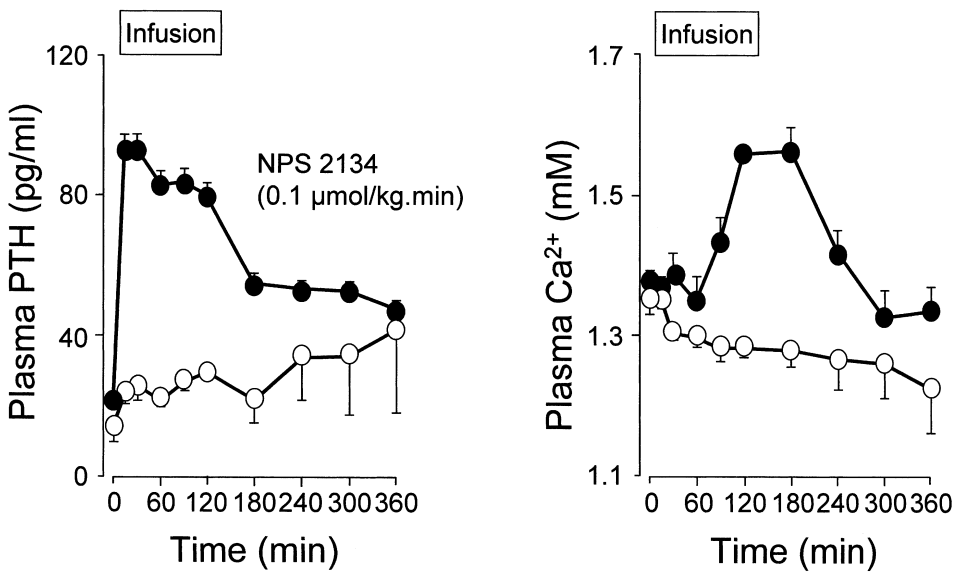

Figure 4 NPS 2143 rapidly increases circulating levels of PTH in normal rats. NPS 2143 was administered by infusing intravenously (0.1 μmol/kg·min; closed symbols) for 2 hr (Fig. 9 in Nemeth *et al.,* 2001).

stimulatory effects of NPS 2143 on PTH secretion *in vitro* and on circulating levels of hormone *in vivo* clearly underscores the key role of the Ca^{2+} receptor in controlling PTH secretion.

Pharmacological Selectivity of Calcium Receptor Ligands

Despite apparently similar Ca^{2+} receptors on parathyroid cells and on C cells, their pharmacological properties are different. An early example of this was noted with Mg^{2+}. Extracellular Mg^{2+} increases $[Ca^{2+}]_i$ in bovine parathyroid cells and in HEK 293 cells expressing the human, bovine, or rat Ca^{2+} receptor (Rogers *et al.,* 1995). Similar increases in the concentration of extracellular Mg^{2+}, however, fail to increase $[Ca^{2+}]_i$ in the MTC cell lines 44–2 or 6–23 (Nemeth, 1990; Fajtova *et al.,* 1991). Nonetheless, increased concentrations of extracellular Mg^{2+} stimulate secretion of calcitonin from authentic C cells *in vitro* (Bell, 1970; Radde *et al.,* 1969) and *in vivo* (Care *et al.,* 1971; Littledike and Arnaud, 1971; Pento *et al.,* 1974). Perhaps this discrepancy results from comparing MTC cells with authentic C cells since these cells are likely to express different phenotypes. This explanation cannot, however, account for the preferential activity of type II calcimimetics on secretion of calcitonin and PTH that is noted in normal rats *in vivo* (Fox *et al.,* 1999b). The Ca^{2+} receptor mRNAs expressed in rat parathyroid cells and C cells are identical and the final protein products are likewise similar (Garrett *et al.,* 1995b). Nonetheless, the type II calcimimetic compounds NPS R-467 and NPS R-568 depress plasma levels of PTH at doses 30- to 40-fold lower than those that increase plasma levels of calcitonin (Figs. 2 and 5).

The mechanisms(s) explaining the preferential inhibitory effects of NPS R-568 on PTH secretion is uncertain. At present, there is no evidence for grossly different posttranslational modifications of the Ca^{2+} receptor in parathyroid cells and C cells. One hypothesis we recently put forward was that perhaps the concentration–response curve for extracellular Ca^{2+} and calcitonin secretion was less steep than that for extracellular Ca^{2+} and PTH (Wada *et al.,* 1999). It appears, however, that the slope (Hill coefficient) of both curves are similar and that maximal secretory responses of both PTH and calcitonin are produced over a very narrow range of plasma Ca^{2+}, even though their ED_{50} values differ (Lavigne *et al.,* 1998; Fox *et al.,* 1999b). Another possibility is that different degrees of receptor occupancy may be required to alter secretion from parathyroid cells and from C cells. In this view, a lower fractional occupancy of the Ca^{2+} receptor is required to depress PTH secretion than is required to stimulate calcitonin secretion. NPS R-568 is correspondingly more potent in depressing plasma levels of PTH. Alternatively, or additionally, the different signaling pathways linking the Ca^{2+} receptor to PTH and to calcitonin secretion might also contribute to the preferential activity of type II calcimimetic compounds on parathyroid Ca^{2+} receptors.

Oligomerization of receptors might also contribute to the differential pharmacology of type II calcimimetic compounds. Many G protein-coupled receptors, including Ca^{2+} receptors (Bai *et al.,* 1998, 1999), have been shown to be expressed as homodimers in heterologous systems although there remain only a few studies in which any G protein-coupled receptor is expressed and functions as a dimer in cellular environments where they normally reside. It is not known if Ca^{2+} receptors are expressed as dimers in their

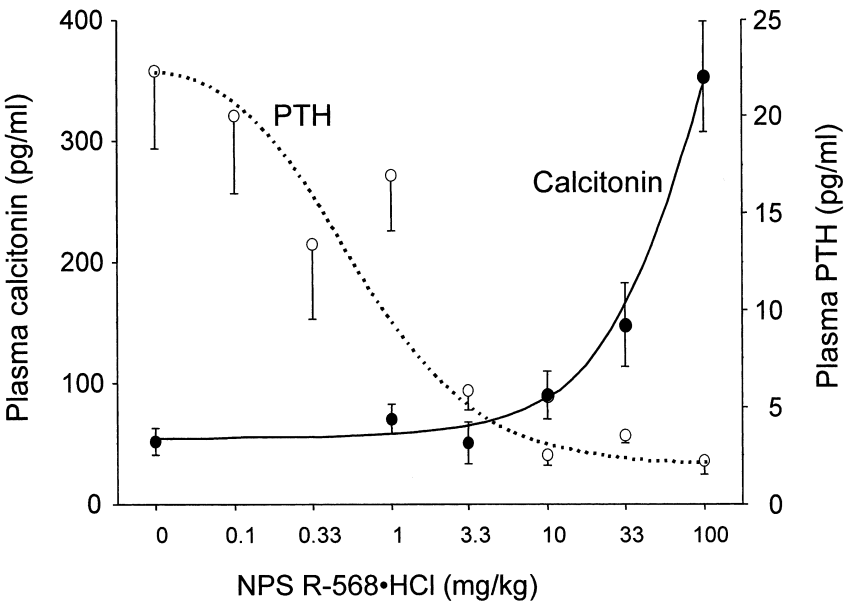

Figure 5 NPS R-568 preferentially acts on the parathyroid Ca^{2+} receptor. NPS R-568 was administered orally to normal rats and the changes in plasma levels of PTH and calcitonin determined 15 to 20 min later. (Redrawn from data in Fox *et al.,* 1999a,b.)

native environment or whether the pharmacological properties of Ca^{2+} receptor monomers and dimers are different.

Regardless of the molecular mechanism underlying the different pharmacology of C cells and parathyroid cells, the important finding is that PTH secretion can be preferentially regulated by a single compound. The preferential actions of type II calcimimetic compounds on parathyroid Ca^{2+} receptors might also apply to renal Ca^{2+} receptors and this would explain why renal Ca^{2+} receptors, although involved in Ca^{2+} transport in the kidney, are not necessary for the type II calcimimetics to cause hypocalcemia.

The Calcium Receptor in Hyperparathyroidism

One of the early uncertainties in developing calcimimetic compounds for treating hyperparathyroidism (HPT) was whether the disease process in some way altered the parathyroid Ca^{2+} receptor so that calcimimetics, while effective in normal animals and humans, were no longer capable of altering plasma levels of PTH in patients with primary or secondary HPT. No mutations, however, have yet been detected in the coding region of the Ca^{2+} receptor gene in pathological parathyroid glands from patients with primary or secondary HPT (Garrett *et al.,* 1995a; Hosokawa *et al.,* 1995; Cetani *et al.,* 2000). A consistent finding, however, is reduced expression of the Ca^{2+} receptor in both adenomatous and hyperplastic tissues (Kifor *et al.,* 1996; Farnebo *et al.,* 1997; Gogusev *et al.,* 1997; Kaneko *et al.,* 1999). This conclusion is based on semi quantitative analyses of Ca^{2+} receptor mRNA and staining intensities of parathyroid tissue using mono- or polyclonal antibodies against the Ca^{2+} receptor. Like the data for the vitamin D receptor (Fukuda *et al.,* 1993), nodular regions showed a greater loss of staining intensity for Ca^{2+} receptor when compared to adjacent areas of diffuse hyperplasia. Recent clinical results obtained in patients with primary HPT reveal a correlation between the level of parathyroid Ca^{2+} receptor expression, as judged by the intensity of staining of tissues with a polyclonal antibody to the Ca^{2+} receptor, and the PTH–Ca^{2+} set point—the set point was higher in those patients with glands having the least staining intensity (Cetani *et al.,* 2000). Thus, in contrast to the altered set points in genetic diseases of the Ca^{2+} receptor that result from changing the structure of the Ca^{2+} receptor, those in hyperparathyroidism result largely from changing the density of otherwise normal Ca^{2+} receptors. The issue was whether there were enough Ca^{2+} receptors expressed to permit regulation of PTH secretion by calcimimetic compounds.

Several different lines of evidence suggest that the apparently reduced expression of Ca^{2+} receptor protein in pathological parathyroid glands does not render such glands insensitive to calcimimetic compounds. First, secretion of PTH from adenomatous or hyperplastic parathyroid glands *in vitro* is inhibited by NPS R-568 (Nemeth, 1996) so, at least in these particular surgical tissue samples, there was enough Ca^{2+} receptor remaining to respond to this calcimimetic compound. Moreover, the potency of NPS R-568 is similar in normal rats or those with secondary HPT resulting from a 5/6 nephrectomy (Fox *et al.,* 2000). Finally, and most significantly, calcimimetic compounds lower plasma levels of PTH in patients with primary or secondary HPT. As detailed below, there has yet to be a patient dosed with a calcimimetic compound who did not show a rapid fall in plasma levels of PTH.

Primary Hyperparathyroidism

Primary hyperparathyroidism is an age-related disorder that afflicts postmenopausal women disproportionally. The disease is characterized by chronically elevated plasma levels of PTH and Ca^{2+} and usually results from a single adenoma or hyperplasia of the parathyroid glands (Heath, 1991). The manifestations of primary hyperparathyroidism may include bone loss, nephrolithiasis and nephrocalcinosis, gastrointestinal distress, muscular fatigue, and mental depression. Although many patients with hyperparathyroidism are apparently asymptomatic, it has been argued that these patients simply have not been followed long enough for symptoms to become manifest (Fischer, 1993). Moreover, because the incidence of primary hyperparathyroidism is age related, many of the rather vague symptoms such as muscular fatigue and depressed cognition may be interpreted as the normal aging process. Whether these so-called asymptomatic patients should be treated is controversial and at present the most widely followed practice is "watchful waiting" (Bilezikian, 1994).

There is somewhat less controversy regarding treatment of moderate and essentially none regarding treatment of severe forms primary hyperparathyroidism. The treatment of choice is surgical ablation of the offending gland(s) and, when performed by a skilled endocrine surgeon, the success (cure) rate is 95% (Clark, 1995). There are, however, many instances when surgery would not be elected if effective medical alternatives existed. These include patients who refuse surgery, are elderly, have comorbid conditions, or have undergone previous unsuccessful surgeries. Many patients with parathyroid carcinoma are not effectively treated by surgery (Shane and Bilezikian, 1982). In addition, there are those situations where it would be desirable to delay surgery or to stabilize plasma levels of Ca^{2+} prior to surgery. At present, there is no safe and effective means of managing these patients although a variety of different medical treatments have been tried (Stock and Marcus, 1994).

Medical treatments such as oral phosphate, calcitonin, or bisphosphonates are effective in temporarily reducing plasma levels of Ca^{2+} but often do not change or may even cause a further increase in PTH levels. For example, in a study of patients with mild primary HPT, 1 year of treatment with alendronate caused a temporary decrease in serum Ca^{2+} at 3 months but returned to hypercalcemic levels by 6 months and remained elevated thereafter. The

temporary decrease in serum Ca^{2+} triggered an increase in PTH levels which returned to pretreatment levels once serum Ca^{2+} rose (Rossini *et al.,* 2001). Another approach to treating primary HPT relies on the use of PTH receptor antagonists which have been shown in animal studies to block increases in plasma Ca^{2+} evoked by infusion of a PTH receptor peptide agonist (Rosen *et al.,* 1997). The competitive peptide antagonist BIM-44002, however, failed to lower plasma levels of PTH or Ca^{2+} when infused into patients with primary HPT. Estrogens and progestins have met with some success in normalizing plasma levels of Ca^{2+} but not those of PTH. All these therapies are aimed at treating the hypercalcemia but not the hyperparathyroidism itself. All these approaches are unlikely to be therapeutically useful because lowering Ca^{2+} will invariably stimulate secretion of PTH which, in turn, will defeat the intent of the treatment.

Additional approaches using drugs that act directly on parathyroid cells have also been explored. Inhibition of the biosynthesis of PTH with 1α-hydroxyvitamin D3 (Lind *et al.,* 1989) transiently lowered plasma levels of PTH but also caused hypercalcemia. Another approach has been to target cell surface receptors like the β-adrenergic or the H2-histaminergic receptors that are linked to a stimulation of PTH secretion. The receptor antagonists propranolol and cimetidine have been unequivocally ineffective in managing primary hyperparathyroidism (Fisken *et al.,* 1982; Gedik *et al.,* 1983; Robinson *et al.,* 1980). The somatostatin analog octreotide has been similarly ineffective in lowering PTH levels in patients with either primary or secondary HPT (Zielke *et al.,* 1997). The only approach that has never been tried is to target the primary physiological mechanism regulating PTH secretion, the Ca^{2+} receptor.

By targeting the Ca^{2+} receptor, parathyroid calcimimetic compounds offer the first "disease-designed" approach for treating primary hyperparathyroidism. The initial clinical trial in normal postmenopausal women showed that the oral administration of NPS R-568 was well tolerated and reduced plasma levels of PTH and Ca^{2+} (Health *et al.,* 1995). Doses of NPS R-568 that maximally depressed plasma levels of PTH were without effect on plasma levels of calcitonin. These findings in humans parallel those obtained in preclinical studies and show that plasma levels of PTH and calcitonin in humans can be differentially affected by a calcimimetic compound.

Calcimimetic compounds have been shown to be effective in reducing circulating levels of PTH and Ca^{2+} patients with primary HPT (Table I; Silverberg *et al.,* 1997; Shoback *et al.,* 2000). The oral administration of NPS R-568 to postmenopausal women with primary HPT caused a rapid and dose-dependent decrease in plasma levels of PTH (Fig. 6). The inhibitory effects of NPS R-568 were readily reversible and, depending on the dose, plasma PTH returned to predose levels with 2 to 8 hr. At low doses, there was a transient decrease in the level of PTH that was unaccompanied by a change in plasma levels of Ca^{2+}. At higher doses, the decrease in plasma PTH levels lasted longer and plasma Ca^{2+} levels started to fall about 1 hr after plasma PTH levels reached a nadir. Paralleling this hypocalcemic response was an increase in urinary Ca^{2+} excretion. This temporal relationship is consistent with a primary action of the calcimimetic to inhibit secretion of PTH and the hypocalcemic response is secondary to this lowering of plasma PTH levels.

Similar findings have recently been obtained in male and in female patients with primary HPT using a newer type II calcimimetic compound, AMG 073 (Shoback *et al.,* 2000). In this study, the calcimimetic compound or a placebo was administered orally twice a day for 15 days. There was a rapid, time- and dose-dependent reduction in circulating levels of PTH which reached a nadir 2 to 4 hr after the first dose and was correlated with plasma levels of AMG 073. Plasma levels of PTH and Ca^{2+} were lower throughout the 15-day treatment period. When dosing was terminated, plasma levels of PTH and Ca^{2+} returned to the abnormally high levels observed in patients prior to study. In this particular clinical study, there was no increase in urinary excretion of Ca^{2+} over the 15-day study period. The clinical experience with type II calcimimetic compounds parallels the results obtained in preclinical studies. Together, they provide compelling evidence showing that these compounds can selectively target the parathyroid Ca^{2+} receptor to preferentially lower plasma levels of PTH. Moreover, the receptor does not desensitize to daily treatment with type II calcimimetics for a period of 2 weeks.

Table I Clinical Experience with the Use of Calcimimetic Compounds in Patients with Primary HPT

Study	Patients and treatment regimen	Dose of calcimimetic	Percentage change in plasma PTH and Ca^{2+} levels
Silverberg *et al.,* 1997	20 Postmenopausal women; single ascending dose	Palcebo or 4 to 160 mg NPS R-568	PTH decreased 26 – 73% in a dose-dependent fashion; Ca^{2+} fell from 1.35 to 1.30 mM
Shoback *et al.,* 2000	22 Male and female patients; 2 doses/day for 15 days	Placebo or 30, 40, or 50 mg AMG 073	PTH decreased 47% and Ca^{2+} decreased 20%
Collins *et al.,* 1998	Male patient with parathyroid carinoma; dose every 6 hr for 600 days	300 or 600 mg NPS R-568/day	PTH decreased from 600 – 1050 to 250 – 600 pg/ml; Ca^{2+} decreased from 1.95 to 1.50 mM

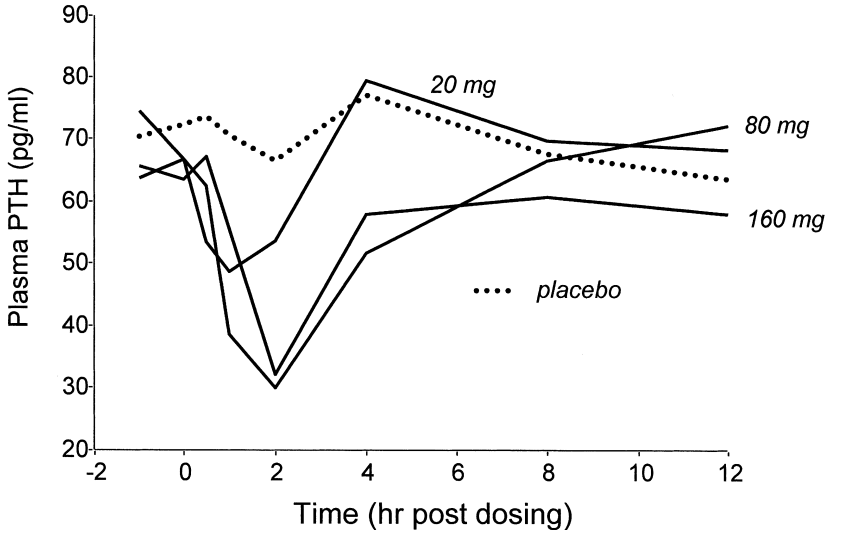

Figure 6 NPS R-568 lowers plasma levels of PTH in postmenopausal women with primary hyperparathyroidism. (Redrawn from data in Silverberg *et al.,* 1997.)

A rare but far more serious form of primary HPT results from a carcinoma of one of the parathyroid glands. In contrast to primary HPT resulting from an adenoma or hyperplasia, that due to a carcinoma is life-threatening. Patients with parathyroid carcinoma present with the signs and symptoms of frank hypercalcemia and often have hyperparathyroid bone disease (Shane and Bilezikian, 1982). Surgical treatment of this malignant form of primary HPT is considerably more difficult because of metastasis; multiple resections are common. In a male patient with severe hypercalcemia resulting from parathyroid carcinoma, NPS R-568 was able to lower plasma levels of PTH and return plasma Ca^{2+} to near normal levels (Collins *et al.,* 1998). Neither calcitonin, furosemide, hydration, nor the bisphosphonate pamidronate, consistently lowered plasma Ca^{2+} levels (Fig. 7) and plasma PTH levels were unaffected by these treatments. In contrast, daily oral dosing with NPS R-568 controlled plasma levels of PTH and Ca^{2+} for almost 2 months. At that time, plasma PTH levels began to increase but plasma Ca^{2+} levels did not. This patient was subsequently maintained on high doses of NPS R-568 (100 mg orally, every 6 hr) for over 600 days without any instances of hypercalcemic crisis or any adverse effects due to drug. At the very least, this study shows that tolerance to hypocalcemic effects of NPS R-568 does not develop within 2 years of daily dosing and that the compound is safe and well-tolerated.

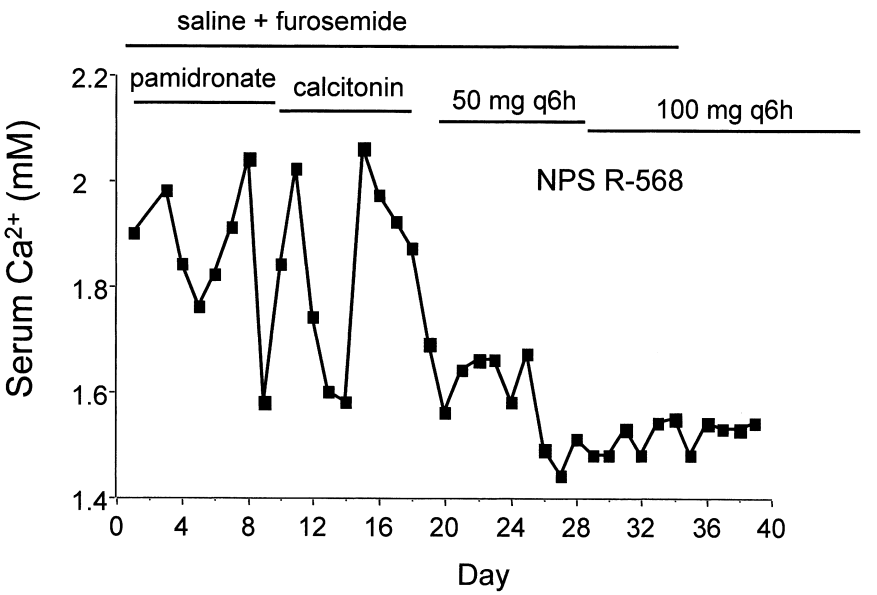

Figure 7 NPS R-568 lowers plasma levels of PTH in a male patient with parathyroid carcinoma (Fig. 1 in Collins *et al.,* 1998).

The initial clinical results obtained in patients with primary HPT arising from either benign or malignant tumors show that targeting the Ca^{2+} receptor with calcimimetic compounds is effective in lowering plasma levels of PTH and Ca^{2+}. In the setting of primary HPT, it is conceivable that a calcimimetic compound could be a stand-alone therapy.

Secondary Hyperparathyroidism

Secondary HPT can result from a variety of circumstances, including the normal aging process, but it is most often associated with chronic renal failure and most patients with end-stage renal disease (ESRD) suffer from secondary HPT (Coburn and Salusky, 1994). Secondary HPT is characterized by enlargement of all four parathyroid glands, principally through hyperplasia but also involving hypertrophy (Drüeke, 1995). The primary factors that lead to enlargement of the parathyroid glands are hypocalcemia, hyperphosphatemia, and lowered plasma levels of 1,25-dihydroxyvitamin D_3 (calcitriol). The mechanisms by which these factors lead to hyperplasia and hypertrophy of the parathyroid glands are not completely understood but each appears capable of acting wholly or partially independent of the others to cause secondary HPT. Resistance to the skeletal effects of PTH also contributes to the development of secondary HPT. Plasma levels of PTH increase very early on in the course of renal failure and may already be elevated when creatinine clearance reaches 60 ml/min (Fajtova *et al.,* 1995), well before the patient would be started on dialysis. The severity of secondary HPT, as indexed by enlargement of the parathyroid glands and increased circulating levels of PTH, often increases as the renal disease progresses from mild chronic renal insufficiency (CRI) to ESRD. Because the etiologies of chronic renal failure are diverse, the relative roles of phosphate, Ca^{2+}, and calcitriol in the development of secondary HPT may vary. This, in turn, may mandate different therapeutic strategies to treat secondary HPT depending on the relative contribution of the factors that cause it.

The management of HPT secondary to chronic renal failure has been covered extensively in recent reviews (Coburn and Salusky, 1994; Fournier *et al.,* 1992; Felsenfeld, 1997). The mainstays in treating secondary HPT are restriction of dietary phosphorous, administration of phosphate-binders and calcitriol or its analogs, and parathyroidectomy. Each of these therapies has met with success in some settings but none are ideal. Hypercalcemia is a common complication with calcium-containing phosphate-binders or with calcitriol and its analogs. Attempts to control plasma levels of PTH with calcitriol alone have not been overwhelmingly successful. Prospective studies have shown that calcitriol fails to lower plasma levels of PTH in many patients and in others it does so only at doses that elicit hypercalcemia and/or hyperphosphatemia (Herrmann *et al.,* 1994; Mazzaferro *et al.,* 1994; Quarles *et al.,* 1994a). The inconsistent response of PTH to calcitriol, which is most prominent in patients with long-established secondary HPT, could reflect variable loss of receptors for calcitriol in parathyroid tissue (Korkor, 1987; Merke *et al.,* 1987). Newer so-called "noncalcemic" analogs of calcitriol, like 19-nor-1-α-dihydroxyvitamin D_2 (Martin *et al.,* 1998) or 1α-hydroxyvitamin D_2 (Tan *et al.,* 1997; Frazão *et al.,* 2000), elicit fewer episodes of hypercalcemia but there is insufficient clinical experience with either analog to determine their efficacies in reducing plasma levels of PTH in patients who do not respond to calcitriol. Different therapies, either alone or in combination, may be required as the renal disease progresses. In general, however, it seems worthwhile to start treatment early in the course of renal failure because treatment in the predialysis period may retard the development of secondary HPT.

Preclinical Studies in Animal Models of Secondary Hyperparathyroidism

A series of collaborative investigations between NPS Pharmaceuticals and the Pharmaceutical Division of Kirin Brewery characterized the effects of calcimimetic compounds in several different rodent models of secondary HPT. CRI was usually produced by surgically removing most of the kidney (5/6 nephrectomy). This lesion could be combined with diets that differed in their calcium-to-phosphate ratios to produce a range of plasma PTH levels characteristic of mild, moderate, or severe forms of secondary HPT. The results that emerged from these studies not only showed the efficacy of calcimimetics in disease models but additionally provided pharmacological evidence showing that the Ca^{2+} receptor regulates proliferation of parathyroid cells.

In rats with secondary HPT resulting from CRI, the oral administration of NPS R-568 caused a dose-dependent decrease in plasma levels of PTH. The magnitude and rate of change of plasma PTH levels following orally administered compound were similar in normal animals or those with secondary HPT (Fox *et al.,* 1999c). Moreover, animals with mild (normal to 150 pg/ml PTH), moderate (150 to 500 pg/ml), or severe secondary HPT (>500 pg/ml) all responded similarly to the intraarterial injection of NPS R-568: the compound reduced plasma PTH levels by 82 to 94% within 20 min of dosing regardless of the initial plasma PTH level (Fox *et al.,* 1999c). Some of these animals had severe hyperphosphatemia and moderate hypocalcemia yet these two stimuli to PTH secretion could not prevent the depressive effect of NPS R-568 on plasma levels of PTH. Thus, activation of the Ca^{2+} receptor with NPS R-568 markedly lowers circulating levels of PTH irrespective of the severity of the secondary HPT or the magnitude of the hyperphosphatemia.

Despite reports showing a decreased expression of parathyroid Ca^{2+} receptor mRNA and protein in such animal models of secondary HPT (Mathias *et al.,* 1998; Brown *et al.,* 1999), there is apparently enough Ca^{2+} receptor

expressed to preserve responsivity to calcimimetic compounds. In fact, the potencies of NPS R-568 for lowering plasma levels of PTH and Ca^{2+} or for increasing those of calcitonin are the same in normal animals and those with CRI and moderate to severe secondary HPT (Fox *et al.,* 2000). Thus, PTH levels can be maximally suppressed by calcimimetic compounds even when Ca^{2+} receptor expression is reduced and during states of chronic hyperphosphatemia and/or hypocalcemia.

More long-term studies in rats with CRI have shown that the decreases in plasma levels of PTH caused by NPS R-568 have beneficial effects on some of the skeletal abnormalities which accompany secondary HPT (Wada *et al.,* 1998). Six weeks following a partial nephrectomy, animals had developed a mild to moderate secondary HPT with profound peritrabecular fibrosis; static and dynamic histomorphometry revealed a high turnover bone lesion. The daily oral administration of NPS R-568 during the last 5 weeks completely prevented the development of osteitis fibrosa and tended to normalize histomorphometric parameters. Daily treatment with NPS R-568 additionally restored the decreases in volumetric cortical bone mineral density and in cortical bone stiffness at the femoral midshaft which was observed in vehicle-treated CRI animals. The results obtained using rodent models of CRI-induced secondary HPT show that NPS R-568 consistently reduces plasma levels of PTH even following prolonged, daily administration. Tolerance to the effects of NPS R-568 do not develop in these animal models of secondary HPT. Moreover, the daily decreases in circulating levels of PTH caused by NPS R-568 translates into positive effects on high turnover bone abnormalities including the prevention or reversal of osteitis fibrosa.

In addition to effectively lowering plasma levels of PTH, calcimimetic compounds also block parathyroid gland hyperplasia resulting from CRI. As noted above, hypocalcemia, hyperphosphatemia, and lowered levels of calcitriol all contribute to parathyroid gland hyperplasia although the mechanisms they use to cause cellular proliferation and hypertrophy are uncertain. In fact, the effect of hypocalcemia on parathyroid gland histology is still controversial and either no effect or a stimulation of cell growth has been reported (Naveh-Many *et al.,* 1995; Wernerson *et al.,* 1991). These discrepant findings possibly reflect the difficulty inherent in any attempt to alter levels of plasma Ca^{2+} without affecting those of phosphate or calcitriol. Type II calcimimetic compounds offer a novel means of reassessing the role of plasma Ca^{2+} in parathyroid gland hyperplasia; they can trick the parathyroid cell into perceiving hypercalcemia despite a prevailing normo- or hypocalcemic state (Nemeth and Bennett, 1998).

Parathyroid gland hyperplasia begins within days following a partial nephrectomy in the rat (Denda *et al.,* 1996) and the initial studies examined the effects of NPS R-568 on parathyroid cell proliferation during the first 5 days of CRI in rats (Wada *et al.,* 1997). Bromodeoxyuridine (BrdU) was infused intraperitoneally throughout and NPS R-568 was administered orally twice a day during the last 4 days. As shown in Fig. 8, there was a threefold increase in cellular labeling with BrdU in partially nephrectomized animals which was blocked in a dose-dependent manner by treatment with NPS R-568. The inhibitory effect of NPS R-568 on BrdU labeling was specific for parathyroid cells and did not affect labeling of C cells, even at the high dose which stimulated calcitonin secretion. These findings suggest that the Ca^{2+} receptor mediates the effects of hypocalcemia on parathyroid cell proliferation. If the glands responded to the prevailing low calcium levels by a mechanism other than the Ca^{2+} receptor, then NPS R-568 would not have prevented the proliferative response to partial nephrectomy. The mechanisms linking the Ca^{2+} receptor to the regulation of cellular proliferation are present in parathyroid cells but not in C cells.

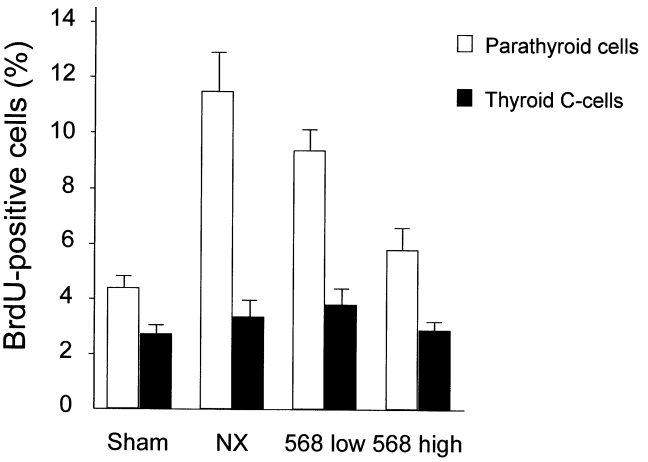

Figure 8 NPS R-568 blocks parathyroid cell proliferation in partially nephrectomized rats. NPS R-568 was administered orally twice a day at a dose of either 1.5 (568 low) or 15 mg/kg (568 high) (Fig. 4 in Wada *et al.,* 1997).

These initial findings prompted two long-term studies in rats with CRI. The first assessed whether initiating treatment with NPS R-568 soon after the development of CRI would prevent parathyroid gland hyperplasia (Wada *et al.,* 2000). CRI was induced by ligating the renal arteries and feeding the animals a diet high in phosphate content. Treatment with NPS R-568 was started 6 days after renal artery ligation and continued for 56 days. The vehicle-treated CRI animals developed severe secondary HPT with 10-fold elevations in circulating levels of PTH compared to sham-operated animals. Parathyroid gland volume was 2.8-fold higher in the former group of animals and this resulted mostly from an increase in cell number rather than cell volume. The enlargement and cellular hyperplasia of the parathyroid glands was completely prevented by treatment with NPS R-568.

The second study assessed whether treatment with NPS R-568 could halt the progression of parathyroid gland hyperplasia in animals with CRI (Chin *et al.,* 2000). Secondary HPT was allowed to develop for 11 weeks following a 5/6 nephrectomy before initiating treatment with NPS

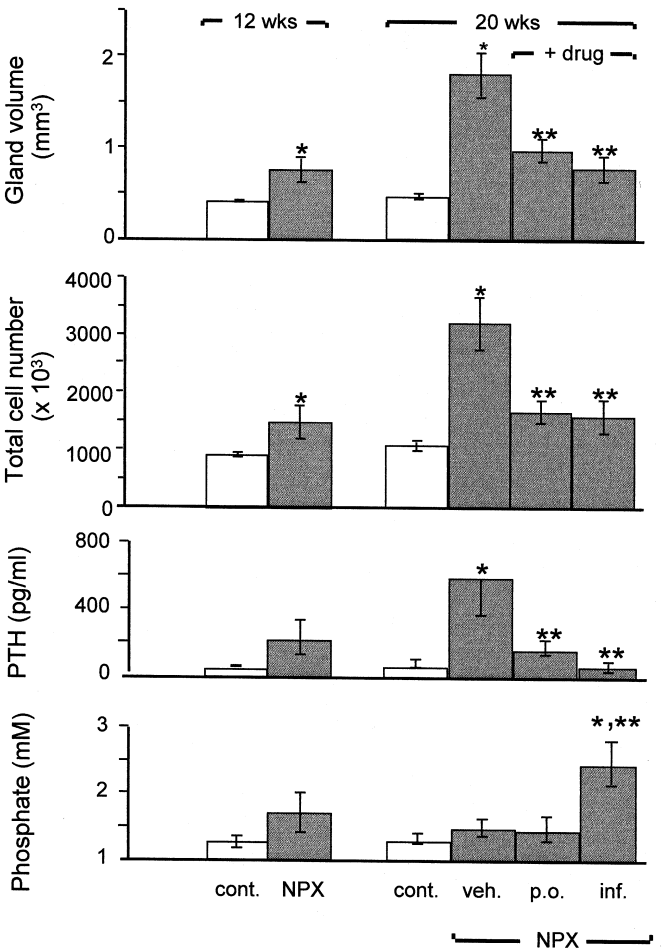

Figure 9 NPS R-568 blocks the progression of secondary hyperparathyroidism in CRI rats. NPS R-568 or vehicle was administered orally once a day (po; 10 μmol/kg) or continuously infused (inf; 20 μmol/kg/day) for 8 weeks beginning 11 weeks after a 5/6 nephrectomy (redrawn from data in Fox *et al.,* Chin *et al.,* 2000)

R-568 for 8 weeks. At 11 weeks following surgery, parathyroid gland volume was 1.9-fold larger in CRI animals and the glandular volume continued to increase in these animals another 2-fold by the end of the study. Parathyroid gland enlargement was paralleled by a 5.4-fold increase in plasma levels of PTH in vehicle-treated CRI animals which continued to increase another 2.6-fold during the last 8 weeks (Fig. 9). Secondary HPT continued to worsen during the course of this study, as indicated by continually rising levels of circulating PTH and progressive enlargement of the parathyroid glands. Daily oral administration of NPS R-568 completely blocked the progression of secondary HPT as indicated by either parameter (Fig. 9). Moreover, progressive increases in plasma PTH levels and parathyroid gland volume were completely prevented by NPS R-568 even when there was profound hypocalcemia, hyperphosphatemia, and lowered levels of 1,25-dihydroxyvitamin D_3 (Wada *et al.,* 2000; Chin *et al.,* 2000). Such findings suggest that the Ca^{2+} receptor plays the dominant role in regulating at least two different parathyroid cell functions: PTH secretion and cellular proliferation. Activating the Ca^{2+} receptor with a calcimimetic compound essentially neutralizes the stimulatory effects of hypocalcemia and dominates those of hyperphosphatemia and calcitriol.

These preclinical findings in rodent models of secondary HPT highlight the therapeutic potential of the parathyroid Ca^{2+} receptor. Because it controls both secretion and proliferation, compounds which activate it have the potential to halt the progression of secondary HPT. It is conceivable that identification of patients with CRI could lead to the prophylactic use of calcimimetic compounds. Treatment with a calcimimetic compound early in the course of renal failure might completely prevent the development of secondary HPT. At the very least, these studies demonstrate the efficacy of calcimimetic compounds in lowering plasma levels of PTH. So far, the results obtained in rodent models of secondary HPT have been reproduced in the clinic with high fidelity.

Clinical Studies in Patients with Secondary Hyperparathyroidism

Studies of the calcimimetic compounds NPS R-568 or AMG 073 in patients with secondary HPT generated results remarkably similar to those obtained in rodent models of this disease. The clinical experience gained with calcimimetic compounds for treating secondary HPT is summarized in Table II.

In the initial prospective, dose-finding pilot study, NPS R-568 was administered orally to seven male patients with secondary HPT at the start of a hemodialysis session and again 24 hr later (Antonsen *et al.,* 1998). Basal levels of PTH in these patients were around 200 pg/ml and fell by 40 or 60% within 1 hr after receiving a single dose of 40 or 80 mg NPS R-568. At the lower dose, plasma levels of PTH returned to basal values 4 hr after dosing but levels were still partially depressed 24 hr after the higher dose. The second administration of either dose when the patients were not receiving dialysis caused a similar percentage decrease in plasma PTH levels. The decreases in plasma PTH levels were accompanied by a lowering of whole blood Ca^{2+} which, even after the high dose of NPS R-568, remained above the lower limit of normal (1.1 mM) and none of the patients developed signs or symptoms of hypocalcemia. With the 80 mg dose, there was a twofold increase in the circulating levels of calcitonin which returned to baseline by 24 hr.

In another pilot study (Akizawa *et al.,* 1998), six male patients on hemodialysis received 100 or 200 mg NPS R-568 on each of two consecutive days, one of which included a dialysis session. The 100-mg dose produced results very similar to those obtained by Antonsen *et al.* (1998): a rapid, reversible decrease in plasma levels of PTH which, in five of the six patients, was unaccompanied by clinically significant hypocalcemia. At the 200-mg dose, plasma PTH levels were suppressed by 80% but, in five of the six patients, this was associated with hypocalcemia. In both of these pilot studies,

Table II Clinical Experience with the Use of Calcimimetic Compounds in Patients with Secondary HPT

Study	Patients and treatment regimen	Dose of calcimimetic	Percentage change in plasma PTH levels
Antonsen *et al.,* 1998	7 Hemodialysis patients with mild SHPT; 1 dose/day for 2 days	40 to 200 mg/day NPS R-568	PTH decreased 37 – 63%
Akizawa *et al.,* 1998	6 Hemodialysis patients with Moderate to severe SHPT; 1 dose/day for 2 days	100 to 200 mg/day NPS R-568	PTH decreased 59 – 73%
Goodman *et al.,* 2000a	21 Male or female hemodialysis patients with moderate to severe SHP; 1 dose/day for 15 days	Placebo or 100 mg/day NPS R-568	PTH decreased 30 – 70%
Goodman *et al.,* 2000b	30 Male or female hemodialysis patients with SHPT; 1 dose/day for 8 days	Placebo or 10 to 50 mg/day AMG 073	PTH decreased 25 – 40%
Coburn *et al.,* 2000	Male and female hemodialysis patients with SHPT; single dose	Placebo or 5 to 100 mg/day AMG 073	PTH decreased 40 – 60%
Lindberg *et al.,* 2000	Male or female hemodialysis patients; 1 dose/day; dose increased every 3 weeks for 12 weeks, final dose was maintained for 6 weeks	Placebo or 10 to 50 mg/day AMG 073	PTH decreased by 26% during the maintenance phase of the study

NPS R-568 was well-tolerated and, other than transient bouts of hypocalcemia at high doses, there were no drug-related adverse events.

In the study of Akizawa *et al.* (1998), plasma levels of PTH before treatment ranged from 1400 to 2350 pg/ml in four of the six patients so these patients had severe secondary HPT. Nonetheless, a single oral 100-mg dose of NPS R-568 decreased plasma PTH levels by about 60% within 2 hr in each of these patients (Fig. 10). These findings in patients on dialysis parallel those obtained in rodent models of secondary HPT and show that the efficacy of calcimimetic compounds is similar in mild or severe cases of the disease.

The results obtained in these pilot studies were confirmed and extended in a randomized, double-blind, placebo-controlled, multicenter trial designed to assess the safety and efficacy of repeated oral doses of NPS R-568 in patients with ESRD and receiving hemodialysis three times per week (Goodman *et al.,* 2000a). Sixteen patients of either gender and with pretreatment serum PTH levels ranging from 300 to 1200 pg/ml received a 100-mg tablet of NPS R-568 once a day for 15 days. All patients treated with NPS R-568 showed a prompt decrease in plasma levels of PTH which reached a nadir within 2 hr and slowly returned to about 80% of pretreatment levels by 24 hr (Fig. 11). By the

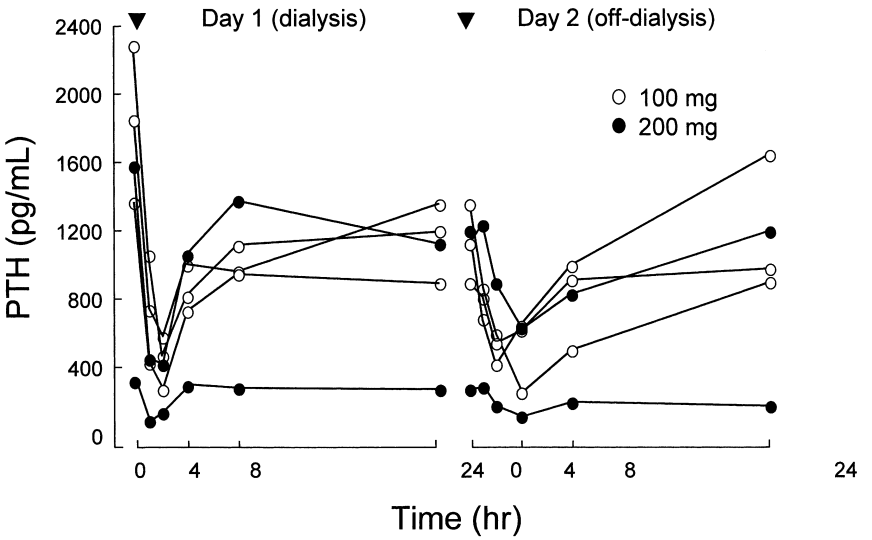

Figure 10 NPS R-568 rapidly lowers plasma PTH levels in patients on hemodialysis. (Redrawn from data in Akizawa *et al.,* 1998.)

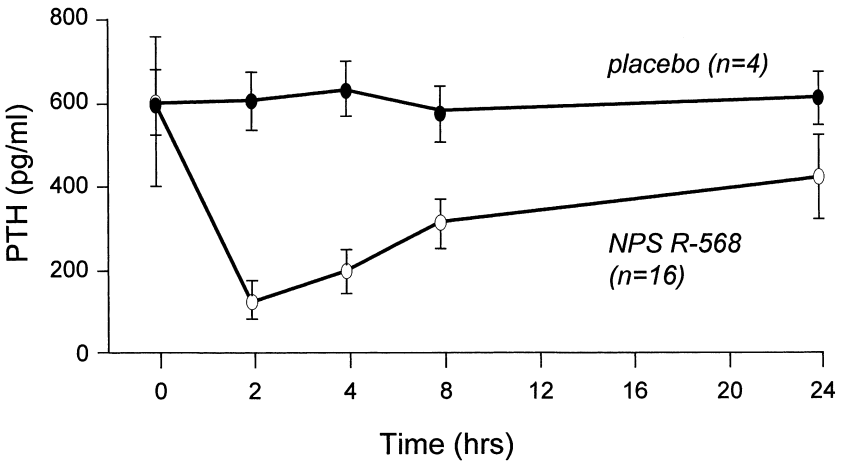

Figure 11 NPS R-568 lowers plasma PTH levels in male or female patients on hemodialysis (Fig. 1 in Goodman *et al.,* 2000a).

second day of dosing, serum PTH levels were significantly lower in calcimimetic-treated patients and, with daily dosing, they continued to decline until around day 10, at which time they were about 30% of pretreatment values. When dosing with NPS R-568 was discontinued on day 15, serum PTH levels began to rise and returned to pretreatment levels within 9 days. In contrast, serum PTH levels in patients receiving placebo continued to rise throughout the 24-day study period.

In general, multiple dosing with NPS R-568 was safe and well-tolerated. The most serious complication was hypocalcemia and at a dose of 100 mg NPS R-568, nearly half the treated patients developed hypocalcemia; it resolved in some when the dose was reduced to 50 mg/day. It is important to note, however, that all the patients were also receiving calcium supplements and vitamin D sterols throughout the course of the study. It is likely that adjusting the dose of one or both of these treatments would have prevented hypocalcemia in patients receiving the calcimimetic compound, a maneuver not permitted by the study protocol. On the other hand, the ability of NPS R-568 to lower circulating levels of PTH even in the face of hypocalcemia underscores the efficacy of the phenylalkylamine calcimimetics. Moreover, these compounds lowered PTH levels in patients already receiving optimal therapy with calcium and vitamin D (*vida infra*).

Despite the profound efficacy and general safety of NPS R-568 in patients with secondary HPT, this compound is not without its blemishes. The bioavailability of NPS R-568 is $<5\%$ and it is metabolized by hepatic cytochrome P_{450} enzymes, specifically the CYP2D6 enzyme. Around 5–7% of the population, depending on race, express an isozyme with substantially reduced enzymatic activity and therefore metabolize NPS R-568 slowly. To avoid these liabilities, clinical trials have continued with a backup compound, AMG 073. This compound, like NPS R-568, is a type II calcimimetic which acts allosterically on the Ca^{2+} receptor. It is similar in potency to NPS R-568 at inhibiting PTH secretion from bovine parathyroid cells *in vitro* (around 30 nM) but has improved bioavailability and is not metabolized by CYP2D6.

The safety and efficacy of AMG 073 in hemodialysis patients has been evaluated in three clinical trials (Table II). The effects of AMG 073 on plasma levels of PTH were similar in rate and magnitude to those described above for NPS R-568 whether administered as a single oral dose (Coburn *et al.,* 2000) or as daily doses for 8 days (Goodman *et al.,* 2000b) or 18 weeks (Lindberg *et al.,* 2000). Together, these three studies describe 102 instances of secondary HPT and every patient treated with AMG 073 showed a prompt fall in circulating levels of PTH. This occurred even in the presence of hypocalcemia and during optimal therapy with phosphate binders and vitamin D. Presumably, circulating levels of PTH had already been suppressed to the maximal extent possible with these conventional therapies. The ability of calcimimetic compounds to further lower PTH in patients already receiving phosphate binders and vitamin D highlights the deficiencies of the current standard of care for ESRD patients.

The decrease in circulating levels of PTH following administration of AMG 073 was associated with a decrease in serum levels of Ca^{2+} and phosphate; thus the Ca^{2+} times phosphate ($Ca \times P$) product decreased (Goodman *et al.,* 2000b; Lindberg *et al.,* 2000). While the reduction of this product is a much desired outcome in treating secondary HPT, the mechanism explaining this reduction is somewhat uncertain. There is no evidence to suggest that calcimimetic compounds lower the $C \times P$ product by allowing these ions to accumulate in soft tissues. In fact, at least in normal rodents, NPS R-568 causes hypocalcemia without affecting the rate of clearance of $^{45}Ca^{2+}$ from the circulation (Fox *et al.,* 1999a), a finding which has been interpreted to reflect diminished bone turnover resulting from a decrease in plasma PTH levels. Thus, understanding how calcimimetics

lower the Ca × P product in ESRD patients might not be a question of asking "Where are Ca^{2+} and phosphate going?" but rather "Where are they not coming from?"

Temporary vs Sustained Decreases in Circulating Levels of PTH

Inherent in the mechanism of action of calcimimetic compounds is their ability to alter plasma PTH levels in a manner that has not been possible previously. The only treatments that directly affect parathyroid cell function act on the synthesis, rather than the secretion of PTH (Naveh-Many and Silver, 1990). This is certainly true for calcitriol and, if phosphate acts directly on parathyroid cells, this might be how it acts as well. Because of this action, the suppressive effects of calcitriol on plasma levels of PTH, when they do occur, are slow in onset and in recovery. In contrast, because it targets the Ca^{2+} receptor, a single oral dose of a calcimimetic compound can lower plasma levels of PTH to a nadir within 30 min and levels can return to near baseline within 24 hr. Thus, a calcimimetic compound causes an intermittent, rather than sustained decrease in circulating levels of PTH (Fig. 12). The overall profile of the change in plasma PTH will vary depending on the dosing regimen and the pharmacokinetic properties of the particular calcimimetic compound being studied. The intriguing issue here is whether oscillating decreases in plasma levels of PTH, when compared to sustained decreases, will have different effects on bone quality.

It is generally accepted that plasma levels of PTH should be maintained at levels two- to three-fold higher than the upper limit of normal to overcome skeletal resistance to PTH and to avoid adynamic bone disease (Goodman *et al.,* 1994; Sherrard *et al.,* 1993). Oscillatory, in contrast to sustained changes in plasma levels of PTH might tend to reduce skeletal resistance to this hormone. Oscillatory decreases in plasma PTH levels might in themselves have different effects on bone than do sustained decreases. As described below, the catabolic effect of PTH on bone resulting from sustained elevations reverses to an anabolic effect when plasma levels of PTH increase intermittently. It has therefore been proposed that intermittent decreases in abnormally raised levels of plasma PTH might have effects on bone quality similar to those achieved by intermittent increases above normal (Nemeth and Bennett, 1998; Nemeth and Fox, 1999). Some evidence supporting this hypothesis has now appeared.

Using a partially nephrectomized rat model of uremia, Schmitt *et al.* (2000) compared the effects of the PTH fragment 1–37 on longitudinal growth when administered intermittently by subcutaneous injection or continuously by infusion. The twice daily administration of PTH fragment 1–37, but not its continuous infusion, improved longitudinal growth in uremic animals.

The effects of intermittent or sustained decreases in plasma levels of endogenous PTH were studied in a rodent model of uremia produced by adriamycin. Rats treated with adriamycin develop glomerular sclerosis which, after several months, results in a mild secondary HPT. Plasma levels of Ca^{2+} and phosphate are normal but vitamin D sterols are greatly reduced and there is massive proteinuria (Ishii *et al.,* 2000). Despite the mild secondary HPT, the renal osteodystrophy is characterized by a low turnover bone lesion with osteopenia and osteomalicia. Circulating levels of PTH were decreased in an oscillatory manner by the daily oral administration of NPS R-568 or they were continuously suppressed by the subcutaneous infusion of this compound. Oral administration, but not continuous infusion of NPS R-568, increased trabecular bone volume and bone mineral density (Fig. 13). Thus, oscillatory decreases in plasma

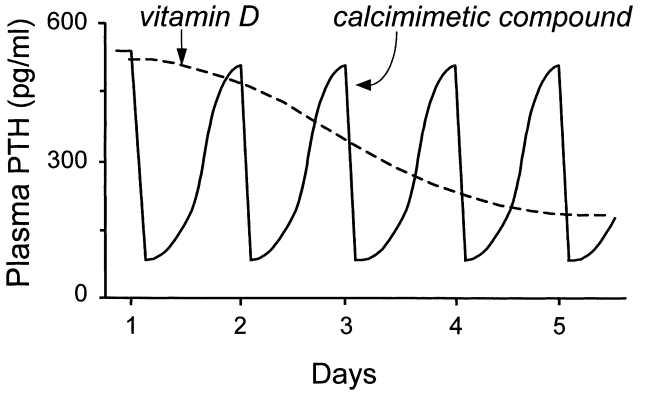

Figure 12 Contrasting effects of calcimimetic compounds and vitamin D sterols on the dynamics of changes in circulating levels of PTH (Fig. 1 in Nemeth and Bennett, 1998).

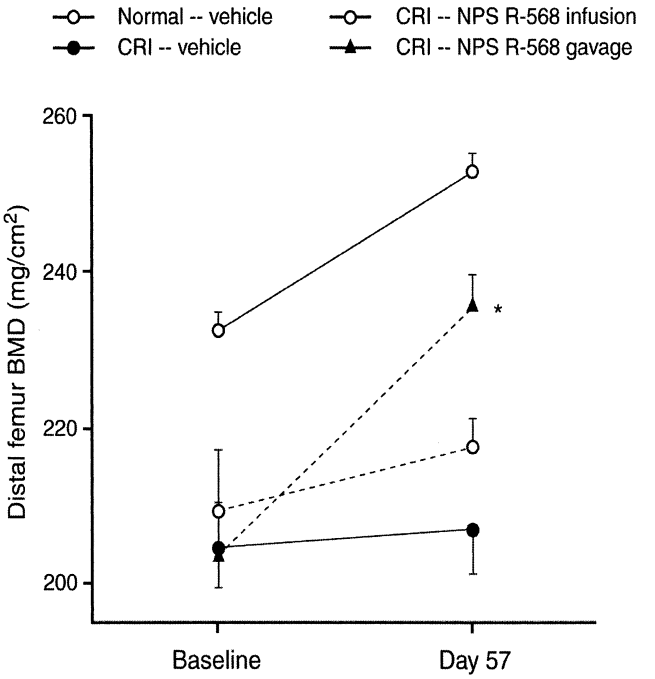

Figure 13 Bone mineral density in the distal femur is increased by daily oral administration of NPS R-568 (10 mg/kg) for 56 days in rats with CRI induced by adriamycin. In contrast, the continuous infusion of NPS R-568 (4.5 mg/kg/day) was without effect (Fig. 3 in Ishii *et al.,* 2000).

levels of PTH have markedly different effects on bone than do sustained decreases and, at least in this animal model of CRI, the intermittent decreases in plasma PTH levels had an "anabolic-like" effect on bone.

Osteoporosis

Because PTH plays such an important role in bone metabolism, there has been considerable interest in determining if abnormal plasma levels of this hormone might contribute to the development or progression of osteopenia. Although the role of PTH in the pathogenesis of type I osteoporosis remains controversial, there is scant evidence to suggest that it plays an important role (Silverberg and Bilezikian, 1994). In women with type I osteoporosis, it has generally been found that plasma levels of PTH are normal or slightly reduced (Gallagher, 1990). In contrast, a number of studies have implicated PTH in the pathogenesis of type II osteoporosis because there is an age-related increase in circulating levels of PTH in both men and women (Blumsohn and Eastell, 1995). This results largely from age-related decreases in renal function resulting in decreased levels of calcitriol, which, in turn, leads to decreased intestinal Ca^{2+} absorption. The systemic result is not that different from that seen in CRI. Consequently, there is increased secretion of PTH and type II osteoporosis could result partly from "senile secondary hyperparathyroidism."

PTH increases bone turnover but the resulting overall effect on bone mineral density is highly dependent on the temporal changes in the circulating levels of PTH. Thus, sustained elevations in plasma PTH levels, such as occur in PTH, lead to increased bone resorption and a net decrease in bone mass at most skeletal sites (Antonsen and Sherrard, 1995; Silverberg *et al.,* 1995). In contrast, temporary increases in plasma levels of PTH or its 1–34 fragment, achieved by the daily (or near daily) injection of either peptide, stimulate new bone formation in animal models of osteopenia (Mosekilde *et al.,* 1991; Dempster *et al.,* 1993; Kimmel *et al.,* 1993; Li and Wronski, 1995; Ejersted *et al.,* 1995a,b; Fox *et al.,* 1997) and in clinical studies of osteoporotic women or men (Reeve, 1996; Lindsay *et al.,* 1997; Hodsman *et al.,* 1997; Lane *et al.,* 1998; Fujita *et al.,* 1999; Rittmaster *et al.,* 2000). The results of these studies using intermittent administration of peptides demonstrate that PTH is a potent anabolic agent that increases bone mineral density and bone strength to a greater extent than that achieved by antiresorptive therapies (Mosekilde *et al.,* 1994; Søgaard *et al.,* 1994; Hodsman *et al.,* 1997; Lindsay *et al.,* 1997; Lane *et al.,* 1998; Fujita *et al.,* 1999; Rittmaster *et al.,* 2000). The profound stimulatory effect of these peptides on bone formation has generated interest in the use of PTH or its fragments as a novel anabolic therapy for osteoporosis. However, the therapeutic use of these peptides is compromised by the need for systemic administration of a costly biological product. Calcilytic compounds might provide an alternative approach to systemic administration of PTH by increasing the circulating levels of endogenous PTH (Nemeth, 1996). Preclinical studies using the calcilytic compound NPS 2143 in ovariectomized (OVX) rat models of osteopenia have generated results consistent with this hypothesis.

Daily oral administration of NPS 2143 for 5 weeks increased circulating levels of PTH and increased bone turnover in osteopenic OVX rats (Gowen *et al.,* 2000). When administered together with 17β-estradiol, the calcilytic compound stimulated new bone formation in the proximal tibial metaphysis and increased bone mass in the distal femur (Figs. 14 and 15). Thus, the increases in circulating levels of endogenous PTH evoked by a calcilytic compound are sufficient to cause an anabolic effect on bone.

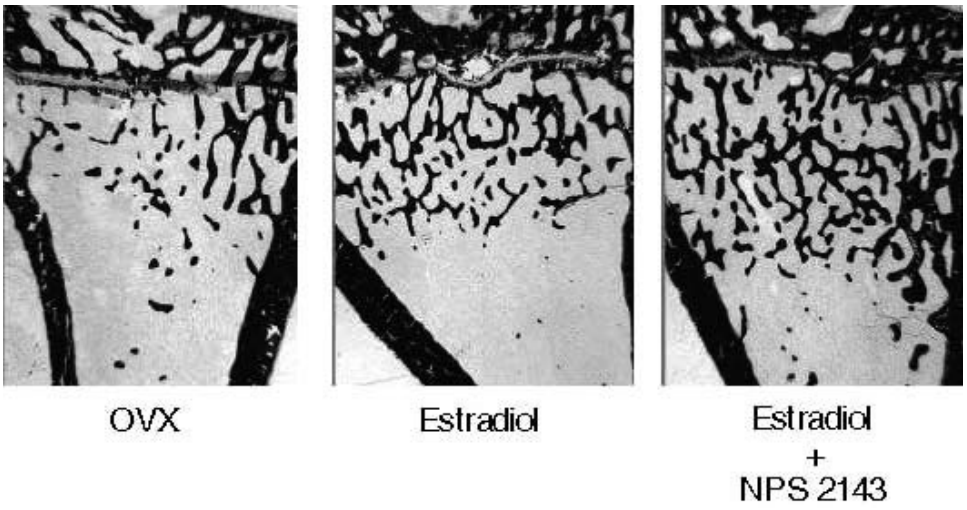

Figure 14 Treatment with NPS 2143 and estrogen increases trabecular bone volume in the proximal tibia of OVX rats. Rats were treated for 5 weeks with vehicle or subcutaneous infusion of estrogen with or without daily oral administration of 100 μmol/kg NPS 2143 (Fig. 6 in Gowen *et al.,* 2000).

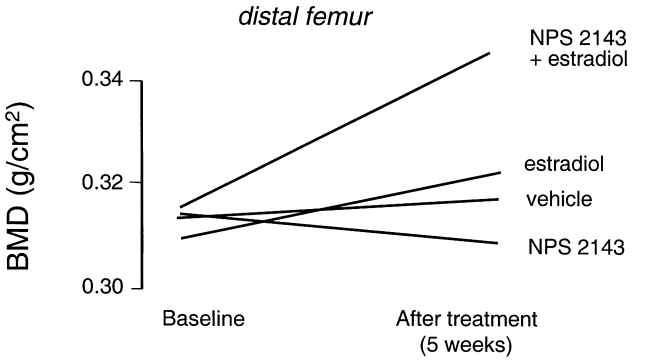

Figure 15 Treatment with NPS 2143 and estrogen increases bone mineral density in the distal femur of OVX rats, treated for 5 weeks as described in the legend to Fig. 14 (Fig. 8 in Gowen *et al.,* 2000).

One concern of repeated daily dosing with a calcilytic compound is the potential for inducing parathyroid cell proliferation. It is well recognized that parathyroid glands are larger in rats fed a diet containing low amounts of calcium (Ham *et al.,* 1940). Weanling rats fed a low-calcium diet for 3 weeks showed markedly increased parathyroid cell proliferation (Naveh-Many *et al.,* 1995). On the other hand, in older rats, comparable in age to those used in studies on OVX, the glandular enlargement accompanying calcium deficiency resulted exclusively from hypertrophy of the parathyroid cells and not from hyperplasia (Wernerson *et al.,* 1991). Daily oral dosing of OVX rats with NPS 2143 for 5 weeks did not increase parathyroid cell proliferation, neither by itself nor when administered together with estrogen (Gowen *et al.,* 2000).

The first calcilytic compounds have entered the clinic so it will soon be known if such compounds can cause an equally large increase in circulating levels of PTH in humans and do so, moreover, only transiently.

Calcium Receptors in Skeletal Tissue

There are a number of unresolved issues which, to various degrees, impact the therapeutic usefulness of any drug acting on the Ca^{2+} receptor. Some of the more basic issues of Ca^{2+} receptor biology, like the cellular distribution of the Ca^{2+} receptor throughout the body or the possibility of other, as yet unidentified Ca^{2+} receptors, are unknown or incomplete. This is a particularly significant issue for the development of calcilytic compounds for treating osteoporosis because there remains some controversy regarding the expression of the Ca^{2+} receptor in skeletal tissue. This issue was covered in detail previously (Nemeth, 1996) and the studies since then have failed to resolve it. There is evidence for extracellular Ca^{2+} sensing mechanisms in some osteoblastic cell lines (Quarles *et al.,* 1994b), osteoclastoma giant cells (Seuwen *et al.,* 1999), and normal rabbit osteoclasts (Steffey and Nemeth, 1993) which are pharmacologically different from that of the parathyroid. Many of the discrepant findings are based on studies using transformed osteoblastic cell lines whose fidelity to the normal phenotype is suspicious. The value of data derived from such cells is always tainted by this caveat. On the other hand, studies using normal tissues or cells have provided rather compelling evidence for expression of the Ca^{2+} receptor at certain skeletal sites. Using a number of different methods, Chen *et al.,* (1989) showed expression of the Ca^{2+} receptor in mouse, rat, and bovine cartilage and bone. In the latter tissue, they were able to detect Ca^{2+} receptor transcripts in osteoblasts, osteocytes, and some marrow cells, but only rarely in osteoclasts. The Ca^{2+} receptor might be expressed only transiently, during certain stages in a cellular lineage, and this effervescent appearance could make its detection problematic.

The possible significance of this issue for the use of calcilytic compounds as drugs resides in the nature of G protein-coupled receptor antagonists. In general, antagonists of these receptors do not show tissue selectively like the corresponding agonists or allosteric activators. Indeed, in rats, the potencies of NPS 2143 for increasing circulating levels of PTH and decreasing those of calcitonin are similar. Thus, there are reasons to suppose that calcilytic compounds will act with similar potencies on Ca^{2+} receptors throughout the body, including those seemingly expressed in skeletal tissues. Although NPS 2143 did not affect a number of indices of bone turnover *in vitro* (Gowen *et al.,* 2000), at this time it's actions on bone *in vivo* cannot be assumed to result solely from changes in circulating levels of PTH.

Conclusion

The Ca^{2+} receptor continues to evolve as a viable therapeutic target for treating hyperparathyroidism and osteoporosis. The discovery and development of potent and selective calcimimetic and calcilytic compounds has demonstrated that circulating levels of endogenous PTH can be altered at will by pharmacologically tuning the sensitivity of the Ca^{2+} receptor to extracellular Ca^{2+}. Additionally, these compounds have permitted Ca^{2+} receptor functions to be studied *in vivo* and this, in turn, has revealed the important role of the Ca^{2+} receptor in regulating parathyroid cell proliferation. Finally, these compounds demonstrate the potential therapeutic benefits to be gained by directly controlling secretion of PTH from the parathyroid glands.

Although discussion of the possible therapeutic significance of drugs acting on the Ca^{2+} receptor has been considered from the viewpoint of changing levels of endogenous PTH, it is possible that this receptor regulates many other functional cellular responses. Conceivably, drugs acting on Ca^{2+} receptors to alter these other responses could be useful in treating disorders beyond HPT and osteoporosis.

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CHAPTER 78

Bisphosphonates

Mechanisms of Action

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Introduction

The bisphosphonates are a class of drugs developed over the past three decades for use in various diseases of bone, tooth, and calcium metabolism. Known to chemists since the mid 19th century, they were first synthesized in Germany (Menschutkin, 1865) and were used mainly in the textile, fertilizer, and oil industries to prevent scaling because of their property to inhibit calcium carbonate precipitation (Blomen, 1995). This chapter will cover the history of the development, chemistry, biological actions, and clinical applications of these compounds, but in the context of this volume will focus primarily on mechanisms of action. Emphasis will be given to newer developments, the reader can consult other recent reviews for additional information (Ebetino *et al.,* 1998; Fleisch, 1998, 2000; Rodan, 1998; Russell *et al.,* 1999; Russell and Rogers, 1999).

History of Bisphosphonate Development for Use in Bone Diseases

Our knowledge of the biologic characteristics of bisphosphonates dates back over 30 years, the first report appearing in 1968 (Fleisch *et al.,* 1968). The concept was derived from earlier studies on inorganic pyrophosphate which were found to prevent both the formation (Fleisch and Neuman, 1961) and dissolution of calcium phosphate *in vitro* (Fleisch *et al.,* 1966). Since pyrophosphate was subsequently shown to prevent ectopic calcification *in vivo* and to be present in urine and plasma (Fleisch and Bisaz, 1962), it was suggested to be a physiological regulator of calcification and perhaps also of decalcification *in vivo* (Fleisch *et al.,* 1966). Due to its rapid hydrolysis, pyrophosphate found therapeutic use only in scintigraphy and in toothpaste, added to prevent dental calculus. This prompted the search for analogs, which would display similar physicochemical properties, but resist enzymatic hydrolysis and metabolism. The bisphosphonates fulfilled these conditions.

Chemistry and General Characteristics

Bisphosphonates are compounds characterized by two C–P bonds. When the two C–P bonds are on a single carbon atom (P–C–P), they are analogs of pyrophosphate (P–O–P) and are called geminal bisphosphonates, because the carbon is at the central or geminal position (Fig. 1). The P–C–P bonds of the geminal bisphosphonate are stable to heat and most chemical reagents and are completely resistant to enzymatic hydrolysis. Only geminal bisphosphonates seem to have a strong activity on the skeleton. For the sake of simplicity they are generally called bisphosphonates.

The P–C–P structure allows a great number of possible variations, either by changing the two lateral chains on the carbon atom or by esterifying the phosphonate groups. Many bisphosphonates have been studied in animals. Each bisphosphonate has its own physicochemical and biologic characteristics, which implies that one cannot extrapolate automatically the findings from one compound to another, with respect to its actions. The ones in Fig. 2 have been investigated in humans in bone disease, eight of them being commercially available today.

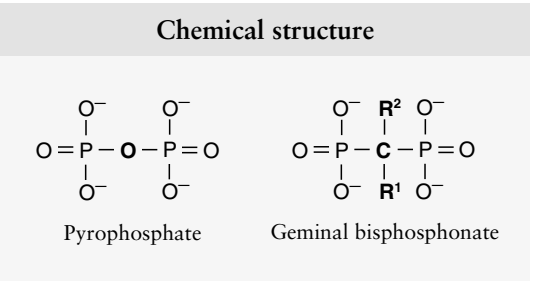


Figure 1 Chemical structure of pyrophosphate and bisphosphonates.

Physicochemical Effects

As anticipated, the physicochemical effects of most bisphosphonates are very similar to those of pyrophosphate. Thus, most inhibit the formation and aggregation of calcium phosphate crystals (Francis, 1969; Francis *et al.,* 1969; Fleisch *et al.,* 1970) and slow down their dissolution (Fleisch *et al.,* 1969; Russell *et al.,* 1970). These effects are related to the marked affinity of these compounds for the surface of solid phase calcium phosphate (Jung *et al.,* 1973), where they interfere with both crystal growth and dissolution. Binding can be bidentate through the two phosphonates, as is the case for clodronate, or it can be tridentate (Barnett and Strickland, 1979) through a third moiety, such as a hydroxyl or a nitrogen attached to the carbon atom, as is the case for most bisphosphonates used clinically today. The third binding site increases the affinity to mineral and hence the inhibitory effect on calcification.

Biological (Pharmacological) Effects

Inhibition of Bone Resorption

Bisphosphonates proved to be very powerful inhibitors of bone resorption when tested in a variety of conditions both *in vitro* and *in vivo.*

IN VITRO

Bisphosphonates block bone resorption induced by various means in organ and cell culture. In the former, they decrease the destruction of bone in embryonic long bones and in neonatal calvaria (Fleisch *et al.,* 1969; Russell *et al.,* 1970; Reynolds *et al.,* 1972). In cell culture, the bisphosphonates inhibit the formation of pits by isolated osteoclasts cultured on mineralized substrata (Flanagan and Chambers, 1989; Sato and Grasser, 1990). With few exceptions, the correlation between bisphosphonate potency *in vitro* and that *in vivo* was rather poor (Green *et al.,* 1994).

IN VIVO

Normal Animals In growing rats, bisphosphonates block degradation of both bone and cartilage, thus suppressing the remodeling of the metaphysis which becomes club-shaped and radiologically denser than normal (Schenk *et al.,* 1973). This effect is used as a model, the "Schenk assay," to study the potency of bisphosphonates (Schenk *et al.,* 1986). The inhibition of endogenous bone resorption has also been documented by ^{45}Ca kinetic studies and by markers of bone resorption (Gasser *et al.,* 1972). Effects occur within 24–48 hr and are therefore slower than that of calcitonin (Mühlbauer and Fleisch, 1990).

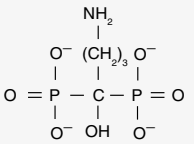
The decrease in resorption caused by bisphosphonates is accompanied by a positive calcium balance (Gasser *et al.,* 1972) and an increase in mineral content of bone. This is possible because of an increase in intestinal absorption of calcium consequent to an elevation of $1,25(OH)_2$ vitamin D. Bone formation also decreases after a certain time, attributed to "coupling" between formation and resorption. The main effect is, therefore, a reduction in bone turnover and some increase in bone mineral content. The increase in bone balance and the decrease in turnover are the reasons for administering these compounds to humans with osteoporosis.

Less is known about the effect in normal adult animals. In dogs and minipigs, long-term administration of alendronate did not lead to an increase in bone mass (Peter *et al.,* 1996). This might be explained by the physiological biomechanical homeostasis of bone structure, which would eliminate an unnecessary excess of bone. A 3-year clinical study of alendronate showed normal bone quality in transiliac bone biopsies (Chavassieux *et al.,* 1997), while a recent 7-year study showed safe and continuous increases in bone mineral density (Tonino *et al.,* 2000). These studies suggest that concerns about dangers of long-term use of therapeutic doses may not be warranted.

Animal Models of Hyperresorptive Diseases Bisphosphonates can also prevent experimentally induced increases in bone resorption. They impair, among others, resorption induced by agents such as parathyroid hormone, $1,25(OH)_2$ vitamin D, and retinoids (Fleisch *et al.,* 1969; Russell *et al.,* 1970). The effect on retinoid-induced hypercalcemia was developed into a rapid screening assay for new compounds (Trechsel *et al.,* 1987). Of special clinical interest are models of osteoporosis and tumor bone disease.

Osteoporosis. The first experimental model in which bisphosphonates prevented bone loss was limb immobilization by sciatic nerve section (Michael *et al.,* 1971; Mühlbauer *et al.,* 1971). Frequently used models also involve ovariectomy (Shiota, 1985; Thompson *et al.,* 1992) or orchidectomy (Wink *et al.,* 1985) in various species. In all osteoporosis models investigated the following bisphosphonates were shown to prevent bone loss: alendronate, clodronate, etidronate, ibandronate, incadronate, minodronate, olpadronate, pamidronate, risedronate, tiludronate, and zoledronate. However, it should be noted that many of these experiments were performed in growing animals, in which it is often difficult to assess to what extent the effect on bone density or bone mass is due to inhibition of sex steroid deficiency-induced bone loss, or of the bone resorption associated with modeling and remodeling during growth. Bisphosphonates were also

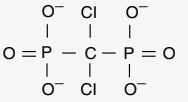
Bishosphonates used in humans



(4-Amino-1-hydroxybutylidene)-bis-phosphonate

alendronate*

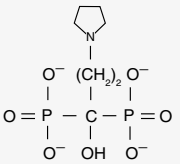
Abiogen; Merck Sharp & Dohme



(Dichloromethylene)-bis-phosphonate

clodronate*

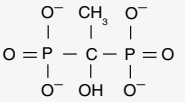
Astra; Abiogen; Leiras; Rhône-Poulenc Rorer



[1-Hydroxy-3-(1-pyrrolidinyl)-propylidene]bis-phosphonate

EB-1053

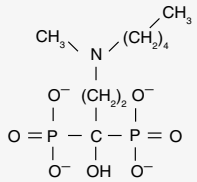
Leo



(1-Hydroxyethylidene)-bis-phosphonate

etidronate*

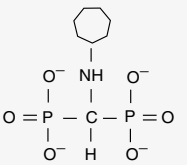
Abiogen; Procter & Gamble



[1-Hydroxy-3-(methylpentylamino) propylidene]bis-phosphonate

ibandronate*

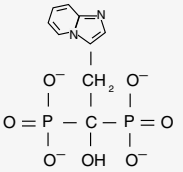
Roche



[(Cycloheptylamino)-methylene]bis-phosphonate

incadronate

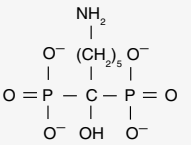
Yamanouchi



[1-Hydroxy-2-imidazo-(1, 2-a) pyridin-3-ylethylidene]bis-phosphonate

minodronate

Yamanouchi – Hoechst



(6-Amino-1-hydroxyhexy-lidene)bis-phosphonate

neridronate

Abiogen

Figure 2 Chemical structure of bisphosphonates investigated for their effect on bone in humans. *Commercially available. Reproduced from H. Fleisch, "Bisphosphonates in Bone Disease — From the Laboratory to the Patient." Academic Press, 2000, with copyright permission from the author. *(continued)*

effective in preventing bone loss in animals treated with corticosteroids (Jee *et al.,* 1981) or thyroid hormone (Yamamoto *et al.,* 1993). In another model, developed to mimic osteolysis and aseptic loosening around total hip arthroplasty, alendronate also inhibited bone destruction (Shanbhag *et al.,* 1997). Finally, of clinical importance is the finding that treatments such as prostaglandins, IGF-1, and PTH, which increase bone formation, remain effective when coadministered with bisphosphonates, sometimes resulting in additive effects on bone mass (Jee *et al.,* 1993; Takano *et al.,* 1996).

The question of bisphosphonate effects on the mechanical properties of the skeleton has also been addressed. This issue is of importance since long-lasting, excessive inhibition of bone resorption might lead to increased fragility. Numerous studies have shown that, when not given in excess, bisphosphonates produce an improvement in bone biomechanical

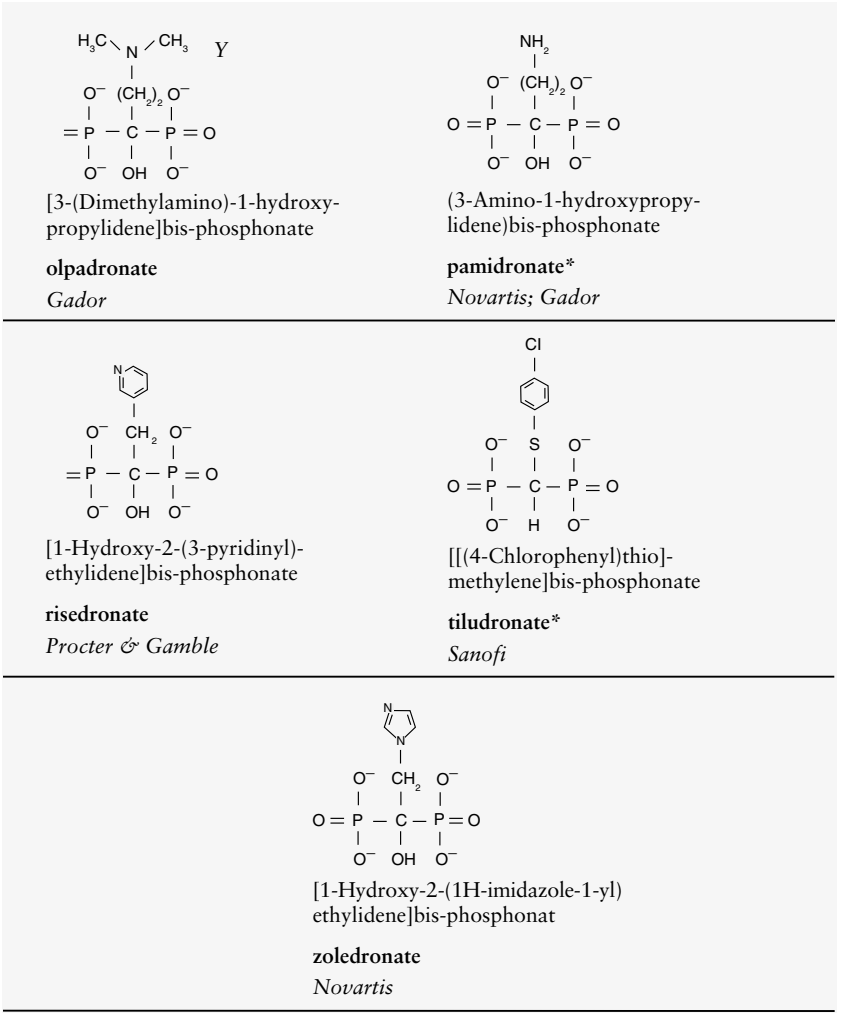


Figure 2 (*continued*)

properties, including torsional torque, ultimate bending strength, stiffness, maximum elastic strength, Young's modulus of elasticity, and others, both in normal animals and in experimental models of osteoporosis. This is the case for all the bisphosphonates mentioned above and was seen in various animals, although most experiments have been performed in the rat (Geusens *et al.,* 1992; Toolan *et al.,* 1992; Ammann *et al.,* 1993; Balena *et al.,* 1993; Guy *et al.* 1993; Ferretti *et al.,* 1993; Motoie *et al.,* 1995; for review see Ferretti, 1995).

Mechanisms of action in osteoporosis. Bisphosphonate improvement of mechanical strength may not be caused uniquely by an increase in bone mass, as previously thought, but also by an improvement in architecture and probably a reduction in bone remodeling. Indeed, a higher number of bone remodeling sites, in which there is excessive osteoclastic destruction of bone, leads to the development of areas of stress concentration and hence to increased fracture risk. Bisphosphonates may serve as a means of reducing these effects, thus reducing the incidence of new fractures. The prevention of bone loss is probably explained to a large extent by the decrease in bone turnover. The initial rise in bone mass is due to "filling of the remodeling space"; in other words bone formation continues to rebuild basic multicellular units (BMUs) initiated prior to bisphosphonate treatment, while fewer new BMUs are initiated. In addition, some bone can be lost at each BMU, because a greater amount of bone is resorbed than formed. The decrease in turnover would therefore slow down total bone loss. Furthermore, bisphosphonates also act at individual BMUs by decreasing the depth of resorption (Balena *et al.,* 1993; Boyce *et al.,* 1995). Both effects will lead to a decreased number of trabecular perforations, thus reducing the decrease in bone strength and the occurrence of fractures.

Another mechanism may be related to the increase in mineralization associated with lower bone turnover (Meunier and Boivin, 1997; Boivin *et al.,* 2000). A lower turnover will lengthen the life span of the BMU, thus permitting it to mineralize more completely, which will increase mineral content, measured as BMD, independent of effects on bone mass. This has been described in alendronate-treated baboons (Meunier and Boivin, 1997) and more recently in osteoporotic women (Boivin *et al.,* 2000). However, the above mechanisms may not fully explain the continuous increase in spinal BMD

observed during 7 years treatment of postmenopausal women with alendronate (Tonino *et al.,* 2000), which led to suggestions that bisphosphonates could stimulate bone formation, possibly by preventing osteoblast apoptosis (Plotkin *et al.,* 1999).

Tumor induced bone resorption. Bisphosphonates very effectively inhibit tumor-induced bone resorption, both *in vitro* and *in vivo,* when osteolysis is induced by circulating humoral factors as well as by local tumor invasion. All bisphosphonates which had previously been found to inhibit bone resorption were active in the tumor models, notably alendronate, clodronate, etidronate, ibandronate, incadronate, olpadronate, pamidronate, and risedronate.

In Vitro When added *in vitro,* bisphosphonates inhibit the bone-resorbing effect of supernatants of various cancers in mice calvaria (Galasko *et al.,* 1980). Inhibition was also seen when the bisphosphonates were injected into the mice before explantation of the calvaria (Jung *et al.,* 1981).

In Vivo Many types of tumor cells have been used in various *in vivo* models, such as bladder tumors, rat mammary adenocarcinoma, prostate adenocarcinoma, myeloma, and melanoma. Perhaps the best host is the nude mouse (Nemoto *et al.,* 1990; Sasaki *et al.,* 1995, 1998, 1999; Hiraga *et al.,* 1996a,b; Yoneda *et al.,* 2000). The first such studies performed showed that the humoral hypercalcemia induced by subcutaneously implanted Walker 256 carcinoma cells or by implanted Leydig tumor cells (Martodam *et al.,* 1983; Jung *et al.,* 1984; Guaitani *et al.,* 1985) could be partially prevented. Bisphosphonates also prevent or slow down bone resorption due to actual tumor invasion, as shown in several models.

One of the questions raised pertains to effects on the skeletal tumor burden. While earlier results showed no effect or even some increase, newer findings in nude mice displayed a decrease (Sasaki *et al.,* 1995, 1998, 1999; Yoneda *et al.,* 1997). This might be secondary to a local decrease in the liberation or production of tumor-stimulating cytokines such as transforming growth factor (TGF-β) and insulin-like growth factors (IGFs) following a reduction in osteolysis, thereby interfering with the "seed and soil" interactions between tumor cells and the bone microenvironment.

The effect on the development of the implanted tumors in the soft tissues is controversial, some studies showing no bisphosphonate effect and others an increase (Kostenuik *et al.,* 1993; Sasaki *et al.,* 1995; Stearns and Wang, 1996). Another open question is the effect on the osteoblastic process initiated by prostate tumor cells. In animals bisphosphonates had no effect (Pollard *et al.,* 1985), although a desirable effect was reported in humans, possibly again acting through inhibition of bone resorption, which is also present in these cancers. It should be stated that, in general, animal models are notoriously poor predictors of the efficacy of antitumor agents.

Mechanisms of action in tumor bone disease. It was generally thought that the bisphosphonates did not directly inhibit the multiplication of tumor cells, and were therefore probably not active on the tumor itself, but exerted their action by inhibiting the osteolytic process. As a secondary consequence, multiplication of tumor cells may be decreased, possibly in part because of a decrease of local cytokines stimulating tumor cell replication, released when bone is resorbed (Guise, 1997, 2000). However, it was recently reported that bisphosphonates induce *in vitro* apoptosis of myeloma cells, albeit at relatively high concentrations (Shipman *et al.,* 1997, 1998). Furthermore bisphosphonates also inhibit the adhesion of breast and prostate tumor cells onto bone matrices *in vitro* (Van der Pluijm *et al.,* 1996; Boissier *et al.,* 1997; Magnetto *et al.,* 1999). Effects on tumor attachment were seen both when the bone matrix was pretreated with and when the tumor cells were exposed to the bisphosphonate. *In vivo,* owing to the rapid accumulation of bisphosphonate in bone, the brief serum half-life, and low serum concentrations (n*M*), effects of bisphosphonates are produced primarily by the bisphosphonate on the bone surface. The degree to which tumor cells are exposed to bone-associated bisphosphonate *in vivo,* therefore, remains to be established.

Other models of bone resorption. Of interest in the dental field is the fact that bisphosphonates also slow down periodontal bone destruction in animal models of spontaneous and induced periodontitis (Shoji *et al.,* 1995; Reddy *et al.,* 1995; O'Uchi *et al.,* 1998). Furthermore, they inhibit tooth movement and alveolar bone resorption induced by orthodontic procedures, and these effects can be achieved when the compounds are administered topically (Adachi *et al.,* 1994; Yaffe *et al.,* 1997; Kaynak *et al.,* 2000).

Last, several bisphosphonates, especially risedronate, were reported to inhibit local bone and cartilage resorption, preserve the joint architecture, and decrease the inflammatory reaction in various types of experimental arthritis (Francis *et al.,* 1989; Österman *et al.,* 1994).

Relative Potency of Various Bisphosphonates on Inhibition of Bone Resorption

The activity of bisphosphonates on bone resorption in the models described above varies greatly from compound to compound (Shinoda *et al.,* 1983), which is not the case for the inhibition of mineralization. For etidronate, one of the first bisphosphonates to be investigated, the dose required to inhibit resorption is relatively high, greater than 1 mg/kg/day parenterally. Since this dose is similar to that which impairs normal mineralization, one of the aims of bisphosphonate research has been to develop compounds with a more powerful antiresorptive activity and lesser inhibition of mineralization. The first of these, clodronate, is more potent than etidronate (Fleisch *et al.,* 1969; Russell *et al.,* 1970) and less active in inhibiting normal mineralization (Schenk

et al., 1973). Later, pamidronate was found to be still more active (Lemkes *et al.,* 1978), and today compounds have been developed that are up to 10,000 times more powerful inhibitors than etidronate in inhibiting bone resorption in animals (reviewed in Fleisch, 2000). Perhaps the best way to compare potency in humans, bypassing the differences in oral bioavailability, is to examine maximally effective intravenous doses in hypercalcemia of malignancy, a condition produced by massive bone resorption. Bisphosphonate treatment is typically given in one or two intravenous doses. As a result, urinary and serum markers for resorption are typically normalized within 48 hr and resorption control can last up to about a month. Maximally effective doses of alendronate (15 mg), ibandronate (6 mg), pamidronate (90 mg), and zoledronate (2–4 mg) results in normalization of serum calcium in 77–100% of patients, depending on the study (Nussbaum *et al.,* 1993a,b; Ralston *et al.,* 1997; Body *et al.,* 1999). This suggests an overall spread in potency of about 40-fold, 6-fold if pamidronate is excluded. In animal studies the spread in potency is approximately 1000-fold for these bisphosphonates (Fleisch, 2000). Therefore, while rank ordering for potency in animals is predictive, the actual potency range is narrower in patients and has to be determined in the clinic.

Structure–Activity Relationship for Bone Resorption

Prior to elucidation of the bisphosphonate molecular target it was difficult to establish a mechanism-based structure–activity relationship. The length of the aliphatic carbon was found to be important and adding a hydroxyl group to the carbon atom at position 1 increases potency and binding to bone (Shinoda *et al.,* 1983; van Beek *et al.,* 1996). Derivatives with an amino group at the end of the side chain are very active, the highest activity being found with a backbone of four carbons, as present in alendronate (Schenk *et al.,* 1986). A primary amine is not necessary for this activity since dimethylation of the amino nitrogen of pamidronate, as seen in olpadronate, increases potency (Boonekamp *et al.,* 1987). Potency is still further increased when other groups are added to the nitrogen, as seen in ibandronate, which is extremely potent (Mühlbauer *et al.,* 1991). Cyclic geminal bisphosphonates are also very potent, especially those containing a nitrogen atom in the ring, such as risedronate (Sietsema et *al.,* 1989). The most potent compounds described so far, zoledronate (Green *et al.,* 1994) and minodronate, belong to this class. The effect of nitrogen has not yet been analyzed by molecular modeling of the interactions with the bisphosphonate target (see below), a three-dimensional structural requirement and intramolecular bonds are obviously involved. Indeed, stereoisomers of the same chemical structure have shown a 10-fold difference in activity (Takeuchi *et al.,* 1993).

Mechanisms of Action on Bone Resorption

General Concepts

Our understanding of the mode of action of the bisphosphonates has made great progress in the past few years. There is no doubt that the action *in vivo* is mediated mostly, if not completely, through mechanisms other than the physicochemical inhibition of crystal dissolution, initially postulated. Many of these mechanisms have been unraveled and more than one mechanism may be operating simultaneously.

The fact that bisphosphonates appear to act, when administered at therapeutic doses, only on bone, is due to their special affinity to this tissue. They are deposited preferentially, not in newly formed bone as previously thought, but under the osteoclasts (Sato *et al.,* 1991; Azuma *et al.,* 1995). The relative deposition on bone forming and bone resorbing areas depends upon the amount of bisphosphonate administered. When the pharmacologically active dose is small, as in the case of the more powerful bisphosphonates such as alendronate, the resorbing surface is preferentially labeled by radioactive bisphosphonate. When larger amounts are given in order to achieve pharmacological effectiveness, as in the case of etidronate, the resorption and formation surfaces are about equally labeled (Masarachia *et al.,* 1996). It has been calculated that the concentration under the osteoclasts can reach relatively high levels in the resorption lacuna, in the range of 0.1–1 mM (Sato *et al.,* 1991).

ACTIONS AT THE MOLECULAR LEVEL

It was shown that in addition to its role in targeting bisphosphonates to bone, the P–C–P backbone also substitutes for phosphate in various enzymatic reactions. The R^2 group on the carbon helps determine the specificity of these interactions. Bisphosphonates were shown to inhibit protein tyrosine phosphatases (PTPs), and inhibition of PTPs with other agents was shown to inhibit bone resorption (Schmidt *et al.,* 1996; Endo *et al.,* 1996; Murakami *et al.,* 1997; Opas *et al.,* 1997; Skorey *et al.,* 1997). However, the IC_{50}s for inhibition of several PTPs by the bisphosphonates tested correlated poorly with their potency for inhibition of bone resorption, suggesting that this was not the major antiresorptive mechanism. Recent studies point more convincingly to other mechanisms of action, which are reviewed below. Regarding the different mechanisms described, it is worth considering the possibility that any primary mechanism of action for a given bisphosphonate may occur in addition to the background inhibition of PTPs or other enzymes, such as the vacuolar ATPase, which was shown to be inhibited by tiludronate (David *et al.,* 1996). Indeed, since tiludronate is poorly metabolized into a nonhydrolyzable ATP analog (described below), or is unstable as such, it remains difficult to prioritize among the three possible mechanisms of action of this bisphosphonate. Furthermore, as intracellular concentrations of bisphosphonates increase, inhibition of secondary enzyme(s) may assume greater prominence in their overall effect.

Several recent breakthrough studies examining bisphosphonate mechanism of action have uncovered the primary sites of inhibition, that account for most of the potency of clinically used bisphosphonates. These studies paint a compelling picture pointing to two general classes of bisphosphonates: those that are metabolized within the cell to form toxic analogs of ATP (e.g., clodronate, etidronate, tiludronate) and those that inhibit farnesyl diphosphate synthase (e.g., alendronate, ibandronate, incadronate, olpadronate, pamidronate, risedronate, zoledronate). The properties that segregate bisphosphonates (BPs) into these two classes appears to be a function of the moieties attached to the geminal carbon at R^2, which can vary in size and complexity (Fig. 2). Without regard to size or shape, the prevailing determinant for mechanism of action of the bisphosphonates used in the clinic relates to the presence or absence of a nitrogen atom located three to five positions away from the geminal carbon of the P–C–P backbone in the R^2 group. Those that contain nitrogen, hereafter referred to as N-BPs, inhibit farnesyl diphosphate (FPP) synthase with IC_{50}s in the nanomolar range, while the non-N-BPs are metabolized to cytotoxic ATP analogs. Throughout the remainder of this section, the salient features of these two classes of bisphosphonate are discussed.

Metabolism of Non-N-BPs to ATP Analogs Studies in the 1970s suggested that clodronate and etidronate could affect a variety of intracellular metabolic processes, including glycolysis, lactate production, fatty acid oxidation, adenylate cyclase and phosphohydrolases (Fast *et al.,* 1978; Felix *et al.,* 1976, 1981). Since bisphosphonates are close structural analogs of inorganic pyrophosphate, it is perhaps not surprising that they can affect a wide variety of enzymes and metabolic pathways. However, none of these effects satisfactorily explain the ability of clodronate and etidronate to inhibit osteoclast function and cause osteoclast apoptosis.

The first clue to the likely molecular mechanism of action of clodronate and etidronate arose from studies using methylenebisphosphonate (medronate) as a marker to study intracellular pH in single-celled amebae of the slime mold *Dictyostelium discoideum* (Klein *et al.,* 1999). Using ^{31}P NMR to study the pH-dependent δ-shift of the phosphonate groups in medronate, new peaks were identified in the ^{31}P NMR spectrum of medronate-treated amebae. These peaks were identified as nonhydrolyzable, methylene-containing analogs of adenosine triphosphate (ATP) and diadenosine tetraphosphate (Ap_4A). The metabolites, $AppCH_2p$ and $AppCH_2ppA$, contained the P–C–P moiety of medronate in place of a P–O–P moiety (Fig. 3), and were thus resistant

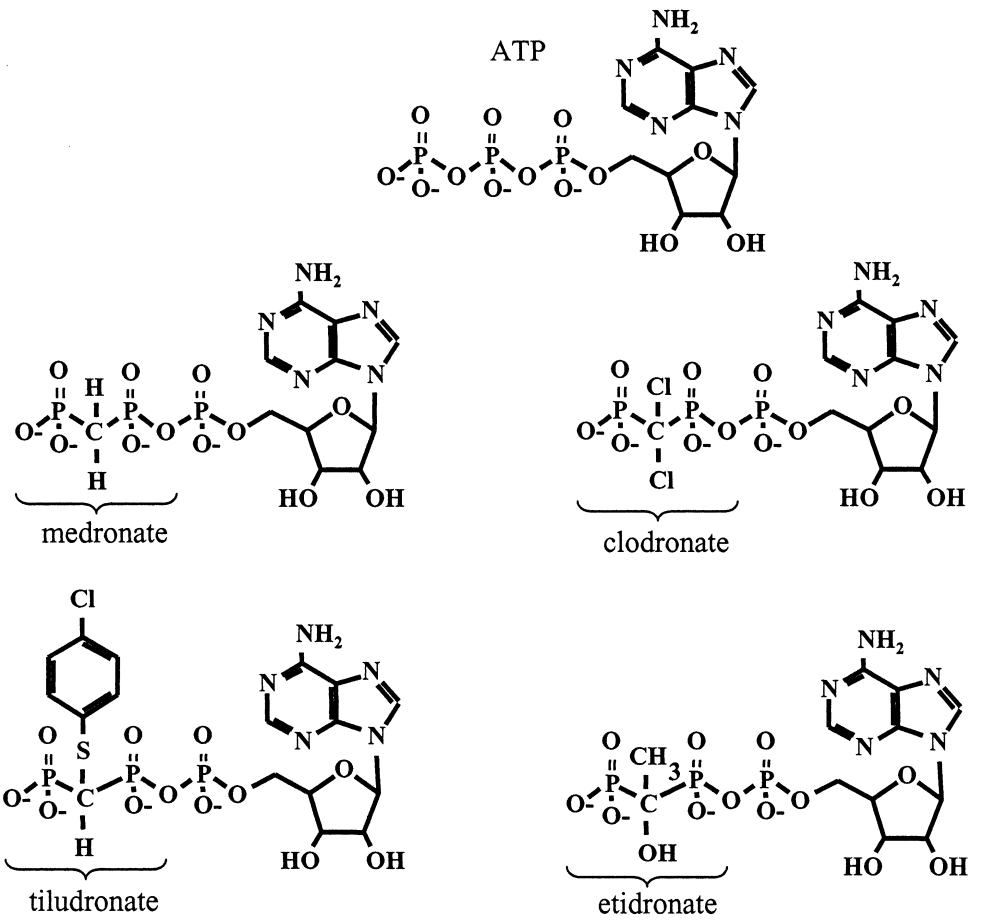


Figure 3 The structure of ATP and the AppCp-type metabolites of medronate, clodronate, etidronate, and tiludronate.

to hydrolysis. More recently, FPLC and UV absorbance as well as ^{31}P NMR analysis showed that clodronate and certain other bisphosphonates (etidronate, fluoromethylene bisphosphonate, difluoromethylene bisphosphonate, hydroxymethylene bisphosphonate) (Fig. 2) are metabolized to methylene-containing (AppCp-type) analogs of ATP, but not to analogs of Ap_4A (Rogers *et al.,* 1992, 1994; Pelorgeas *et al.,* 1992). Interestingly, bisphosphonates with larger R^2 side chains (such as the amino- alkyl bisphosphonates and heterocycle-containing bisphosphonates), which poorly resemble pyrophosphate, are not metabolized (with the exception of tiludronate). Similar results were obtained using cell-free extracts of human HL60 cells as well as human cell lines, in particular J774 macrophages which (like osteoclasts and *Dictyostelium* amebae) are highly endocytic and may internalize bisphosphonates more efficiently than most other cell types (Rogers *et al.,* 1996, 1997; Frith *et al.,* 1997; Benford *et al.,* 1999). Liposome-encapsulated bisphosphonates are internalized even more effectively by phagocytic cells, leading to greater accumulation of intracellular AppCp-type metabolites (Frith *et al.,* 1997). Preliminary studies suggest that up to 50% of clodronate internalized intracellularly is metabolized by macrophages *in vitro* to $AppCCl_2p$, which may reach an intracellular concentration as high as 1 mM (Mönkkönen *et al.,* 2000a).

A much more sensitive approach than FPLC/UV absorbance or ^{31}P NMR for the detection of AppCp-type metabolites of bisphosphonates has recently been reported, using a combination of ion pairing HPLC and electrospray ionization mass spectrometry (Auriola *et al.,* 1997; Mönkkönen *et al.,* 2000b). This method can detect $\geqslant 0.5\ \mu M$ AppCp-type metabolites in cell extracts. Using this method, the metabolites of clodronate, etidronate and tiludronate have been unequivocally identified, since the metabolites fragment upon collision-induced dissociation (bombardment with electrons), giving rise to a characteristic pattern of ions that are detected by ion-pairing electrospray mass spectrometry (Benford *et al.,* 1999; Auriola *et al.,* 1997; Mönkkönen *et al.,* 2000b) (Fig. 3). Using this method of analysis, N-BPs, such as alendronate, pamidronate, and ibandronate, do not appear to be metabolized by macrophages (Benford *et al.,* 1999).

The incorporation of bisphosphonates into AppCp-type nucleotides is probably achieved through interactions with the family of Type II aminoacyl-tRNA synthetases (which utilize the amino acids Asn, Asp, Gly, His, Lys, Phe, Ser) (Rogers *et al.,* 1994, 1996). These enzymes play an essential role in protein synthesis (Fig. 4), since they catalyze the condensation of an amino acid with ATP (Appp) to form an aminoacyl-adenylate (amino acid-AMP), releasing pyrophosphate (P–O–P) in a reversible reaction (reaction I). The aminoacyl-adenylate then condenses with a molecule of tRNA to form aminoacyl-tRNA, utilized for ribosome-dependent translation of mRNA into protein (reaction II). It appears that tiludronate and bisphosphonates with short side chains (but not more potent bisphosphonates that contain a nitrogen in the R^2 side chain) can replace P-O-P in the active site of Type II aminoacyl-tRNA synthetases, but not Type I enzymes. Since bisphosphonates (P–C–P) resemble pyrophosphate (P–O–P) in structure, the reverse reaction of (I) can occur with P–C–P in place of P–O–P, to form an analog of ATP (AppCp) containing the bisphosphonate (reaction III), a reaction demonstrated more than 30 years ago (Zamecnik and Stephenson, 1968). For medronate, difluoromethylenebisphosphonate, and hydroxymethylenebisphosphonate (but not clodronate or etidronate), AppCp can be further metabolized (to AppCppA, an analog of AppppA), by the condensation of ATP with AppCp to form AppCppA + AMP (reaction IV) (Rogers *et al.,* 1994; Zamecnik, 1983).

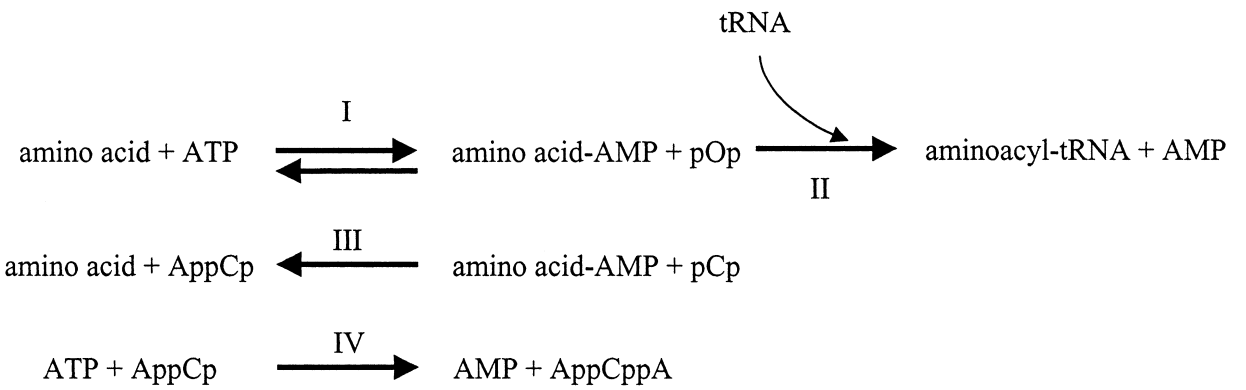


Figure 4 The mechanism by which aminoacyl-tRNA synthetase enzymes catalyze the formation of AppCp-type metabolites of bisphosphonates. An amino acid condenses with ATP (Appp) to form an aminoacyl-adenylate (amino acid-AMP), releasing pyrophosphate (pOp) in a reversible reaction (I). The aminoacyl-adenylate then condenses with a molecule of tRNA to form aminoacyl-tRNA (reaction II). Since bisphosphonates (pCp) resemble pyrophosphate in structure, the reverse reaction of (I) can occur with pCp in place of pOp, to form an analog of ATP (AppCp) containing the bisphosphonate. $AppCH_2p$, the metabolite of medronate, can condense with another molecule of ATP (reaction IV) to form an AppCppA-type nucleotide (i.e., $AppCH_2ppA$).

Although it was considered for many years that bisphosphonates were metabolically inert, the discovery that some could be metabolized intracellularly to AppCp-type nucleotides by cells *in vitro* suggested the possibility that their anti-resorptive effect may be due to the accumulation of these metabolites in osteoclasts *in vivo* (Frith *et al.,* 1997). The ability of osteoclasts to metabolize clodronate and etidronate *in vitro* has recently been confirmed, using HPLC-electrospray mass spectrometry to identify $AppCCl_2p$ in extracts of purified rabbit osteoclasts. Furthermore, the $AppCCl_2p$ metabolite of clodronate was found in extracts of osteoclasts purified by magnetic bead separation following injection of rabbits with clodronate, confirming that osteoclasts can metabolize clodronate *in vivo* (Frith *et al.,* 2000).

Inhibition of the Mevalonate Pathway by N-BPs It was while searching for squalene synthase inhibitors that the N-BPs incadronate (YM175), ibandronate, pamidronate, and alendronate were found to inhibit the mevalonate/cholesterol biosynthetic pathway (Amin *et al.,* 1992, 1996). Etidronate and clodronate, which lack nitrogen in the R^2 side chain, were inactive in the same assays. Whereas incadronate and ibandronate were shown to potently inhibit squalene synthase, alendronate and pamidronate were inactive, yet nonetheless inhibited sterol biosynthesis with IC_{50}s of 170 and 420 nM, respectively. This suggested the presence of a target upstream in the mevalonate pathway (Fig. 5, see also color plate).

FPP Synthase as the Molecular Target of the N-BPs In recent studies, (van Beek *et al.,* 1999a,b; Bergstrom *et al.,* 2000; Dunford *et al.,* 2001) the upstream enzyme inhibited by all of the N-BPs examined to date was identified as FPP synthase (Table I). The most potent anti-resorptive N-BPs such as zoledronate and minodronate (YM-529) are extremely potent inhibitors of FPP synthase (IC_{50} 3nM with respect to recombinant human FPP synthase; Dunford *et al.,* 2001). The discovery of these inhibitory activities revealed several interesting findings: (A) While alendronate is a specific inhibitor of FPP synthase, ibandronate and incadronate inhibit both squalene synthase and FPP synthase (Amin *et al.,* 1992; van Beek *et al.,* 1999b; Bergstrom *et al.,* 2000; Dunford *et al.,* 2001). The specificity of the other N-BPs remains to be determined. (B) While in enzymatic assays risedronate is 90-fold more potent than alendronate for inhibition of purified FPP synthase (Bergstrom *et al.,* 2000), it is only 5-fold more potent in crude enzyme preparations (Dunford *et al.,* 2001) and approximately equipotent for increasing bone mineral density after oral administration (5 mg po qd, 2 years) in the clinic (Bone *et al.,* 1997; Fogelman *et al.,* 2000). This suggests that differences in pharmacokinetics, pharmacodynamics, binding to bone (van Beek *et al.,* 1994, 1998), osteoclast targeting, cell permeability, or intracellular protein binding between these mechanistically similar bisphosphonates contribute significantly to their ultimate potency. (C) While risedronate is a potent inhibitor of FPP synthase, modifications (e.g., addition of a methyl group) to the structure of the R^2 side chain that influence the position of the nitrogen group in relation to the phosphonate groups give rise to analogs that are markedly less potent inhibitors of FPP synthase and less effective inhibitors of bone resorption *in vivo.* Furthermore, for a wide range of N-BPs, there is a significant correlation between potency for inhibition of FPP synthase and anti-resorptive potency *in vivo,* pointing to FPP synthase as the major molecular target of the N-BPs (Dunford *et al.,* 2001). (D) Consistent with the initial findings (Amin *et al.,* 1992), neither clodronate nor etidronate showed any significant inhibitory activity against FPP synthase (van Beek *et al.,* 1999b; Bergstrom *et al.,* 2000; Dunford *et al.,* 2001). Detailed enzymological studies will be needed to better understand the kinetics and reversibility of N-BP inhibition of FPP synthase.

Recently, computer modeling has been used to suggest that N-BPs may inhibit FPP synthase by acting as isoprenoid transition-state analogs (Martin *et al.,* 1999). The phosphonate groups of bisphosphonates appear to fit into the diphosphate binding site of the enzyme, thus explaining why modifications to either or both of the phosphonate groups (such as methylation) prevents these compounds from inhibiting protein isoprenylation (discussed below) and reduces anti-resorptive potency (Ebetino and Jamieson, 1990; Luckman *et al.,* 1998a). Importantly, this would confirm the view that the two phosphonate groups have a dual purpose (Rogers *et al.,* 1995; Luckman *et al.,* 1998a), being required for the molecular mechanism of action as well as for targeting to bone mineral (as discussed above). Furthermore, it has been suggested that the nitrogen in the R^2 side chain may act as a carbocation transition state analog, which could be stabilized by oxygen atoms in the active site cleft of FPP synthase (Martin *et al.,* 1999). The length and orientation of the bisphosphonate R^2 side chain could affect the interaction of the nitrogen in the side chain with amino acid residues in the active site cleft, hence explaining why minor changes to the structure or conformation of the side chain also affect the ability to inhibit protein isoprenylation (Luckman *et al.,* 1998b) and markedly influence anti-resorptive potency (Shinoda *et al.,* 1983; Schenk *et al.,* 1986; Sietsema *et al.,* 1989; van Beek *et al.,* 1994; Rogers *et al.,* 1995). However, the exact manner in which bisphosphonates bind to and inhibit FPP synthase remain to be clarified.

Inhibition of FPP Synthase Blocks Protein Isoprenylation and Sterol Synthesis The mevalonate pathway is not only responsible for the production of cholesterol but also isoprenoid lipids such as isopentenyl diphosphate (IPP) (five carbon), FPP (15 carbon), and geranylgeranyl diphosphate (GGPP) (20 carbon), the latter two being substrates for protein isoprenylation. Isoprenylation involves the transfer of a farnesyl or geranylgeranyl lipid group onto a cysteine residue in characteristic carboxy-terminal (e.g., CAAX) motifs (Zhang and Casey, 1996; Sinensky, 2000), giving rise to farnesylated and geranylgeranylated proteins. Most of the isoprenylated proteins identified to date are small GTPases

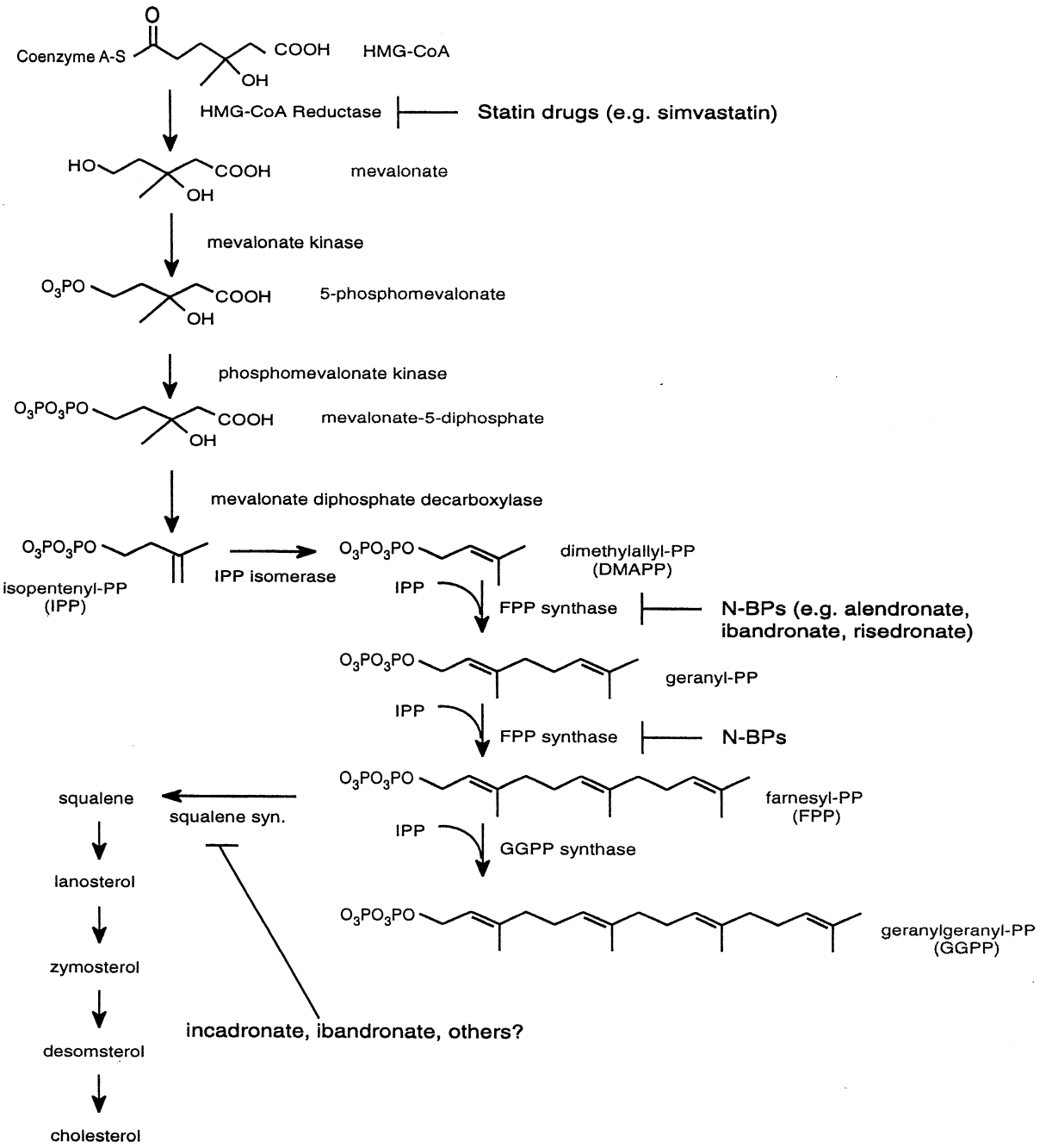


Figure 5 Metabolites in red may act via feedback to suppress HMG-CoA reductase expression in the osteoclast *in vivo*. N-BP inhibition of FPP synthase blocks (1) farnesylation, (2) geranylgeranylation, and (3) cholesterol synthesis. GGPP, required for geranylgeranylation, is the critical lost metabolite in the osteoclast. (See also color plate.)

(the majority of which are geranylgeranylated) (Zhang and Casey, 1996), which are important signaling proteins that regulate a variety of cell processes important for osteoclast function, including cell morphology, integrin signaling, membrane ruffling, trafficking of endosomes, and apoptosis (Ridley *et al.,* 1992; Ridley and Hall, 1992; Zhang *et al.,* 1995; Clark *et al.,* 1998). Isoprenylation is required for the correct function of these proteins, since the lipid isoprenyl group serves to anchor the proteins in cell membranes and may also participate in protein–protein interactions (Zhang and Casey, 1996).

The ability of N-BPs to inhibit protein isoprenylation was first directly demonstrated using J774 macrophages, where the N-BPs risedronate, incadronate, ibandronate, and alendronate were found to prevent the incorporation of [^{14}C]mevalonate into isoprenylated proteins (both farnesylated and geranylgeranylated proteins), whereas the bisphosphonates that lack a nitrogen in the R^2 side chain (clodronate and etidronate) had no effect (Benford *et al.,* 1999; Luckman *et al.,* 1998b). Risedronate almost completely inhibited protein isoprenylation at a concentration of

Table I Inhibition of FPP Synthase by Various Bisphosphonates

Bisphosphonate	IC_{50} (nM) Purified recombinant human[a]	IC_{50} (nM) Recombinant human[b]	IC_{50} (nM) Murine endogenous[c]
Alendronate	340	50	500
Ibandronate		20	310
Incadronate		30	
Minodronate		3	
Pamidronate	500	200	850
Risedronate	3.9	10	100
Zoledronate		3	20
Clodronate	NA $\leqslant$ 350,000	NA $\leqslant$ 100,000	NA $\leqslant$ 100,000
Etidronate	80,000	NA $\leqslant$ 100,000	NA $\leqslant$ 100,000

Note. Analyses of FPP synthase inhibition were performed using purified [a]recombinant human enzyme (Bergstrom *et al.,* 2000), [b]recombinant human enzyme in *E. coli* lysate (Dunford *et al.,* 2001), or [c]endogenous murine enzyme in J774 macrophage lysate (Dunford *et al.,* 2001). NA, not active/negligible inhibition.

10^{-5} M, which is similar to the concentration that affects osteoclast viability *in vitro* (Sato and Grasser, 1990; Carano *et al.,* 1990; Breuil *et al.,* 1998) and, based on findings for alendronate, could be achieved within the osteoclast resorption lacuna (Sato *et al.,* 1991). N-BPs (zoledronate, risedronate, ibandronate, alendronate, pamidronate) also inhibit protein isoprenylation in osteoclasts *in vitro.* Alendronate has been shown to inhibit incorporation of [^{14}C]mevalonate into either isoprenylated proteins or sterols (nonsaponifiable lipid) in purified murine osteoclasts (Bergstrom *et al.,* 2000), while another study found that the N-BPs alendronate, ibandronate, pamidronate, risedronate, and zoledronate, but not the non-N-BPs clodronate, etidronate or tiludronate, prevented incorporation of mevalonate into isoprenylated proteins in purified rabbit osteoclasts (Coxon *et al.,* 2000). Inhibition of protein isoprenylation in osteoclasts was dose-dependent, with alendronate inhibition at $\geqslant$15 μM, while zoledronate was effective at $\geqslant$10 μM (Bergstrom *et al.,* 2000; Coxon *et al.,* 2000).

Evidence of Bisphosphonate Action in Vivo The molecular actions of the bisphosphonates, described above, has recently been confirmed *in vivo* (Fisher *et al.,* 2000; Frith *et al.,* 2000). Direct demonstration of inhibition of FPP synthase *in vivo* is not possible, thus necessitating the use of surrogate markers for measuring this effect. In one study, the previously documented feedback regulation of HMG-CoA reductase expression by mevalonate pathway metabolites, illustrated by the induction of HMG-CoA reductase in the liver of lovastatin treated rats (Singer *et al.,* 1988), was examined (Fisher *et al.,* 2000). Rats were therefore administered bisphosphonate and expression of HMG-CoA reductase was examined in the proximal tibia. The N-BPs alendronate, ibandronate, and risedronate, but not clodronate or etidronate (which lack nitrogen), suppressed expression of HMG-CoA reductase in the osteoclast. While all three N-BPs induced changes in HMG- CoA reductase expression in the osteoclast, no changes were seen in other bone- or marrow-associated cells. This is consistent with the observed targeting of alendronate to the osteoclast and not the osteoblast, observed previously (Sato *et al.,* 1991; Masarachia *et al.,* 1996). This effect on HMG-CoA reductase expression appeared to be mediated, in part, by the accumulation of metabolites upstream of FPP synthase, since coadministration of simvastatin along with alendronate partially blocked the effect. The loss of HMG-CoA reductase expression along with inhibition of FPP synthase in the osteoclast, could potentially have additive effects on the mevalonate/cholesterol biosynthetic pathway.

In another study (Frith *et al.,* 2000), osteoclasts were examined for the actions of both alendronate and clodronate. In the case of alendronate, geranylgeranylation of the small GTPase Rap1A was measured as a surrogate marker for inhibition of FPP synthase in osteoclasts isolated and purified from rabbits that had been administered with either alendronate or clodronate *in vivo.* Whereas alendronate suppressed Rap1A geranylgeranylation in osteoclasts, but not other marrow- and bone-derived cells, clodronate was without effect. Furthermore, osteoclasts purified from clodronate-treated rabbits were found to contain the metabolite App-CCl_2p consistent with the evidence described above that this bisphosphonate has a distinct mechanism of action which does not involve inhibition of the mevalonate pathway. In light of these convincing proofs and the sizable body of *in vitro* evidence, the data strongly support the division of bisphosphonates so far into two general classes, based on their interaction with FPP synthase to suppress protein isoprenylation (the N-BPs) or their interaction with aminoacyl-tRNA synthetases to form cytotoxic ATP analogs (the non-N-BPs).

Cellular Level Actions The relationship between molecular action and anti-resorptive effects is perhaps best

documented for the N-BPs, whereby inhibition of FPP synthase appears to be integral to inhibition of bone resorption (Luckman *et al.,* 1998b, Fisher *et al.,* 1999; van Beek *et al.,* 1999c). This was clearly demonstrated by the complete restoration of bone resorption in the presence of inhibitory concentrations of alendronate and of osteoclast number in ibandronate-treated mouse metacarpal bones *in vitro* by addition of components of the mevalonate pathway, which bypass the site of bisphosphonate enzyme inhibition (Fisher *et al.,* 1999; van Beek *et al.,* 1999c). Among several downstream metabolites, only geranylgeraniol, a lipid alcohol that can replenish geranylgeranyl diphosphate, prevented inhibition of osteoclast formation and bone resorption by these N-BPs. Furthermore, the effects of lovastatin and mevastatin (which also inhibit bone resorption, prevent osteoclast formation in bone marrow cultures, and disrupt the actin cytoskeleton of osteoclasts), can also be completely blocked by addition of geranylgeraniol, but not farnesol or squalene, to osteoclast cultures (Fisher *et al.,* 1999; Woo *et al.,* 2000). Finally, a selective inhibitor of protein geranylgeranylation (GGTI-298) also inhibits bone resorption *in vitro* (Coxon *et al.,* 2000). Taken together, these observations strongly suggest that the ability to inhibit bone resorption is a consequence of loss of geranylgeranylated proteins.

The inhibition of FPP synthase by the N-BPs causes a block in the formation of isoprenoids and sterols as discussed above. This leaves several possible downstream pathways that might be implicated in N-BP inhibition of bone resorption. A number of pathways leading away from FPP synthase are linked to the regulation of gene transcription via nuclear transcription factors such as SREBP, LXR, FXR, and PPARα (Forman *et al.,* 1995; Janowski *et al.,* 1996; Hanley *et al.,* 2000; also reviewed in Edwards and Erickson, 1999). FPP and GGPP can serve as precursors for the isoprenylation of proteins, such as small GTPases, which regulate cell growth, differentiation, and survival, cytoskeletal organization and vesicular trafficking. Indeed it was originally hypothesized (Luckman *et al.,* 1998b) that inhibition of the mevalonate pathway by N-BPs and loss of isoprenylation of small GTPases such as Rho, Rac, cdc42, and Rab might account for many or all, of the various effects on osteoclast function, including loss of the ruffled border and disruption of the actin cytoskeleton (Sato *et al.,* 1991; Sato and Grasser, 1991; Selander *et al.,* 1994), altered trafficking of membranes and intracellular proteins such as the osteoclast proton ATPase (Carano *et al.,* 1990; Zimolo *et al.,* 1995), disrupted intracellular signaling by integrins (Fong and Ingber, 1996) and the induction of osteoclast apoptosis (Hughes *et al.,* 1995; Ito *et al.,* 1998, 1999; Reszka *et al.,* 1999). At the cellular level four mechanisms could be involved in bisphosphonate action (Fig. 6). These are discussed below in the context of how molecular action triggers these responses.

1. Inhibition of Osteoclast Recruitment Several bisphosphonates inhibit osteoclast differentiation in various systems of cells (Hughes *et al.,* 1989) and bones (Boonekamp *et al.,*1987) in culture. Some experiments suggest that the effect occurs at the terminal step of the differentiation process (Löwik *et al.,* 1988). At least for alendronate, addition of geranylgeraniol, a lipid alcohol used to replace the isoprenylation substrate geranylgeranyl diphosphate, was shown to partially block the inhibitory effects of N-BP on the osteoclast differentiation process (Fisher *et al.,* 1999).

2. Inhibition of Osteoclast Adhesion to the Mineralized Matrix One study *in vitro* reports that osteoclast adhesion to bone can be inhibited by alendronate (Colucci *et al.,* 1998), although the mechanism involved is unknown. Whether this takes place *in vivo* is not yet established, however, the number of osteoclasts on the bone surface increases shortly after bisphosphonate treatment (Fisher *et al.,* 2000). There is excellent evidence that bisphosphonates can inhibit the adhesion of tumor cells *in vitro* (Van der Pluijm *et al.,* 1996; Boissier *et al.,* 1997).

3. Shortening of the Osteoclast Lifespan It was reported that bisphosphonates induce osteoclast apoptosis, both *in vitro* and *in vivo,* both in normal mice and in mice with increased bone resorption (Hughes *et al.,* 1995). A recent study showed that the action of the bisphosphonates in inducing osteoclast apoptosis is direct, i.e., resulting from intracellular action within the osteoclast (Reszka *et al.,* 1999). This is true for both classes of BP. There can be no doubt that a postapoptotic osteoclast is incapable of bone resorption. However, it is important to note that apoptosis may only be the primary mechanism of bone resorption inhibition by clodronate and etidronate but not the N-BPs (Halasy-Nagy *et al.,* 1999).

For the non-N-BPs such as clodronate, the induction of apoptosis is likely due to the formation of toxic ATP analogs. In accord, the chemically synthesized metabolite of clodronate (AppCCl_2p), when liposome-encapsulated, has been shown to reduce cell viability much like clodronate itself (Frith *et al.,* 1997). The ability of AppCCl_2p to cause osteoclast apoptosis may be related to inhibition of the adenine nucleotide translocase (Lehenkari *et al.,* 2000), a component of the permeability transition pore complex in the inner mitochondrial membrane. This could lead to disruption of the mitochondrial membrane potential (Benford *et al.,* 2001) and the release of Apaf-1 and cytochrome C, causing the subsequent activation of pro-apoptotic caspases (Cohen, 1997; Crompton *et al.,* 1999; Nicholson, 1999).

The likelihood that N-BPs cause apoptosis by interfering with signaling pathways downstream of isoprenylated proteins in osteoclasts was bolstered by the observation that protein synthesis was necessary for the induction of apoptosis by N-BPs and statins (which also prevent isoprenylation) (Coxon *et al.,* 1998). N-BP-induced and statin-induced macrophage apoptosis occurs after a lag period of 15–24 hr, while in the osteoclast apoptosis is observed at $\geqslant$12 hr, although other inducers can act within 2–4 h (Reszka *et al.,* 1999). Ultimately, the importance of inhibition of FPP

synthase and the identification of possible downstream pathways was established through the demonstration that pro-apoptotic activities of the N-BPs are blocked simply by replacing lost mevalonate pathway metabolites. By adding back the metabolites, FPP and GGPP (or the more cell-permeable alcohol forms, farnesol and geranylgeraniol respectively) in J774 or JJN-3 cells, alendronate- or incadronate-induced apoptosis is blocked (Luckman *et al.,* 1998b; Shipman *et al.,* 1998; Benford *et al.,* 1999). Induction of osteoclast apoptosis by alendronate and risedronate, but not clodronate or etidronate, was blocked by addition of geranylgeraniol, but not farnesol, suggesting that only geranylgeranylation was critical (Reszka *et al.,* 1999). Consistent with this, an inhibitor of geranylgeranylation (GGTI-298), but not farnesylation (FTI-277), can induce osteoclast apoptosis *in vitro* (Coxon *et al.,* 2000).

The signaling pathways involving geranylgeranylated small GTPases such as Rho, Rac, and cdc42 that are affected by bisphosphonates and that lead to osteoclast apoptosis remain to be determined. However, Mst1 has been identified as one pro-apoptotic signaling intermediate downstream of the bisphosphonates that activated during apoptosis by N-BPs, lovastatin and clodronate (Reszka *et al.,* 1999). This kinase acts as both a substrate for caspases 3 and 7 and as an activator of these caspases (Graves *et al.,* 1998). Caspase cleavage of Mst1 results in a highly active kinase that is itself capable of initiating apoptosis. It has been very recently shown that caspase 3 is the major effector caspase activated in osteoclasts undergoing apoptosis following treatment with a range of bisphosphonates *in vitro* (Benford *et al.,* 2001).

4. Direct Inhibition of Osteoclast Activity While the former three mechanisms will lead to a decrease in the number of osteoclasts which is usually seen after longer treatment, the fourth will lead to inactive osteoclasts, often seen already at the beginning of treatment. It was reported long ago that, following bisphosphonate administration, osteoclasts show changes in morphology and appear inactive (Schenk *et al.,* 1973). The changes are numerous (Sato and Grasser, 1990) and include alterations in the cytoskeleton, including actin (Sato *et al.,* 1991; Selander *et al.,* 1994; Murakami *et al.,* 1995) and vinculin (Murakami *et al.,* 1995) as well as disruption of the ruffled border (Schenk *et al.,* 1973; Sato *et al.,* 1991). Another observation that defines the N-BPs as a class and calls to question the roles of recruitment, apoptosis, or adhesion to bone in N-BP inhibition of bone resorption, is the increase in osteoclast number found *in vivo* after treatment with alendronate, ibandronate, and risedronate, but not clodronate or etidronate (Fisher *et al.,* 2000). Unlike in previous studies where increased osteoclast numbers seen after alendronate treatment were associated with increased bone surface (Seedor *et al.,* 1991; Bikle *et al.,* 1994), the increase in osteoclast number was recently observed within 48 hr of treatment (Fisher *et al.,* 2000), which is generally considered to be the first time point at which inhibition of bone resorption occurs.

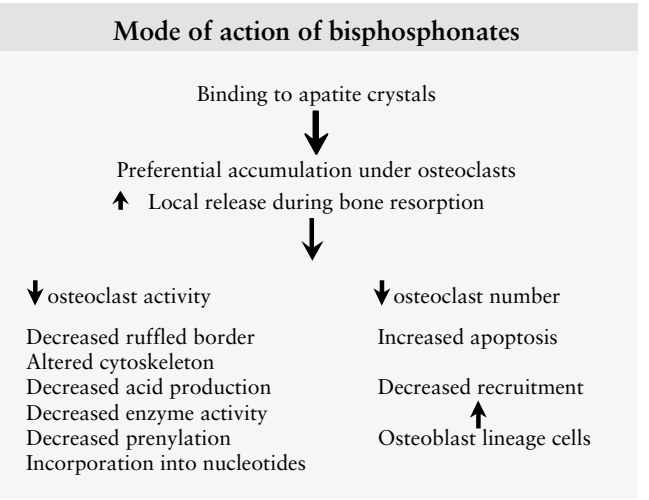


Figure 6 Summary of the effects of bisphosphonates on the osteoclast. Reproduced from H. Fleisch, "Bisphosphonates in Bone Disease — From the Laboratory to the Patient." Academic Press, 2000, with copyright permission from the author.

For the various cellular effects of the bisphosphonates on the osteoclast described above (Fig. 6), it is necessary for the bisphosphonates to either bind to or enter the cells, as has been shown to be the case *in vivo* (Sato *et al.,* 1991; Masarachia *et al.,* 1996). However, another possibility that cannot be excluded is that in addition bisphosphonates could act through the intermediary of other cells such as the osteoblast. Low amounts of bisphosphonates were shown to induce osteoblasts to secrete a factor that inhibits osteoclastic recruitment (Sahni *et al.,* 1993; Nishikawa *et al.,* 1996; Vitté *et al.,* 1996). Such an effect on osteoblasts *in vitro* is consistent with the observation that bisphosphonates can be taken up by various mammalian cell types *in vitro.* The cellular uptake is mostly into the cytosol and *in vitro* the concentration reached can be very high (Felix *et al.,* 1984). However, intracellular levels in osteoblasts *in vivo* were below the limit of autoradiographic detection, in radioisotope studies which visualized alendronate in rat osteoclasts (Sato *et al.,* 1991; Masarachia *et al.,* 1996).

Inhibition of Calcification

Like pyrophosphate, bisphosphonates can inhibit calcification *in vivo.* Thus, among others, they prevent experimentally induced calcification of many soft tissues when given parenterally or orally (Fleisch *et al.,* 1970), as well as ectopic ossification. Topical administration leads to a decreased formation of dental calculus (Briner *et al.* 1971).

If administered in sufficiently high doses, bisphosphonates can also impair the mineralization of normal calcified tissues such as bone, cartilage, dentine, and enamel (King *et al.,* 1971; Schenk *et al.,* 1973). The amount required to produce this effect varies somewhat according to the bisphosphonate

used, the animal species, and the length of treatment. However, in contrast to bone resorption where the different compounds vary greatly in their activity, they inhibit mineralization at similar doses. For most species that dose is in the order of 1–10 mg of compound phosphorus per kg per day parenterally. There is a close relationship between the ability of a bisphosphonate to inhibit calcium phosphate precipitation *in vitro* and its effectiveness at inhibiting calcification *in vivo* (Fleisch *et al.,* 1970; Trechsel *et al.,* 1977; van Beek *et al.,* 1994). Therefore, the mechanism for this effect is likely to be a physicochemical one. It is possible that other cellular effects, for example changes in the bone matrix, are involved but this remains to be proven. The inhibition of mineralization can lead to fractures (Flora *et al.,* 1980) and to impaired healing of the fractures (Fleisch, 2001). Although the inhibition is eventually reversed after discontinuation of the drug, the propensity to inhibit the calcification of normal bone has hampered the therapeutic use of bisphosphonates for ectopic calcification. However, this is not the case for their use to inhibit bone resorption, since bisphosphonates have now been developed that can inhibit resorption at doses 1000 times lower than those inhibiting mineralization. The doses used to inhibit resorption do not impair fracture healing either, when assessed by estimating biomechanical properties (Fleisch, 2001).

Other Effects

It has been suggested that bisphosphonates may also increase bone formation. Thus very low concentrations of bisphosphonates were found to increase the multiplication of various cells, including bone cells (Guenther *et al.,* 1981). Bisphosphonates were also reported to stimulate colony formation, nodule formation, mineralization, and osteocalcin synthesis in cell cultures of animal and human bone (Tsuchimoto *et al.,* 1994 Giuliani *et al.,* 1998). Furthermore, it was shown that the amount of bone formed in an individual BMU is somewhat increased during bisphosphonate treatment (Balena *et al.,* 1993; Boyce *et al.,* 1995). Thus, bisphosphonates might, under certain conditions, increase bone formation *in vivo*. However, this still needs to be confirmed.

Some reported bisphosphonate effects are difficult to explain based on their action on bone. Thus several bisphosphonates inhibit local cartilage resorption, preserve the joint architecture, and decrease the inflammatory reaction in several types of experimental arthritis, e.g., induced by Freund's adjuvant, carrageenin or collagen (Francis *et al.,* 1972, 1989; Dunn *et al.,* 1993). This effect is especially pronounced when the bisphosphonates (in particular clodronate) are encapsulated in liposomes (van Lent *et al.,* 1994; Kinne *et al.,* 1995). The fact that not only bone resorption is decreased, but also the inflammatory reaction in the joint and in the paw itself is diminished (Österman *et al.,* 1994), suggests that mechanisms other than those acting on bone are at work, possibly involving the mononuclear phagocyte system. These results open the exciting possibility of using bisphosphonates in inflammatory arthritis, given either systemically or locally, possibly encapsulated in liposomes. A recent study by Barrera *et al.* (2000) confirmed that a single intraarticular injection of lipo-clodronate caused depletion of synovial macrophages and was well tolerated in patients with rheumatoid arthritis.

Pharmacokinetics

Bisphosphonates are synthetic compounds, not found to occur naturally in animals or humans. No enzymes capable of cleaving the P-C-P bond have been discovered. The bisphosphonates studied and reported so far, including alendronate, clodronate, etidronate, pamidronate, and tiludronate, appear to be absorbed, stored, and excreted from the body unaltered, except for the incorporation of etidronate, clodronate, and pamidronate into ATP inside the osteoclast, described above. (For reviews see Papapoulos, 1995; Lin, 1996; Porras *et al.,* 1999).

Intestinal Absorption

The bioavailability both in animals and in humans is low, probably because of low lipophilicity and high negative charge. It ranges from <1 to 10% of an oral dose, is generally higher in the young, sometimes higher at higher doses, and shows a great inter- and intraspecies variation (Michael *et al.,* 1972; Recker and Saville, 1973; Yakatan *et al.,* 1982). It is generally lower for the more potent bisphosphonates. Absorption occurs to the largest extent in the small intestine and appears to occur by passive diffusion, possibly through a paracellular pathway (Boulenc *et al.,* 1993). It is diminished when the drug is given with meals, in the presence of calcium, and interestingly, also when given with coffee, tea, or orange juice (Gertz *et al.,* 1995).

Distribution

About two-thirds or more of etidronate and clodronate, half of pamidronate, but a much smaller fraction of other bisphosphonates, such as alendronate, are ultrafiltrable when in the blood. The values are strongly species dependent. The remainder is either bound to proteins, especially albumin (Lin *et al.,* 1994), or present in very small aggregates (Wiedmer *et al.,* 1983).

Between 30 and 70% of absorbed bisphosphonates are taken up by bone, the remainder being rapidly excreted in the urine (Michael *et al.,* 1972; Yakatan *et al.,* 1982). The uptake varies with species, gender, and age and can differ among compounds. While it is below 30% or less for clodronate, it is about 50% for etidronate and higher for pamidronate and alendronate. Sometimes bisphosphonates can deposit in other organs such as the stomach, liver, and

spleen. Part, if not all, of this extraosseous deposition appears to be due to the formation of complexes, occurring when too much compound is infused too rapidly. If given at therapeutic doses and infused slowly, extraosseous deposition seems to be negligible.

The half-life of circulating bisphosphonates is short, in the order of minutes in the rat (Bisaz *et al.,* 1978) and around 0.5 to 2 hr in humans. The rate of uptake in bone is very fast, bone clearance being compatible with complete first passage extraction from the circulation by the skeleton (Bisaz *et al.,* 1978). Skeletal uptake might therefore be determined to a large extent by the bone vascularization. Soft tissues are thus exposed to these compounds for short periods only, which explains their bone-specific effects.

When bisphosphonates are given at clinical doses to humans, there seems to be no saturation of the total skeletal uptake, at least not within periods as long as years or decades. In contrast, the anti-resorbing activity rapidly reaches a maximum, both in animals (Reitsma *et al.,* 1980) and in humans (Garnero *et al.,* 1994). The fact that a plateau of activity is reached despite continuous bisphosphonate accumulation in the skeleton, is consistent with entombment of the compound, where it is inactive since it is not accessible to the osteoclasts on the bone surface.

From the skeleton the bisphosphonates are liberated to a small extent by physicochemical mechanisms, such as ion exchange, but for the largest part they are released only when the bone in which they are deposited is resorbed (Kasting and Francis, 1992). Thus the half-life in the body depends upon the rate of bone turnover. It has been estimated to be up to 1 year in mice or rats (Mönkkönen, *et al.,* 1990), and much longer, up to 10 years, in humans (Kasting and Francis, 1992). It is possible that some of the administered bisphosphonate can stay in the skeleton for life. This is also true for other "bone seekers" such as tetracyclines, heavy metals, and fluoride.

Renal Clearance

The renal clearance of bisphosphonates is high. When taking into account that they are only partially ultrafiltrable, it can be as high as that of inulin or even higher, suggesting a secretory component (Troehler *et al.,* 1975; Lin *et al.,* 1992). Urinary excretion is decreased in renal failure and the removal by peritoneal dialysis is poor, which has to be accounted for when the compounds are administered to patients with kidney disease.

Clinical Use and Efficacy

As mentioned in the Introduction, in the context of this volume this chapter focuses primarily on the mechanism of action of bisphosphonates; however, we would be remiss not to briefly review the extensive current clinical use of these compounds in the treatment of many skeletal disorders. There are now safe and effective treatments for osteoporosis in both women and men; for glucocorticoid-induced osteoporosis, Paget's disease, bone metastases, and hypercalcemia of malignancy. Treatment of other conditions, e.g., osteogenesis imperfecta, are under investigation. In the following section, we briefly summarize the use and efficacy of bisphosphonates for these indications.

Postmenopausal Osteoporosis

Bisphosphonates Increase BMD

The three bisphosphonates currently available in many countries for the prevention and/or treatment of osteoporosis include (cyclical) etidronate, daily alendronate or risedronate and weekly alendronate. Etidronate administered at 400 mg per day orally for 2 weeks, followed by 11 weeks calcium alone increases BMD at the lumbar spine (LS) by 2.3 to 5.2%, at the femoral neck (FN) by up to 1.9%, and at trochanter (TR) by up to 2.9% relative to baseline (Watts *et al.,* 1990; Meunier *et al.,* 1997; Herd *et al.,* 1997). In the placebo groups, there were typically decreases in BMD at these sites (vs baseline) by up to −2.3 to + 1.2%, −2.0% and −0.6%, respectively. After treatment discontinuation, BMD declined by 1.4% (LS) and −0.9% (FN) at 12 months. In a separate 3-year etidronate study BMD increased from baseline by 5.7% (LS), 1.4% (FN), and 7.1% (TR), while in placebo, changes were 0.17% (LS), −2.97% (FN), and 1.5% (TR) (Montessori *et al.,* 1997). Relative risk of fractures at the hip or spine were not determined in the aforementioned studies, owing in part to the small population size.

Alendronate is perhaps the most extensively studied drug so far for the prevention and treatment of postmenopausal osteoporosis. The longest patient follow-up (Tonino *et al.* 2000) reported the 7-year findings of the twice extended original phase III study (Liberman *et al.,* 1995; Tucci *et al.,* 1996). The 7-year cumulative increases in BMD (versus baseline) were 11.4% (LS), 4.9% (FN), and 9.5% (TR) with daily 10-mg dosing, which was significantly higher at LS than the 5-year BMD (9.4%). Increases in BMD similar to those produced by 10 mg per day were observed when patients were given 20 mg daily for 3 years followed by 2 years with 5 mg daily. Most importantly, upon discontinuation, there was no change in BMD in the 2-year follow-up period.

Treatment for shorter duration showed rapid dose-dependent increases in BMD at the various sites. For example in a 2-year study 5 mg oral alendronate daily, increased BMD (from baseline) by 6.2% (LS), 1.9% (FN), and 4.1% (TR), while with 2.5 mg, the increases were 4.1% (LS), 0% (FN), and 1.8% (TR) (Bone *et al.,* 1997). A similar study comparing responses to 2.5 or 5 mg oral dosing in women of either Asian or Caucasian descent showed similar changes in spinal BMD in the two groups (Wasnich *et al.,* 1999). These findings were consistent across multiple studies (e.g., Adachi *et al.,* 2001; Tucci *et al.,* 1996; Liberman *et al.,* 1995). Alendronate

administered orally once weekly at 70 mg for 1 or 2 years produced very similar increases in BMD to 10 mg daily dosing, 5.1% (once weekly) vs 5.4% (daily) at LS, 2.3% vs 2.9% (FN), and 3.9% vs 4.4% (TR) observed after 12 months of treatment, which fell within equivalence bounds (Schnitzer *et al.,* 2000). The increases seen at 24 months remained comparable and were even closer.

Risedronate has also been studied extensively, using either 2.5 or 5 mg daily oral dosing. Two years of continuous daily treatment with 5 mg oral risedronate increased LS BMD by either 1.4% (vs −4.3% in placebo) or to 4.1% (vs 0% in placebo), resulting in a consistent increase of 4.1 to 5.6% over placebo (Mortensen *et al.,* 1998; Fogelman *et al.,* 2000). Discontinuation for 12 months resulted in a decline in LS BMD of 3.7% (−2.3% vs baseline), while in the placebo group BMD had further declined by 1.3% (to −5.6% vs baseline) (Mortensen *et al.,* 1998). At FN and TR, increases were 1.3 to 2.6% and ≈0.5 to 2.7%, respectively (vs baseline), while in the placebo group, BMD had declined by 1.0 to 2.4% and 0.6 to 2.8%, respectively. The 2.5-mg oral dosing increased BMD by1.4% (vs 0%, in placebo) at LS, 0.9% (vs −1.0% in placebo) at FN, and 1.7% (vs −0.6% in placebo) at TR (Fogelman *et al.,* 2000). After 3 years with 5 mg oral dosing, increases in BMD were 5.4% (LS), 1.6% (FN), and 3.3% (TR), while changes in the placebo group were 1.1% (LS), −1.2% (FN), and −0.7% (TR) (Harris *et al.,* 1999).

Bisphosphonates Reduce Fracture Risk

The best evidence for treatment-related fracture reduction reported so far was obtained with the oral bisphosphonates alendronate and risedronate. A 3-year study with alendronate showed 47 and 49% reductions in vertebral fractures and 51 and 56% reductions in hip fractures in women with a *T* score of ⩽−2.1 and −2.5, respectively (Black *et al.,* 1996, 2000). After 4 years treatment, women with an existing vertebral fracture showed a 47% reduction in vertebral fractures and a 51% reduction in hip fractures (Black *et al.,* 2000).

Analysis of the cumulative proportion of new fractures in women treated with alendronate, that already had a vertebral fracture or a BMD of $T \leqslant -2.5$, showed significant risk reduction of 59% for clinical vertebral fractures by 12 months, 27% for any clinical fracture, and 63% reduction for hip fracture by 18 months, 26% for nonvertebral fractures by 24 months, and 34% reduction for wrist fractures by 30 months (Black *et al.,* 2000).

Three years of treatment with risedronate of women who had two or more prevalent vertebral fractures at baseline reduced the risk of vertebral fracture by 49% and the risk of nonvertebral fractures by 33% in Reginster *et al.* (2000). In the HIP study, women with a *T* score of ⩽−4.0 or a combination of a *T* score of ⩽−3.0 with a hip axis length of ⩾11.1 cm showed a significant 40% reduction in hip fracture risk (McClung *et al.,* 2001). Within this group, the presence of a prevalent vertebral fracture at baseline reduced the risk of hip fracture with treatment by a significant 60%, while those lacking any vertebral fracture at baseline had a 40% (NS) risk reduction. In a separate arm, women aged ⩾80 years with ⩾1 clinical risk factor for hip fracture showed a 20% (NS) reduction in risk of hip fracture. The combined effect in all subgroups was a significant 30% reduction in the risk of hip fracture.

Several mechanisms are probably responsible for the reduction in fracture risk produced by bisphosphonate treatment (see above under mechanism of action) including increase in BMD, caused in part by increased mineralization, and the reduction in the number of bone resorption sites, reflected in reduced turnover markers.

Glucocorticoid-Induced Osteoporosis

Although glucocorticoid induced osteoporosis is caused, at least in part, by inhibition of bone formation, bisphosphonates have been very effective in treating and preventing this condition and several are approved for this indication (for review see Adachi and Ioannidis, 2000). Cyclical etidronate increased BMD in the lumbar spine by 3.8% relative to placebo in 1 year, daily alendronate by 3.3% in 1 year and 4.7% in 2 years, and daily risedronate by 2.6% in 1 year. Each of these bisphosphonates reduced vertebral fractures relative to placebo (Adachi *et al.,* 2001; Wallach *et al.,* 2000).

Paget's Disease

The earliest studies demonstrating the effectiveness of bisphosphonates for normalizing bone resorption in Paget's disease were performed with etidronate (Smith *et al.,* 1971). Several bisphosphonates have since been approved for the treatment of Paget's disease and are today the treatment of choice for this condition (for review see Grauer *et al.,* 1999). Since Paget's disease is characterized by highly active bone resorption, virtually every bisphosphonate considered for clinical use has been evaluated in this disease using various oral (tiludronate, olpadronate) or intravenous (pamidronate, zoledronate, ibandronate) regimens (Laroche *et al.,* 1999; Gonzalez and Mautalen, 1999; Buckler *et al.,* 1999).

They are administered at higher doses than those prescribed for osteoporosis, e.g., 40 mg per day alendronate or 30 mg per day risedronate, given for a limited period of time, 6 and 3 months, respectively, for these two agents. This regimen normalized alkaline phosphatase (ALP) in 63% of the patients on alendronate and 77% of those on risedronate. Most interestingly, the patients entered a long-term remission; after 18 months 86% of alendronate patients and 53% of risedronate patients still had normal ALP and were free of symptoms.

Tumor Bone Disease

The most common human tumors, breast and prostate cancer, as well as many other solid tumors, e.g., lung carcinoma

and melanoma, have frequent skeletal complications: osteolytic or osteoblastic metastases and tumor-induced hypercalcemia. Bisphosphonate treatment, in addition to antitumor therapy, has become the standard of care for these patients (for recent reviews see Major *et al.,* 2000; Diel *et al.,* 2000).

In tumor-induced hypercalcemia, most often due to the secretion by the tumor of parathyroid hormone-related peptide, bisphosphonates are given intravenously after adequate hydration. Calcium levels drop significantly after 48 hr and are fully normalized at 10 days in 69.7% of patients receiving 90 mg pamidronate i.v. and 88.4% of patients given 4 mg zoledronate i.v. The median duration of complete response was 18 days for pamidronate and 32 days for 4 mg and 43 days for 8 mg zoledronate, respectively (Major *et al.,* 2001).

The efficacy of bisphosphonates in the treatment of bone metastases is not as well established. Randomized studies with 1600–3200 mg oral clodronate per day yielded conflicting results, the positive ones showing fewer skeletal complications, but no survival advantage (with the exception of Diel *et al.,* 1998). Intravenous pamidronate given to patients with multiple myeloma was reported to decrease pain and slow down the deterioration in performance (Berenson and Lipton, 1999) without extending survival. When given to breast cancer patients, the median time to first skeletal complication is delayed by about 7 months and bone pain is reduced, again without extending survival (Hortobagyi *et al.,* 1996, 1998). It is possible that the more potent bisphosphonates currently in development, such as zoledronate or ibandronate, will have more pronounced effects on bone metastases. A most desirable goal is to reduce their incidence to begin with.

Other Diseases

There are many other conditions in which osteoclastic bone resorption is increased, either due to inflammation, such as in rheumatoid arthritis and periodontal disease, or to genetic mutations, such as in osteogenesis imperfecta and fibrous dysplasia. So far, no placebo-controlled randomized studies have shown bisphosphonate suppression of bone loss in either the periarticular lesions of rheumatoid arthritis or in periodontal disease. On the other hand, observational studies have shown significant improvement in osteogenesis imperfecta (Plotkin and Glorieux, 2000) and polyostotic fibrodysplasia (Zacharin and O'Sullivan, 2000) with intravenous pamidronate treatment.

Animal Toxicology

Toxicological animal studies have been published on alendronate, clodronate, etidronate, incadronate, pamidronate, and tiludronate. Acute, subacute, and chronic administration in several animal species have, in general, revealed little toxicity. Teratogenicity, mitogenicity, and carcinogenicity tests have been negative. When bisphosphonates are administered subcutaneously, local toxicity can occur, with local necrosis. This is especially the case for the N-containing derivatives. Mild hypocalcemia due to inhibition of bone resorption was observed at high doses. Acute systemic toxicity and hypocalcemia can also be caused following intravenous administration of high doses of less potent bisphosphonates by the formation of complexes with calcium. Some renal alterations can occur.

The chronic toxicity of the bisphosphonates is extremely low. This is probably due in part to their strong affinity for bone, which allows them to be cleared rapidly from blood. The toxic effects occur in general when doses substantially larger than those which inhibit bone resorption are used. As for polyphosphates and phosphate itself, the first alterations are usually seen in the kidney (Alden *et al.,* 1989; Cal and Daley-Yates, 1990). The liver, as well as the testis, the epididymis, the prostate, and possibly the lung, can in some cases also show changes.

Following the appearance in humans of gastrointestinal adverse events, after oral administration of N-containing bisphosphonates (De Groen *et al.,* 1996), the effects of oral bisphosphonates were studied in animals. Alendronate, given orally to rats at suprapharmacological doses, has been reported to occasionally induce gastric and esophageal erosions and ulcerations and delay healing of indomethacin-induced gastric erosions. These effects are not attributable to changes in gastric acid secretion, or prostaglandin synthesis, but are thought to be due to a topical irritant effect. Similar effects were reported with etidronate, risedronate, and tiludronate when given at pharmacologically equivalent doses. These effects were obtained at doses equivalent to (Peter *et al.,* 1998a) or much larger than the ones given in humans (Blank *et al.,* 1997; Elliott *et al.,* 1998; Peter *et al.,* 1998b).

To address the issue of mechanism, recent studies have examined apoptosis and suppression of cell growth in *in vitro* models for the esophageal stratified epithelium and the large intestine (Mönkkönen *et al.,* 2000c; Reszka *et al.,* 2000, 2001). In CACO-2 intestinal epithelium cells and in Ch1.Es esophageal fibroblasts, the N-BPs-induced apoptosis, which could be blocked with the addition of geranylgeraniol (Mönkkönen *et al.,* 2000; Reszka *et al.,* 2000). This suggested that N-BP inhibition of protein geranylgeranylation was instrumental in the apoptotic response. In normal human epidermal keratinocytes (Reszka *et al.,* 2001) and, as a second phenotype, in CACO- 2 cells, growth suppression was observed in response to N-BP treatment. In the keratinocyte, used as a model for stratified squamous epithelium lining the esophagus, this was associated with both suppression of cholesterol biosynthesis and protein geranylgeranylation, and was linked to a block in the phosphorylation of the growth regulatory protein retinoblastoma, loss of cdk2 and cdk4 expression, and enhanced association of these cdks with the inhibitors $p21^{waf1}$ and $p27^{kip1}$ (Reszka *et al.,* 2001). These recent *in vitro* studies, suggest that the N-BP-induced gastrointestinal irritation and/or delayed repair of irritation are

mediated by inhibition of FPP synthase in the affected tissues. These findings await *in vivo* validation.

The most relevant toxicity associated with the low potency bisphosphonate etidronate is the inhibition of bone and cartilage calcification, as described above (King *et al.,* 1971; Schenk *et al.,* 1973). This effect starts to occur in animals at parenteral doses of approximately 5–10 mg P/kg daily. The radiological appearance resembles rickets or osteomalacia, although there are some histological differences. Fractures can occur after long-term administration of high doses and are probably the result of defective mineralization (Flora *et al.,* 1980). Developmental disturbances of enamel can also appear at high systemic doses. This problem is not observed with the more potent bisphosphonates in current use, as these are administered at far lower doses.

Last, at least certain bisphosphonates, such as etidronate and pamidronate, cross the placenta and can affect the fetus. Very large doses can lead to a decrease in the number of live pups, to fetal abnormalities of the skeleton and the skin, and to malformations and hemorrhages (Eguchi *et al.,* 1982; Graepel *et al.,* 1992; Okazaki *et al.,* 1995). In view of these results, bisphosphonates should not be administered to pregnant women.

To summarize this section, bisphosphonates are the most effective anti-osteoporosis treatment to date, are the treatment of choice for Paget's disease and hypercalcemia of malignancy, are the standard of care for bone metastases, and have potential uses in several other skeletal conditions, all with a very good safety profile.

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CHAPTER 79

Fluoride in Osteoporosis

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Introduction

Fluoride is an indispensable trace element with a recommended daily dose of 1.5 to 4 mg (Mertz, 1981). As is true for other essential trace elements, deficiency or excess has clinical consequences: intakes below the recommended daily dose result in growth and development retardation, while long-term high intake leads to hyperostosis or skeletal sclerosis. The latter was described for the first time in the 1940s after observation of cases with severe endemic or industrial fluorosis (Möller and Gudjonsson, 1932; Roholm, 1937; Shortt *et al.,* 1937). The same is true for the teeth. Excessive intake of fluoride may produce dental fluorosis while adequate supply reduces the risk of caries. In this latter regard, a highly successful water and topical fluoridation program has dramatically reduced the prevalence and severity of dental caries.

Success with the use of fluoride as a therapeutic agent for the skeleton has been harder to accomplish. There is no doubt that pharmacological doses of fluoride stimulate osteoblastic bone formation and significantly augment bone mass. In fact, fluoride is the most potent anabolic substance for bone available today (Caverzasio *et al.,* 1998). Positive effects on bone mass in human subjects are well documented. It is relatively bone specific, very inexpensive, and can be administered orally.

The seemingly narrow therapeutic window for the effects of fluoride on the skeleton, however, has hampered progress in this field. Despite nearly four decades of clinical experience with fluoride, uncertanties still exist about the quality of the newly formed bone tissue and the effectiveness of fluoride treatment in reducing vertebral fracture rate (Heaney, 1994). Large studies to evaluate definitively vertebral fracture rates are not available due, in part, to the low price of fluoride salts and the lack of patent protection of the product. Ringe and Meunier (1995) have therefore labeled fluoride as an orphan drug.

This point has kept major pharmaceutical companies from investing in fluoride therapy despite its obvious potential. (Kleerekoper, 1998)

Pharmacology

Gastrointestinal Absorption

A clear distinction should be made between the two mainly used fluoride salts: sodium fluoride (NaF) and monofluorophosphate (MFP) (Delmas *et al.,* 1990). NaF has been studied in the plain generic form, different types of enteric-coated tablets, or as a slow-release preparation. Since the application of plain NaF is accompanied by a high rate of mainly epigastric side effects (Riggs *et al.,* 1990; Kleerekoper *et al.,* 1991), this fluoride salt is usually given as enteric-coated and/or sustained-release tablets.

In the stomach, fluoride is absorbed by passive nonionic diffusion in the form of hydrogen fluoride (HF), the amount of HF formed being dependend on gastric acidity. Absorption is more rapid and more complete in the fasting state, when the gastric contents are more acidic. Fluoride not absorbed in the stomach is readily absorbed from the upper small intestine. Following a single oral dose of NaF administered after an overnight fast, peak serum fluoride levels are achieved rapidly with the plain form, at approximately 30 min, while this peak is delayed by up to 2–3 hr with some slow-release preparations. While intestinal absorption of NaF is as high as 95% when used as a plain preparation, different preparations may reduce the bioavailability of the fluoride ions to variable degrees (Table I). The major advantage of delayed-release and/or enteric-coated preparations lies in their fewer side effects.

Disodium-monofluorophosphate (Na_2PO_4F) usually called MFP has the same high intestinal absorption rate as plain NaF (95–100%), but a significantly lower rate of gastrointestinal

Table I Differentiated Dosage of Fluoride Using Sodium Fluoride (NaF) or Monofluorophosphate (MFP) Based on Fluoride Content and Bioavailability from the Two Salts

	NaF	MFP
Range of usual dosages/day (mg)	50–80	100–200
Fluoride content per 100 mg (mg)	45.5	13.2
Mean bioavailability (%)	60[a]	95
Average absorbed fluoride from 100 mg substance (mg)	27.3	12.5
Equivalent total amount needed if 15 mg fluoride lons is to be absorbed (mg)	55	120

[a]Average estimated bioavailability; depends on type of enteric-coated or sustained-release preparation.

side effects (Müller *et al.,* 1992). This fluoride compound dissociates in a acqueous milieu into Na+ and $(FPO_3)_2$ ions. The monofluorophosphate ion is absorbed before hydrolisis mainly in the upper small intestine. It then becomes hydrolyzed by alkaline phosphatases in the intestinal mucosa into fluoride and phosphate ions. The low rate of epigastric side effects of MFP are due to the fact that no irritating free fluoride ions are released in the gastrointestinal tract before absorption (Müller *et al.,* 1992). New sustained-release formulations of MFP are being investigated (Erlacher *et al.,* 1995).

Effects of Food and Calcium

Another important difference between the two fluoride salts is the effect of food and the presence of calcium in the gut on their respective absorption and bioavailability. Given the marked gastric irritability of plain NaF, and the need to provide adequate calcium during long-term treatment of osteoporosis with fluoride, interference by calcium with fluoride absorption has major therapeutic implications. Ekstrand and Spak (1990) demonstrated that after ingestion of a solution containing 20 mg of NaF, peak plasma concentration achieved was 848 ± 116 ng/ml at 0.46 ± 0.17 hr. When NaF was supplied as a 20-mg sustained-release tablet, absorption was delayed (1.85 ± 0.63 hr) and a lower peak plasma level was achieved (336 ± 58 ng/ml). Absorption was further delayed (2.86 ± 1.75 hr) and lower peak plasma levels were achieved (252 ± 86 ng/ml) when the tablet was coadministered with 500 mg of calcium carbonate. In a separate study, enteric-coated NaF was administered in two divided doses of 10 mg each, 12 hr apart. Peak plasma levels after the morning dose taken following an overnight fast were obtained at 3.3 hr. Peak plasma levels following the second dose given after just 4 hr without food were not obtained until 6.1 hr later. Assuming 100% bioavailability from the solution of fluoride, they concluded that bioavailability from sustained-release NaF was approximately 90%, reducing to approximately 65% with enteric-coated tablets, and further reduced to 30–40% if calcium and/or food had been previously ingested.

Pak *et al.* (1986, 1989) have evaluated the absorption of a slow-release preparation of NaF (SR-NaF). Not only was there a delayed absorption, but total absorption as assessed by the area under the curve (AUC) of plasma F levels as a function of time postadministration was markedly decreased. This resulted in a flatter pharmacokinetic dynamic for F than with other preparations, a potential advantage since it seems to be important to avoid high peak serum levels (Table VI).

It is recommended that NaF be taken separate from the time of administration of calcium. In contrast, MFP is generally administered together with calcium in the same tablets because there is no interaction with calcium in the upper gastrointestinal tract. When MFP is taken with meals, a slower kinetics of fluoride absorption ensues, which helps to avoid excessively high plasma levels.

Serum Levels

In normal adults, who are not knowingly ingesting fluoride fasting plasma levels range from 0.5 to 2.3 μM (8.5–45 ng/ml). Concentrations progressively increase with age (Husdan *et al.,* 1976). The "therapeutic window" has been estimated at between 5 and 10 μM (95–195 ng/ml) (Taves, 1970) but the evidence to support this therapeutic window is quite limited. On the basis of different studies relating the dose of NaF to the annual rate of increase in bone mass Kleerekoper and Balena (1991) found that a dose less than 30 mg/day (=13.7 mg fluoride ions) is the minimum dose at which an effect of bone mass is seen. Doses between 30 and 50 mg/day cause is a rapid increase in anabolic effect of fluoride; above that dose, a flattening of the dose–response curve occurs. This relatively narrow dose–response range is consistent with a narrow therapeutic window, such as the postulated twofold concentration range of 5–10 μM. The major concern about exceeding the therapeutic window for serum F is predominantly related to potential long-term deleterious effects on the skeleton.

Renal Excretion

After absorption fluoride is either deposited in bone or excreted by the kidney. Renal excretion of fluoride is positively correlated with serum fluoride levels (Ekstrand *et al.,* 1978). Some investigators have suggested that renal fluoride excretion can be used as an index to predict the skeletal response to fluoride therapy. Duursma *et al.* (1987, 1990) found a significant positive correlation between changes in average BMD values and 24-h urinary excretion of F in patients treated with fluoride for 1 year or more. The positive relationship could reflect simply the higher serum concentrations of fluoride achieved (Kraenzlin *et al.,* 1990).

The extrarenal clearance of fluoride (about 80 ml/min) reflects mainly accretion into bone (Ekstrand *et al.,* 1978). On average, about 50% of the absorbed fluoride dose is deposited in the skeleton, and most of the remainder is excreted in the urine. The uptake of fluoride by the bone tissue is not homogeneous, with a higher proportion taken

up by cancellous sites than by cortical sites. This may account for the heterogeneous response of different skeletal regions observed in clinical studies.

It should be emphasized that, inasmuch as the kidney is the major excretory route for fluoride, patients with impaired renal function should be given fluoride cautiously. Fluoride is not contraindicated but the dosage must be carefully adjusted depending upon the fluoride serum level.

Effects on Bone Cells

There is general agreement that fluoride stimulates bone formation directly without the need of prior bone resorption, a point that may help to explain why this element is so effective in increasing bone mass. The first *in vitro* evidence that fluoride acts directly on osteoblasts to stimulate proliferation was reported by Farley *et al.,* in 1983. These studies were prompted by earlier bone morphometric studies showing that the positive effect of fluoride treatment on bone balance was due mainly to augmented bone formation without increased bone resorption. The increased formation rate was related to an increased number of osteoblasts (Briancon and Meunier, 1981).

The *in vitro* mitogenic activity of fluoride was confirmed subsequently by several groups studying for bone cells of different species, including humans (Bellows *et al.,* 1990; Marie *et al.,* 1990; Khokher and Dandona, 1990). The optimal *in vitro* mitogenic dose of fluoride varies depending on culture conditions ranging between 5 and 100 μM, which encompasses the effective serum fluoride level of about 10 μM in treated patients (Libanati *et al.* 1996). It has been demonstrated that fluoride treated bone tissue is more resistant to resorption than untreated bone (Grynpas and Cheng, 1988). This is more likely to be the consequence of a more stable crystal lattice of fluorapatite as compared to hydroxyapatite than any direct inhibitory effect of F on osteoclasts (Eannes and Reddi, 1979). In isolated systems, doses of NaF up to 500 μM (substantially greater concentrations than are seen with NaF therapy) are without effect on osteoclasts. Higher doses are cytotoxic to both osteoclasts and osteoblast (Bellows *et al.,* 1990). Except at these very high concentrations, osteoclast number, resorption depth, and other histologic features of bone resorption are affected minimally by NaF therapy.

Molecular Mechanisms of Action

The precise mechanism(s) of the bone-specific mitogenic potential of fluoride is still unknown. Several hypotheses have been proposed, but none explains all observations made so far. Fluoride requires the presence of a bone cell growth factors, such as insulin-like growth factor I (IGF-I) or transforming growth factor β (TGF-β) to stimulate bone cell proliferation (Libanati *et al.,* 1996). Therefore, fluoride may be considered as a mitogen enhancer rather than a bone cell mitogen per se. Furthermore, the mitogenic effect of fluoride is dependent on the phosphate concentration of the medium. Finally, there is evidence that fluoride acts more on progenitor cells and preosteoblasts, which produce large amounts of growth factors, with only small effects on mature osteoblasts (Kassem, 1994). Still unexplained also is the cell and tissue specificity of fluoride's mitogenic action. Recent studies indicate that an increased level of tyrosyl phosphorylation of a protein that regulates mitosis (MAP kinase) could be involved (Lau and Baylink, 1998). The most likely explanation for the increased tyrosyl phosphorylation of key mitogenic proteins is the ability of fluoride to inhibit a specific osteoblastic phosphotyrosyl protein phosphatase (PTPP). Interestingly, this growth factor dependent tyrosine phosphorylation pathway of fluoride action is enhanced further in the presence of aluminum (Caverzasio *et al.,* 1996).

Side Effects

No teratogenic effects attributable to fluoride have been observed at therapeutic doses in human subjects. The two main adverse effects during fluoride therapy are gatrointestinal and osteoarticular effects. Gastrointestinal side effects occur more frequently when non-enteric-coated NaF is used. A typical example was provided by the two aforementioned American double-blind studies (Riggs *et al.,* 1990; Kleerekoper *et al.,* 1991) using simple capsules containing plain NaF. In contrast, epigastric symptoms are rarely reported with sustained-release and/or enteric-coated NaF tablets (Pak *et al.,* 1986), and when they do occur they are mild in nature. When MFP is used, it is rare to note gastric side effects. The better gastric tolerance of MFP compared with NaF has also been documented by a gastroscopic study (Müller *et al.,* 1992).

Osteoarticular symptoms mostly affecting the ankles or the calcaneus are referred to as "lower limb pain syndrome" (O'Duffy *et al.,* 1986). Pronounced cases appear on the roentgenogram as low-contrast intraosseous coarsening (Fig. 1) and as hot spots on the scintigram. They tend to correspond to mineralization defects located in the microcallus of trabecular microfractures (Boivin *et al.,* 1991). If treatment is discontinued for 4 to 6 weeks, symptoms abate and radiological findings return to normal. Therapy may then be started again with an initially lower dose.

Calcium Deficiency and Stress Fractures.

Very rapid and large increases in bone formation in response to fluoride may lead to calcium deficiency, undermineralization of newly formed osteoid, and secondary hyperparathyroidism (Duursma *et al.,* 1987; Dure-Smith *et al.,* 1996). The evidence that osteomalacia can occur during fluoride treatment is documented by morphometric studies Libanati *et al.,* 1996). Low urinary calcium excretion developing during fluoride therapy is a marker for calcium deficiency. Fluoride therapy should always be combined with calcium supplements (500–1000 mg/day) and in

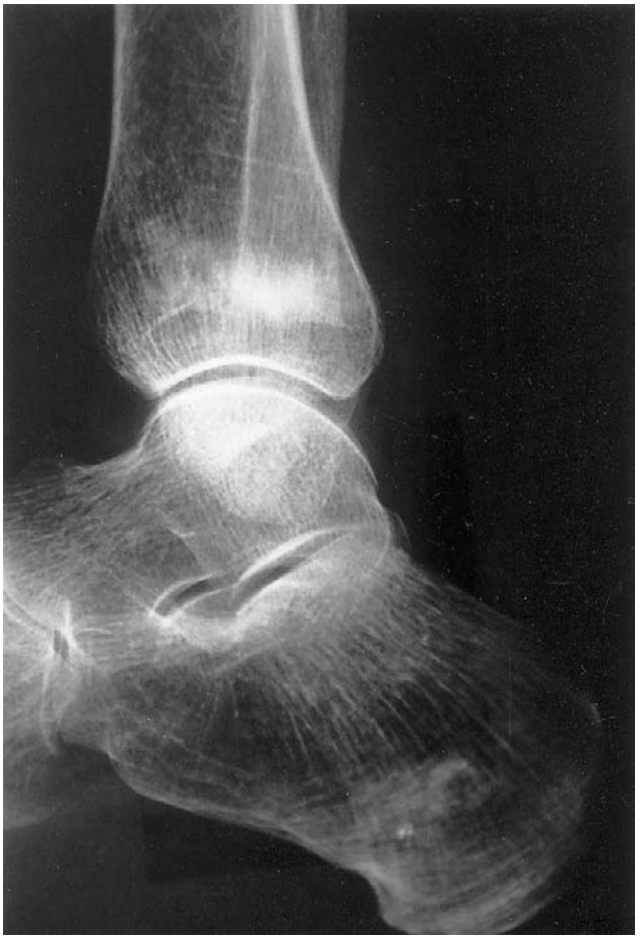

Figure 1 Lateral X-ray of ankle region of 74-year-old patient with postmenopausal osteoporosis with severe "osteoarticular pain" during treatment with MFP/calcium. In the distal tibia a horizontal intraosseous area of coarsening can be identified parallel to the joint surface (lower limb pain syndrome).

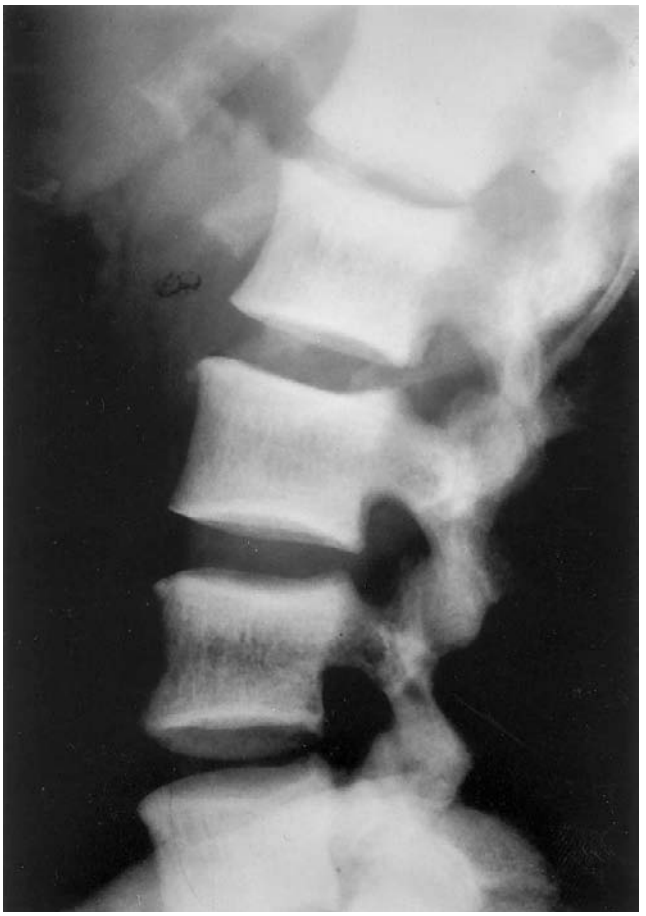

Figure 2 Lateral X-ray of the lumbar spine of a 58-year-old woman. Iatrogenic fluorosis already 3 years after start of fluoride treatment (fluorosis type I, see text).

elderly persons combined also with vitamin D supplements (e.g., 800 IU vitamin D, 1200 mg calcium per day). In some cases, a short-term treatment with an active metabolite (alfacalcidol 1 μg/day) may be indicated to normalize bone mineralization more rapidly.

When calcium deficiency is severe, fluoride-induced osteomalacic stress fractures may occur. Classical Looser zones may be seen radiologically. With adequate calcium/vitamin D, fluoride is unlikely to be associated with osteomalacia. In studies reporting an increased risk of peripheral fractures during fluoride therapy very often no clear distinction was made between true fractures, stress fractures, Looser zones, or subjects with lower limb pain syndrome (Riggs *et al.,* 1990)

Iatrogenic Fluorosis

Iatrogenic fluorosis is not a drug side effect as such but the result of incorrect therapy. We observed two forms. The first type very quickly (often in less than 24 months) produces a distinct sclerosed bone structure in the lateral roentgenogram or the spine. In such cases it has always been found in retrospect that the diagnosis of osteoporosis was based on a poor X-ray film; i.e., that osteoporosis was probably not present at the start of fluoride treatment. An individually strong response to fluoride therapy might be involved or a potentiation of fluoride effect by aluminum (Caverzasio *et al.,* 1996). The second type of iatrogenic fluorosis occurs in patients with established osteoporosis only after excessively long-term and/or high dosage fluoride treatment, i.e., usually after 6–10 years. While in the first type normally shaped vertebrae show a more or less white, ivory-like appearance (Fig. 2), for the second type, coarsening of the previously impressed end plates and a remarkable thickening of the trabeculae are characteristic (Fig. 3).

Prevention of Osteoporosis

Over the past three decades, the classical indication for fluoride was mainly established postmenopausal osteoporosis (Ringe, 1997), with the aim of continuously increasing

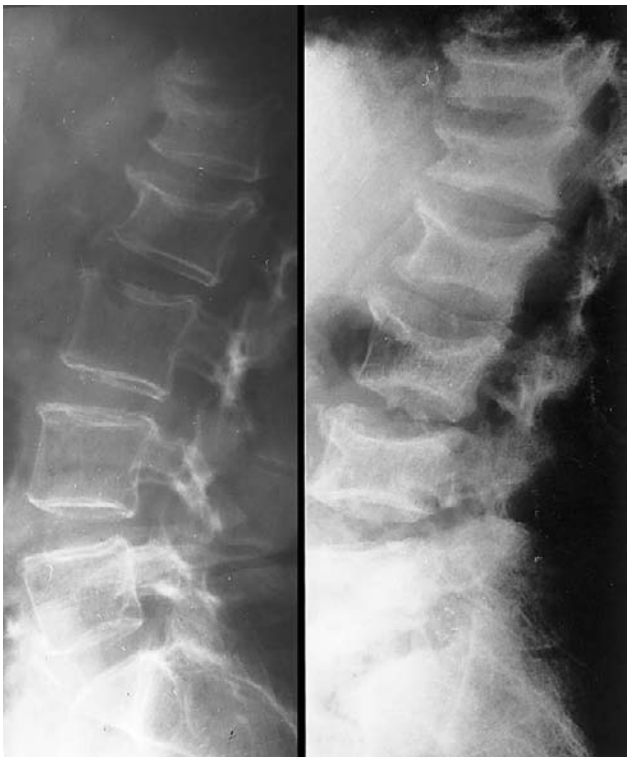

Figure 3 Lateral X-rays of the lumbar spine of a 68-year-old woman with established osteoporosis. (Left) At onset of treatment, fractures at L1 and L2. (Right) After 8 years high-dose fluoride therapy (36 mg fluoride ions per day) iatrogenic fluorosis and new fractures at L3 and L4 (fluorosis type II, see text).

bone mineral density (BMD) at the lumbar spine and thereby decreasing vertebral fracture incidence. Theoretically bone-forming drugs should be able to increase bone mass above the critical fracture threshold in all stages of osteoporosis. It must be taken into consideration, however, that the lower the initial bone mass the more advanced is the microarchitectural deterioration of spongy bone. If an anabolic treatment is only able to increase the diameter of the remaining trabeculae this treatment should start also as early as possible and would be of limited value in the very advanced cases of osteoporosis. On the other hand, in healthy postmenopausal women with mild osteopenia (i.e., rather well-preserved trabecular bone connectivity and still extended endosteal surfaces) an anabolic substance might be extremely effective (Kleerekoper and Mendlovic, 1993; Ringe, 1997). This interesting concept of an early therapy of postmenopausal spinal osteoporosis has never been tested in sufficiently large trials.

Only three small fluoride studies have been performed to prevent osteoporosis, or increase BMD, in early postmenopausal women with moderate osteopenia (Pouilles *et al.,* 1991; Affinito *et al.,* 1993; Sebert *et al.,* 1995). Consistent increases in lumbar spine BMD were recorded, but the studies were too small to evaluate fracture events.

Combinations of fluoride with hormone replacement therapy (HRT) are more effective than fluoride alone. Gambacciani *et al.* (1995) were able to show that a dose as low as 10 mg fluoride ions per day given as MFP was associated with a significant additive effect on lumbar spine BMD in early postmenopausal women in comparison with that seen with HRT alone. Alexandersen *et al.* (1999) studied 100 healthy postmenopausal women treated with four different regimens: HRT, MFP, HRT + MFP, or placebo. In the combined treatment group they found a strong synergistic effect of HRT and MFP and biochemical markers, indicating a dissociation of bone formation and resorption.

Water Fluoridation

Although the benefit of fluoridation of drinking water in the prevention of dental caries has been overwhelmingly substantiated (McDonagh *et al.,* 2000), the effects on BMD and fracture rates turned out inconsistent (Ringe, 1998).

The majority of evidence regarding the effect of fluoride in drinking water on fracture incidence is based on comparisons of fracture rates by geographic regions with different concentrations of fluoride in drinking water either naturally or adjusted.

In 1955, Leone *et al.* observed that radiographic signs of osteoporosis were substantially less severe in people living in Bartlett County, Texas, with a water fluoride content of 8 ppm (ppm = mg/kg) than in Framingham, Massachusetts, with a water fluoride content of 0.09 ppm. This observation led to a study in two communities in North Dakota with different water fluoride levels. Higher BMD by X-ray in 300 subjects living in the high-fluoride area was noted. They also noted a significant reduction in osteoporotic fractures in the lumbar vertebrae in the women in this community. However, there was no difference in the prevalence of these fractures in the men in the two areas (Bernstein *et al.,* 1966).

A rather large number of studies comparing rates of fractures specific for age and sex between fluoridated and nonfluoridated communities have variously found that exposure to fluoride by water increases the risk of hip and other nonvertebral fractures (Danielson *et al.,* 1991; Jacobsen *et al.,* 1990), has no effect on fracture incidence (Madans *et al.,* 1983; Cooper *et al.,* 1990), or decreases the risk of hip fractures (Simonen and Laitinen, 1985; Jacobsen *et al.,* 1993).

In a more recent study from Germany, data from two towns of the former German Democratic Republic could be compared (Lehmann *et al.,* 1998). In the city of Chemnitz drinking water had been fluoridated with 1 ppm (1 mg/liter) for a period of 30 years, while the town of Halle had no fluoridation during the same interval. Calculating age-adjusted incidences, significantly fewer hip fractures occurred in Chemnitz in both men and women (Fig. 4). However, the authors found no significant differences in BMD values in the populations of the two towns and speculated that with 1 ppm fluoride no anabolic blood level of fluoride can be reached. The lower fracture rate, however, could be

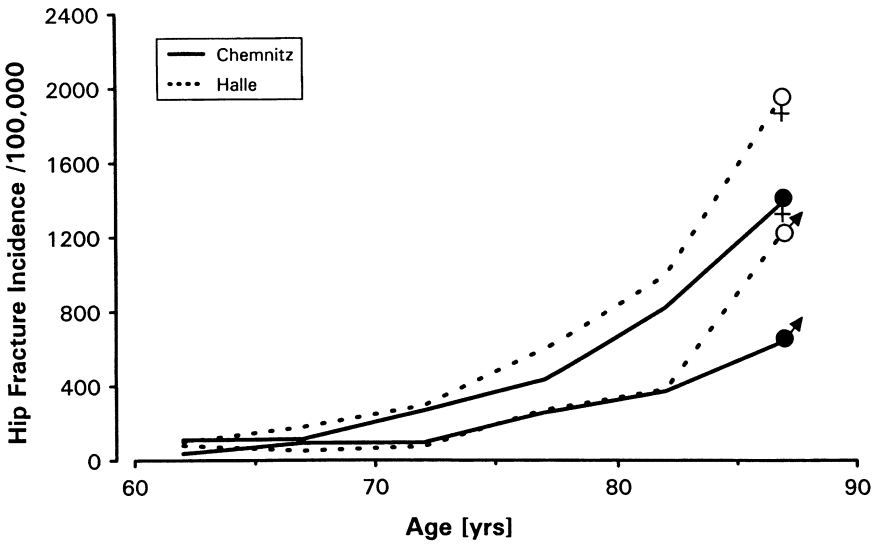

Figure 4 Annual incidences of hip fractures given in 5-year groups for men and women in the towns of Chemnitz with water fluoridation, and Halle without fluoridation (solid line, Chemnitz; dotted lines, Halle). (According to Lehmann *et al.,* 1998.)

explained by a higher content of less soluble fluorapatite in the skeletons of the population of Chemnitz, i.e., a long-lasting lowered rate of bone resorption (Allolio and Lehmann, 1999). Very recently a large prospective multicenter study on 9704 elderly women was published (Phipps *et al.,* 2000). The aim of this study was to determine whether, on an individual level, older women with long-term exposure to fluoridated water had different BMD and fracture rates compared with women with no exposure. The women, all ambulatory and without bilateral hip replacement, had been enrolled during 1986–1988. Of the total, 7129 could provide information on exposure to fluoride for each year from 1950 to 1994. The BMD results of this study are given in Table II. In women with continuous exposure (group C) BMD was 2.5% higher at the lumbar spine, 2.6% higher at the femoral neck, and 1.9% lower at the distal radius ($P < 0.001$ for all three sites). Women in group C had a significantly reduced risk of hip fractures (RR, 0.69; 95% confidence interval, 0.50 to 0.96; $P = 0.028$). The relative risk for vertebral fractures was reduced to 0.73 (confidence interval, 0.55–0.97; $P=0.033$). There was no significant effect on fracture incidence in women with intermittent "mixed exposure" (Table III).

These findings may have enormous importance for public health. It is the first prospective study with adequate power to examine the risk of specific fractures associated with fluoride on an individual rather than community basis. The study shows that long-term exposure to fluoridated water will lead to a significant reduction in the incidence of hip and vertebral fractures in elderly women. The authors conclude that fluoridation may be one of the most cost effective methods for reducing the incidence of fractures related to osteoporosis (Phipps *et al.,* 2000).

Table II Multivariate Adjusted Bone Mineral Density (g/cm^2) by Exposure to Fluoride in Drinking Water 1971–1990 (Phipps *et al.,* 2000)

Category	A No exposure	B Mixed exposure	C Continuous exposure
Patients (*n*)	2563	1348	3218
Mean age (years)	74,5	74,2	73,9
Lumbar spine	0.849	0.853	0.871*
Fem. neck	0.647	0.652	0.664*
Distal radius	0.371	0.362	0.364*

*P value A vs C. 0.001.

Treatment of Established Postmenopausal Osteoporosis

Earlier Clinical Trials

There is a large body of radiologic and densitometric evidence that long-term fluoride treatment increases bone mass mainly at bone sites with a high proportion of trabecular

Table III Reduced Risk of Incident Fractures According to Exposure to Fluoride in Drinking Water (Phipps *et al.,* 2000)

	Mixed exposure	Continuous exposure
Spine	$P = 0.096$	$P = 0.033$
Hip	$P = 0.126$	$P = 0.028$
Wrist	$P = 0.628$	$P = 0.051$

bone (Ringe *et al.,* 1978; Eriksen *et al.,* 1988; Kleerekoper and Mendlovic, 1993).

The first controlled study showing a positive effect of 50 mg/day NaF on vertebral fracture incidence was published by a French group in 1988 (Mamelle *et al.,* 1988). These findings were supported by a large retrospective American study showing a correlation between increase in vertebral mineral density and decrease in fracture rate (Farley *et al.,* 1992). In 1990–1991, however, two placebo-controlled studies reported a discrepancy between gain in BMD and fracture rates (Riggs *et al.,* 1990; Kleerekoper *et al.,* 1991). In the very often cited study of Riggs *et al.* (1990), 75 mg/day of plain NaF had been given over 4 years. The gain of bone density at the spine was 36% at the end of the study (= 9% increase per annum). There was also a small increase at the proximal femur but a significant loss at the radius although 1500 mg calcium was supplemented daily. The reason was obviously the very high fluoride dose corresponding to approximately 34 mg bioavailable fluoride ions per day. Doubts concerning fluoride therapy were fueled by this widely publicized study and the other study with similar design and results (Kleerekoper *et al.,* 1991), because the rate of vertebral fractures after 4 years was not significantly lower in the fluoride–calcium group than in the placebo–calcium group.

An extension and new analysis of the Riggs study, however, published 4 years later, showed a reduction of the vertebral fracture rate with lower fluoride doses; i.e., it was conceded that the original dosage of fluoride was too high (Riggs *et al.,* 1994).

More Recent Studies

Several important studies have appeared since 1994, demonstrating positive effects of a long-term fluoride therapy on fracture incidence in postmenopausal osteoporosis (Pak *et al.,* 1994, 1995; Farrerons *et al.,* 1997; Reginster *et al.,* 1998). These studies were performed with NaF or MFP at different doses but for all the range of daily applicated bioavailable fluoride ions was between 10 and 20 mg.

The 4-year study of Reginster *et al.* (1998) was a randomized, double-blind, controlled trial in 200 cases of postmenopausal osteoporosis (initial lumbar spine BMD <-2.5 *T* score). There was a progressive but moderate increase at the spine reaching 10% after 4 years. The rate of new vertebral fractures after 4 years was significantly lower for the MFP group with 2.4% than in the calcium group with 10%. Obviously a slow and moderate increase of BMD appeared to be better than a rapid and high gain in BMD (e.g., 36% after 4 years in the Riggs study (1990)).

Despite these very positive results, the discussion of the definitive clinical value of fluoride treatment is not at its end. In a randomized controlled French study of 2 years duration with four different treatment arms, no significant effect of fluoride on fracture rate could be documented in comparison to vitamin D/calcium supplementation (Meunier *et al.,* 1998). On the other hand, a prospective controlled study from our group performed on 134 women with established postmenopausal osteoporosis showed a dose-dependent fracture-reducing potency (Ringe *et al.,* 1999).

In this three-arm study, all patients received 1000 mg calcium per day and group A additionally a low-dose intermittent fluoride schedule (114 mg MFP = 15 mg fluoride ions, 3 months on, 1 month off), group B a higher continuous fluoride dosage (152 mg MFP = 20 mg fluoride ions), and group C no fluoride. Table IV shows the yearly and total incidences of new vertebral fractures for the three groups. Although the annual increase in lumbar spine BMD was lower in group A than in group B (4.2% versus 6.5%) the lowest fracture rate was found in group A. The statistical analysis proves significantly lower fracture rates for both fluoride groups as compared to calcium alone, but no significant difference between the two fluoride regimens (Ringe *et al.,* 1999). Furthermore the incidence of the lower limb pain syndrom was significantly lower in group A with lower dose intermittent application of fluoride. Intermittent fluoride therapy had previously found significant advantages of intermittent administration by histomorphometric analysis (Schnitzler *et al.,* 1997).

Altogether today much more positive results are documented in the literature than negative experiences. A meta-analysis, however, is difficult because of the aforementioned differences in the fluoride salts used, preparations, and

Table IV Effects of Two Different Fluoride/calcium Regimens or Calcium Alone on the Incidence of New Vertebral Fractures (Ringe *et al.,* 1999)

	Group A (MFP/Ca intermittent)	Group B (MFP/Ca continuous)	Group C (Ca only)
After 1 year	4/4 (10.5)	10/6 (16.2)	9/9 (21.9)
After 2 years	4/4 (11.4)	6/6 (18.7)	16/14 (35.9)
After 3 years	1/1 (3.1)	1/1 (3.3)	11/11 (32.4)
Within 3 years	9/8	17/12	36/30
Patient-years	105	100	114
New fractures per 100 patient-years	8.6	17.0	31.6

Note. Values are number of fractures/number of patients (%).

dosages in the different studies. As an additional explanation for discrepant study results, Kleerekoper (1998) suggests that the trials have included patients with different severity of disease and that there may be a point in the bone loss spectrum at which even a potent bone-stimulating agent such as fluoride is ineffective.

Effect on Cortical Bone

Hodsman and Droost reported that patients who did not respond to NaF with an increase in spinal bone mass had no change in forearm BMD, while those who did increase spinal bone mass lost bone from the forearm at the remarkable rate of 7.7% per year (Hodsman and Droost, 1989). Similarly, Christiansen *et al.* (1980) reported a 3.6% annual rate of loss of forearm bone mass in their fluoride-treated patients, but this was not different from the 3.3% annual rate of loss in their placebo-treated patients. In this preventive trial comparing fluoride and HRT the fluoride dose of 20 mg NaF per day (= 9.1 mg fluoride ions) was obviously below the therapeutically effective threshold of 10 mg fluoride ions per day. Most earlier studies had reported no significant changes in forarm BMD during fluoride therapy. In our own first study from 1978 there was even an increase at the radius shaft and distal radius (Ringe *et al.,* 1978). In the two placebo-controlled American studies the one of Riggs *et al.* (1990) reported a decrease at the radius while the one of Kleerekoper *et al.* (1991) found no effect, although the same fluoride regimen was used in both studies. Those who report an increased loss of appendicular (cortical) bone in fluoride-treated patients have postulated that the increase in spinal bone mass occurs at the expense of the appendicular skeleton (Hodsman and Droost, 1989; Riggs *et al.,* 1990). They have postulated further that this phenomenon may explain the alleged increased occurrence of appendicular fractures in fluoride-treated patients. There are several reasons to question this hypothesis. The appendicular fractures that have been attributed to fluoride therapy are predominantly incomplete stress fractures of the lower extremities. These fractures (see Side Effects above) are felt not to result from accelerated bone loss but from excess osteoid accumulation.

Taken together we conclude that the effect of fluoride on cortical bone may be in part dose dependent. With high fluoride dosage and very rapid increase of trabecular bone formation, slight decreases in cortical bone may occur, especially in patients with simultaneous calcium/vitamin D insufficiency. Important advantages of the low dose fluoride therapy are a continuous moderate increase of trabecular bone and no decreases or even slight increases of cortical bone (Peichl *et al.,* 1995; Ringe *et al.,* 1999).

Hip Fractures

An increased rate of hip fractures during fluoride therapy was reported in 1984 and 1990 by Gutterigde *et al.,* and again by Hedlund and Gallagher in 1989. These studies were confounded because most individual cases showed significant problems contributing to an increased hip fracture risk (e.g., advanced age, frequent falls, renal insufficiency, osteomalcia). Especially renal insufficiency may be a risk if the fluoride dosage is not adjusted.

A multicenter retrospective analysis undertaken by Riggs *et al.* (1987) evaluated more than 1000 patient years on fluoride treatment and did not confirm an augmented risk for hip fractures. In all larger controlled studies that appeared since that analysis, again no increased rate of hip fractures could be documented (Ringe, 1998). In summary there is no evidence at this time that fluoride increases or decreases the incidence of hip fractures.

Fluoride in Male and Corticoid-Induced Osteoporosis

Bone mineral density results from individual male subjects or small male cohorts in mixed patient groups suggested that the anabolic effect of fluoride is similar in men and women (Ringe, 1997). There is only one larger prospective controlled study which included only male patients (Ringe *et al.,* 1998). In that study of 64 men with primary osteoporosis without fractures at onset we used a rather low fluoride dose giving 114 mg/day MFP (=15 mg fluoride ions) intermittently 3 months on, 1 month off plus 1000 mg/day calcium continuously. We found a moderate but highly significant increase of lumbar spine BMD, amounting to 9% after 3 years and even small increases at the radius shaft and femoral neck (Fig. 5). In comparison to the controls this treatment was able to reduce significantly the rate of vertebral fractures over 3 years; i.e., fluoride treatment with the adopted schedule was able to reduce the rate of patients developing established osteoporosis. The moderate increase of 3% per year at the lumbar spine is consistent with the results of Reginster *et al.* (1998) in postmenopausal osteoporosis.

The therapeutic potential of fluoride salts in glucocorticoid-induced osteoporosis on bone density has been shown in different studies (Meys *et al.,* 1993; Rizzoli *et al.,* 1995; Guaydier-Souquieres *et al.,* 1996; Lippuner *et al.,* 1996) but fracture data are not available. In these studies again mixed female/male patient groups were evaluated.

Fluoride in Combined Treatment Regimens

A combined treatment with an anabolic and antiresorptive drug may have additive effects on BMD and could improve microarchitectural quality of the newly formed bone. In an early study from the Mayo Clinic with five different treatment groups the one with NaF + HRT + calcium + vitamin D was the most effective in terms of reducing the vertebral fracture rate (Riggs *et al.,* 1982). As mentioned above (see prevention) fluoride given together with HRT led to very encouraging results (Gambacciani

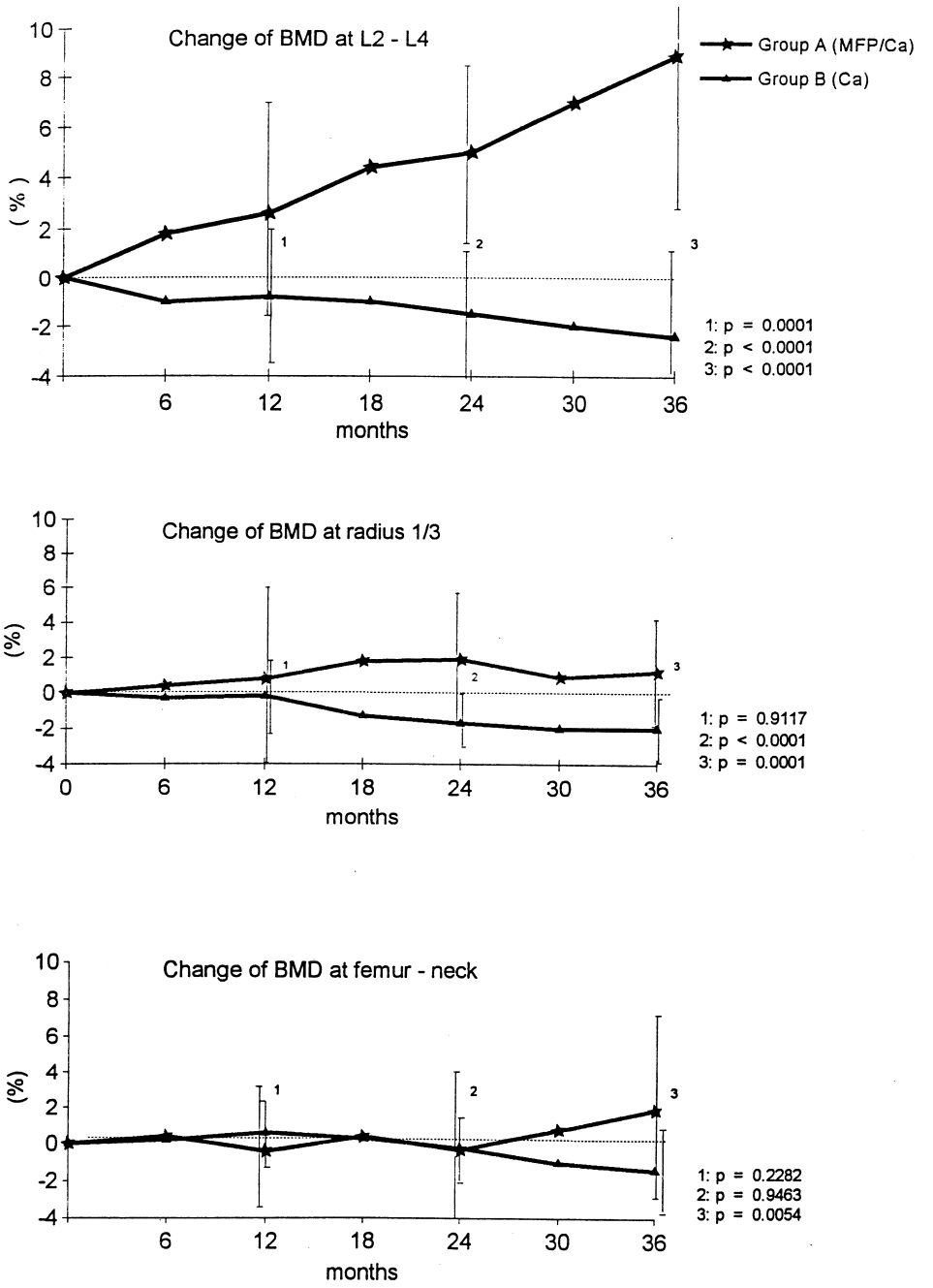

Figure 5 Percentage BMD changes from baseline in osteoporotic men treated for 3 years with low-dose intermittent fluoride plus calcium or calcium alone (Ringe *et al.,* 1998).

et al., 1995; Alexandersen *et al.,* 1999). Our results from a trial in 60 women with established postmenopausal osteoporosis using a low dose intermittent MFP-regimen plus HRT confirm these very positive results (Ringe *et al.,* 1999b). A multicenter trial studying the combination fluoride/raloxifen will soon be finished.

The combination fluoride/etidronate was studied for the first time by Lems *et al.* (1997) in 47 patients with corticoid-induced osteoporosis. After 2 years lumbar spine BMD increased 9.3% in the etidronate/fluoride patients and only 0.3% in the etidronate monotherapy group. A pilot study included 35 postmenopausal women with advanced osteoporosis: average initial lumbar spine BMD, −3.9 *T* score; average number of vertebral fractures, 7 per patient (unpublished results). Patients were treated cyclically either with 14 days 400 mg etidronate followed by 76 days with 20 mg MFP plus 1200 mg calcium and 800 IU vitamin D (EFCaD group) or the same without fluoride (ECaD group). Table V shows the average BMD changes at the lumbar spine, total hip, and femoral neck after 12 and 24 months of therapy for both groups. At all sites the EFCaD group shows significantly higher increase rates than the ECaD

Table V Average Percentage Changes of BMD at the Lumbar Spine, Total Hip, and Femoral Neck after 12 and 24 Months of Therapy in 35 Postmenopausal Women with Severe Osteoporosis Treated Either with Etidronate/Calcium/Vitamin D (ECaD) or Etidronate/Fluoride/Calcium/Vitamin D (EFCaD) (own Unpublished Results)

	ECaD $n = 17$			EFCaD $n = 18$		
Months	L2–L4	Total hip	Femur neck	L2–L4	Total hip	Femur neck
12	1.6	1.5	0.3	10.2	5.1	3.6
24	2.6	1.7	0.6	14.5	5.6	3.4

group. In this context the study of Guanabens *et al.* (2000) on 118 patients with severe postmenopausal osteoporosis is of great interest. The authors directly compared the therapeutic effects of 50 mg enteric-coated NaF with the classical intermittent etidronate/calcium regimen. At 36 months the mean change from baseline of the lumbar spine bone density in the fluoride group was 8.5% and in the etidronate group 3.6% ($P < 0.01$). The total number of new vertebral fractures was significantly lower in the fluoride group but the difference in the number of patients with new fractures did not reach statistical significance.

Another ongoing trial from our group studied the combination fluoride/alendronate in men and women again in selected severe cases of osteoporosis. The results so far from combined fluoride/bisphosphonate therapies are very encouraging in terms of BMD effects. To prove the superiority of fluoride/bisphosphonate versus fluoride or bisphosphonate alone a large study with fractures as the primary end point is needed. Histological examinations at least in a subgroup would be of high interest.

Persisting Problems and Future Perspectives of Fluoride Therapy

It must be emphasized that the existing therapeutic data on fluoride treatment of osteoporosis do not reach the level of evidence-based medicine. As already mentioned, the big problem with fluoride is the low price and the lack of protection by a patent. That means that very large studies with enough power to assess definitely the therapeutic efficacy will probably never be performed. A metaanalytic approach looking at all randomized controlled studies may come to wrong conclusions due to considerable differences in dosage and bioavailability of fluoride between studies depending on used fluoride salts and the respective galenic preparations. Accordingly one recent analysis on 11 trials published between 1980 and 1998 including older studies with high dose fluoride (35 mg ions per day) and newer trials with lower doses (15–20 mg ions per day) concluded that fluoride increases lumbar spine BMD but does not reduce the incidence of vertebral fractures (Haguenauer *et al.,* 2000). Furthermore, is was stated that increasing the dose of fluoride will increase the risk of nonvertebral fractures and gastrointestinal side effects without any effect on the vertebral fracture rate, i.e., a possible advantage of lower fluoride doses can be anticipated. A metaanalysis including only studies with low fluoride dosage published between 1994 and 2000 will come to positive effects on lumbar spine BMD and vertebral fracture rate (unpublished results). Obviously the selection of included papers is crucial in every metaanalytic approach.

As long as no other potent osteoanabolic substances are available fluoride should be kept within the choice of therapeutics for osteoporosis. Accepting a certain therapeutic value of a low dose and well controlled fluoride therapy, the low price may be an important advantage, especially in countries with limited resources for the health care system. In some European countries and especially in Germany, where fluoride was approved for treatment of osteoporosis more than 20 years ago, several groups have long-time favorable clinical experience with fluoride and there is accumulated evidence that with enterocoated NaF or with retarded preparations of NaF or MFP using lower doses than in the past (between 10 and 20 mg bioavailable fluoride ions per day), fluoride therapy is rather safe and may lead to a significant decrease in vertebral fractures.

With doses below 10 mg per day no significant effects on BMD can be expected (Christiansen *et al.,* 1980; Grardel, 1995). The increase of BMD at the spine should be only moderate, between 4 and 6% per year (Riggs *et al.,* 1994). With the recommended dosage the risk of hip fractures is not increased (Ringe, 1998). Some studies show even moderate positive effects on cortical bone. Taking into account the recommendations of Table VI the therapeutic results with fluoride can be improved. The most important point is to reach a stimulation of osteoblasts with the lowest possible

Table VI Strategies for Improving Fluoride Treatment and Practical Recommendations

1. Avoid calcium deficiency and secondary hyperparathyroidism (calcium and vitamin D supplementation or vitamin D-hormone)
2. Avoid high fluoride content of bone:
   - —use lowest fluoride dose that stimulates osteoblasts (serum fluoride ride 5–10 μM?)
   - —intermittent fluoride regimen?
   - —slow-release fluoride? (no plasma peaks after ingestion)
3. Combination with antiresorptive drugs (e.g., HRT, SERMs, BP)
4. Control treatment carefully and stop fluoride application after 3 to 4 years
5. Adapt fluoride dosage or stop it in renal insufficiency
6. Reduce fluoride dosage if alkaline phosphatase increases more than 50% of pretreatment value
7. Monitoring of serum fluoride and/or bone fluoride content?

dose and thereby to keep the fluoride content of bone tissue at a low level. Furthermore there is increasing evidence that the combination of fluoride with calcium and vitamin D is very important to improve safety and therapeutic results (Dure-Smith *et al.,* 1996; Orcel and Sebert, 1997; Ringe, 1997). Future studies with fluoride should also focus on early stages of postmenopausal, corticoid-induced or male osteoporosis. It can be assumed that the better preserved the connectivity of trabecular bone is, the better the therapeutic results could be with an osteoanabolic treatment. Another interesting aspect for the future of fluoride remains the possibility of combining this potent anabolic substance with one of the different existing antiresorptive drugs.

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CHAPTER 80

The Pharmacology of Estrogens in Osteoporosis

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Introduction

Control of bone remodeling is intimately linked to estrogen status in the human female and perhaps also in the human male. Consequently, loss of estrogen production by the ovary, which occurs in all women at menopause, is associated with increased activation of remodeling and an alteration in the equilibrium between bone resorption and formation (Lindsay, 1995). The consequence is loss of bone tissue with accompanying microarchitectural damage that leads to increased bone fragility, commonly called osteoporosis (Consensus Development Conference, 1993). Estrogen intervention restores bone remodeling to its premenopausal equilibrium and prevents bone loss. Observational and some clinical trial data suggest that estrogens also reduce the risk of fractures (Weiss *et al.,* 1979; Hutchinsen *et al.,* 1979; Paganini *et al.,* 1981; Kreiger *et al.,* 1982; Kiel *et al.,* 1987; Lindsay, 1995; Lindsay, 1980; Komulainen, 1998; Herrington, 2000), the only clinical consequence of osteoporosis. The precise mechanism by which estrogens control bone remodeling is still obscure, although significant advances in our understanding have been made in recent years. In this chapter, we will review the current status of our knowledge of the actions of sex steroids in both prevention and treatment of osteoporosis in postmenopausal women.

Responses to Estrogen Administration

Biochemistry

When estrogens are administered to estrogen-deficient women, there is a reduction in bone remodeling that can be monitored biochemically (Siebel *et al.,* 1993). During the initial weeks of therapy, there is a gradual decline in biochemical resorption such that of as the pyridinoline cross-linking molecules (Delmas, 1995). There is a similar but more delayed decline in biochemical measures of bone formation, such as osteocalcin and bone-specific alkaline phosphatase. The timing of these biochemical changes is presumed to represent an initial rapid reduction in activation of new remodeling sites. Since resorption is the initial event in remodeling, a decline in the rate at which new remodeling sites are coming "on-line," initiated at the start of treatment, would produce a reduced supply of the products of resorption into the circulation. Formation follows resorption and is a more prolonged process. Consequently, formation would be expected to continue unabated in those remodeling sites activated before treatment until the osteoblast teams complete their work. Thus, markers of formation in serum decline more gradually (Delmas, 1995). These modifications in skeletal remodeling may occur in part through direct effects of estrogens on bone cells and indirectly by increased resistance to the bone-resorbing effects of parathyroid hormone. Accompanying these changes in skeletal metabolism are modifications in calcium homeostasis, including reduced renal excretion of calcium, improved hydroxylation of 25(OH)D to $1,25(OH)_2D$, and improved calcium absorption across the intestine. In some studies, declining remodeling within the skeleton is also associated with a small fall in serum calcium and a rise in PTH which might affect the renal and intestinal calcium handling.

Bone Density

The consequence of these alterations in skeletal homeostasis is preservation of bone mass, which has been detailed in

many controlled studies (Lindsay, *et al.,* 1976, 1978, 1980, 1984; Horsmann *et al.,* 1977; Christiansen and Rodbro, 1983). Most studies in which estrogens are given during active bone loss provide evidence of a gradual increase in measured bone mass or density that occurs primarily during the first 1–2 years of treatment. It has been assumed that this phenomenon results from the reduced activation of remodeling, which would mean that at any time point after initiation of therapy, there would be a smaller proportion of the skeleton undergoing the remodeling process. The consequence would be an apparent gradual increase in bone mass, without the necessity of an anabolic effect of estrogen. This increment would be time dependent during the early months of treatment as completing remodeling sites gradually filled with new bone tissue, and bone mass would be expected to be relatively stable thereafter. (Delmas, 1995). Indeed, all agents that act by reducing bone remodeling produce the same effect during the first 1–2 years of treatment. The magnitude of the effects vary dependent upon the site measured (the spine usually produces greater increases) and the magnitude of the reduction in remodeling (bisphosphonates and estrogen are most potent).

In long-term studies with estrogens, there are somewhat divergent results, possibly due to a variety of issues. First, it is clear that the long-term compliance with estrogen is less than perfect, and consequently bone loss, which is known to restart when treatment is stopped, might be expected to be seen among some individuals who are not compliant with treatment (Lindsay *et al.,* 1978; Christiansen and Christiansen, 1981). Second, the response of the skeleton may be variable and dependent upon the bone measured. For example, the vertebral bodies contain the highest proportion of cancellous bone and produce the greatest initial response to estrogen. Some data suggest that there is very little subsequent loss in the vertebrae, while the hip may be more sensitive to other factors, especially physical activity (Lindsay, 1995; Orwoll *et al.,* 1996). Thus, although initial cross-sectional data suggested very little loss of bone from any skeletal sites, more recent longitudinal data has confirmed some slow loss (~0.5% per year) from the hip in patients who have been on long-term estrogen (<5 years).

When estrogen therapy is discontinued, there is an immediate increase in bone remodeling that is reminiscent of ovariectomy (Lindsay *et al.,* 1978; Christiansen and Christiansen, 1981). The consequence of this is an increase in the rate of bone loss (Lindsay *et al.,* 1978; Greenspan *et al.,* 1999). The longer the process of bone loss continues the more the treated population will begin to resemble an untreated population in terms of bone density. More than 20 years ago we demonstrated that the effects of 4 years of estrogen intervention were virtually lost 4 years after estrogens had been discontinued (Lindsay *et al.,* 1978).

Effects on Fracture

The consequence of reduced bone turnover and prevention of bone loss is a reduction in the risk of fracture. Numerous epidemiological studies have shown that estrogen exposure is associated with a reduction in the risk of fractures of the hip and wrist (Lindsay *et al.,* 1976, 1978a,b; 1980a; 1984; Horsman *et al.,* 1977; Christiansen and Christiansen, 1981; Christiansen and Rodbro, 1983). In general, there appears to be about a 50% reduction in hip fracture risk. More recent data indicate that the estrogen effect is greatest among those currently taking treatment, and those who began earliest (Cauley *et al.,* 1995). These epidemiological data are compatible with the prospective effects on bone mass and emphasize the importance of continued therapy to obtain the maximum effects on fracture. Since menopause occurs at the average age of 50 years, and hip fracture affects women over 70 years mostly, this may mean 20–30 years of treatment or even treatment for life may be necessary. Controlled clinical trial data suggest a reduction of 50–80% in the risk of vertebral fractures, when estrogens are used for prevention (Lindsay *et al.,* 1980a), or treatment (Lufkin, 1992). One recent clinical trial has confirmed the effects of HRT on clinical nonvertebral fractures in a recently menopausal population (Komulainen 1998). However, in one study of HRT's effects on cardiovascular disease, the largest outcome study completed as yet using HRT, there was an equal number of fractures in the HRT and placebo arms, casting doubt on fracture efficacy (Hulley *et al.,* 1998). It is important to emphasize that that study recruited women based upon cardiac history without regard to skeletal status, which was largely unknown. Moreover, there was a high incidence of obesity in that population which might have reduced the likelihood of a significant estrogen effect on fractures. There was no comprehensive evaluation of vertebral compression fracture, since spine radiographs were not routinely performed. However there did not appear to be a reduction in height loss. In another smaller study in which cardiac outcomes were also assessed by cardiac catherization, while there was no effect on atheroma, there was a reduction in the number of clinical fractures. Thus, despite one negative study, most data support a fracture benefit for estrogens.

Route of Administration

The evidence suggests that the route of estrogen administration required to obtain a skeletal effect is irrelevant and that if adequate doses are given, reduced bone remodeling results (Christiansen and Lindsay, 1991). Thus, data obtained from transdermal, percutaneous, subcutaneous, as well as oral administration all confirm skeletal conservation. In contrast, vaginal estrogen administration does not appear to result in high enough estrogen levels in circulation to exert an effect on skeletal remodeling. For oral doses the equivalent of 0.625 mg of conjugated estrogen is sufficient for most individuals, but effects on bone remodeling and skeletal mass can be seen at lower doses (Lindsay *et al.,* 1984). Whether fractures can be reduced by lower doses is not known. For transdermal estrogens, the 50-mg patch, which results in estradiol levels in circulation of ~50 pg/ml, appears sufficient (Lufkin *et al.,* 1992). Some data suggest that higher doses of estrogen produce greater effects on bone mass, but no head-to-head comparisons with lower doses have been attempted (Savvas *et al.,* 1992).

Presently, data demonstrate that all estrogens used in the postmenopausal population produce skeletal effects. For some, such as conjugated equine estrogens, the data include dose–response and fracture outcomes. For others, more limited data on bone mass are available, often without good dose–response data. Synthetic estrogens such as those used in the oral contraceptive also produce skeletal effects in estrogen-deficient individuals (Lindsay *et al.,* 1976). Indeed ethinyl-estradiol in doses between 5 and 10 μg/day prevents bone loss. In addition at the 5 μg dose there appears to be a modest increase when norethindrone (1 mg/day) is combined with the estrogen. However, whether the oral contraceptive, with doses of ethinyl-estradiol between 20 and 30 μg per day, produces much in the way of alterations in skeletal homeostasis in premenopausal women is far from known.

Reduction in skeletal remodeling is an effect that can be produced with other steroids. Progestins given to postmenopausal women also produce reductions in bone remodeling and prevention of bone loss (Lindsay *et al.,* 1978). The required doses are greater than those normally used in estrogen-deficient women (20 mg/day for medroxyprogesterone acetate and 5 mg/day for norethindrone) (Lindsay *et al.,* 1978; Gallagher and Kable, 1991). The addition of a C-21 progestin in more conventional doses to estrogen therapy, does not modify the estrogen response significantly (PEPI). As noted in the previous paragraph there are some data suggesting an additive effect of the 19-nortestosterone derivative norethindrone when given in combintaion with 5 μg of ethinyl-estradiol (Speroff, 1996). Whether this occurs with other combinations is not known and there are no fracture data with combinations to determine if there is an additive effect on fractures. On the other hand, some evidence suggests that the progestins, when used by themselves for contraceptive purposes in premenopausal estrogen-replete women, will actually be detrimental to skeletal health, presumably by reducing endogenous production of estrogen by the ovary (Mark, 1994). There is no evidence that progestin creams can result in high enough circulating levels of progesterone to produce skeletal effects.

The activity of progestins on bone is also evident in the data published on tibolone (Lindsay *et al.,* 1980; Milner *et al.,* 2000; Castelo-Branco, 2000). This steroid is related to norethindrone and thus is a 19-nortestosterone derivative. It has androgenic, progestogenic, and weak estrogenic activity. In doses that do not appear to stimulate the endometrium, it produces estrogen-like effects in bone. Thus, tibolone can be considered the first tissue-selective compound, although its tissue spectrum of activity produces some effects related to its androgenicity and progestin activity rather than its estrogen action.

Estrogens are potent hormones with effects on multiple tissues. Some of these effects confer significant benefit and some significant risk to the postmenopausal women. One clear-cut benefit of hormone replacement therapy (HRT) is on suppression of menopausal symptoms, such as hot flashes and vaginal dryness. Many of the other potential benefits of HRT, however, have not been proven. It is unclear whether estrogens affect the risk of cardiovascular disease. A large body of clinical trial data indicates that estrogen improves intermediate markers of cardiovascular health, including cholesterol and other lipoproteins, and the vasodilatory coronary artery response to acetylcholine. Observational data suggest a clinical benefit as well, however two separate clinical trials (mentioned above) showed a lack of efficacy against clinical heart disease outcomes (HERS) (Hulley *et al.,* 1998) and a lack of regression of coronary artery plaque (Herrington *et al.,* 2000), in those women assigned to estrogen or HRT. There are insufficient clinical trial data to connect HRT to cognitive function, dementia, and urogenital symptoms such as incontinence. HRT increases the risk of venous thromboembolic disease threefold and the risk of gallbladder disease twofold.

Estrogens also cause endometrial hyperplasia and increase the risk of endometrial malignancy (Jick *et al.,* 1979; Shapiro *et al.,* 1985). Protection of the endometrium is the only rationale for the prescription of a progestin along with estrogen to postmenopausal women (Woodruff and Pickar, 1994). There is also concern that long-term therapy with estrogen may increase the risk of breast cancer, although the epidemiological data available are by no means consistent on this point (Barrett-Connor, 1989). In general, however, it appears as though long-term estrogen intervention ($<$10–15 years) produces a modest increase in breast cancer risk, particularly in thin women. From the few epidemiological data available, the point estimate of relative risk appears to be between 1.15 and 1.40 and is probably related to dose as well as duration. This raises concern when long-term therapy for prevention of hip fracture is considered.

Consequently there has been considerable interest in the idea that estrogen analogs might be found that would provide the beneficial effects of estrogen on bone, brain, and heart, without endometrial or breast stimulation. Since there appears to be a single estrogen receptor, it is at first glance difficult to believe that separation of estrogen effects by tissue might be possible. However, with improved understanding of the molecular biology of estrogen action, it has become possible to hypothesize how estrogens might act differently in different tissues.

Estrogen Actions

The molecular actions of estrogens have been reviewed in detail elsewhere in this book. Estrogen receptors are members of the steroid receptor family that include thyroid hormone, vitamin D, and retinoids among their ligands as well as sex steroids and adrenal steroids (Auchus and Fugue, 1994). In cells that express estrogen receptors, estrogens produce a potential cascade of gene activation that is dependent on a variety of intracellular factors. Generally responsive cells contain 10,000–100,000 receptors, and clearly the concentration of receptors is one variable that might affect response (in general lower levels appear in nonreproductive cells) (Brown, 1994). The receptor–ligand complex binds to DNA with high affinity that is dependent on the integrity of the amino acid sequence related to the

base of the first zinc finger on the C region of the receptor (Picard *et al.,* 1990). Ligand binding alters the conformation of the receptor, exposing the DNA binding domain. In addition, while ligand binding is usual for dimerization, DNA binding and subsequent transcription activity, the ER can activate genes in the absence of ligand. Other transcription activators may also modulate the responsivity of the genome to ER even in the presence of ligand (Ronies and Spelsberg, 1989; Brown, 1994).

Given the complexity of the system, it is not difficult to suggest how estrogen responsivity might be modulated. Abnormal receptors, or abnormal conformational changes in the receptor upon ligand binding, the absence of cofactors, or the alteration in the response elements of target genes all might modify the cellular responses to estrogen (Auchus and Fugue, 1994). Tissue selectivity of estrogen response could be obtained by differences in receptor number or the expression of factors within the cells of that tissue influencing the cascade, for example, the capability to phosphorylate the ER ligand complex.

Several genes are targets for estrogen actions including nuclear proto-oncogenes (c-*fos,* c-*myc,* and c-*jun*), and the estrogen responses include expression of several genes including growth factors and cytokines (Murphy and Murphy, 1994).

The estrogen target that is responsible for the effects of these agents in bone is still not clearly understood. Osteoblasts have been shown to have estrogen receptors (Eriksen *et al.,* 1988; Komm *et al.,* 1988), but very variable responses to estrogen in osteoblasts or osteoblast cell lines have been published (Turner *et al.,* 1994). Indeed some evidence suggests that only cells transfected with a functional estrogen receptor will respond to estrogens (Ernst *et al.,* 1991). Some data suggest that osteoclasts also respond directly to estrogens (Oursler *et al.,* 1991). The strongest evidence of estrogen receptors in osteoclasts comes from studies of avian osteoclasts. Rat osteoclasts probably do not respond to estrogens, and there is almost no information on human osteoclasts (Arnett *et al.,* 1996). The most likely cell targets for estrogen appear to be other constituent cells of bone marrow. Release of second messengers, such as TGF-β, or reduction in secretion of cytokines, such as IL-1 or IL-6, could produce estrogen effects by reducing the recruitment and maturation of osteoclasts (Oursler *et al.,* 1991; Turner *et al.,* 1994). Estrogen has also been reported to cause osteoclast apoptosis, although not in all systems (Arnett *et al.,* 1996). Finally, estrogens also have effects on calcium homeostasis. Estrogens reduce urinary calcium loss and, at the renal level, cause an increase PTH activity. Estrogens, however, increase skeletal resistance to the resorptive stimulatory effects of PTH without appearing to interfere with the anabolic effects of PTH (Cosman *et al.,* 1993). Estrogens also stimulate intestinal absorption of calcium, perhaps directly or by modulating the effects of PTH on the renal 1α-hydroxylase, thereby increasing the supply of 1,25-dihydroxyvitamin D. Whether these effects of estrogen on the calcium homeostatic system are primary or secondary to the skeletal effects of estrogens is not clear.

The knowledge that estrogen responsivity may vary from tissue to tissue has clearly heightened interest in the concept of the ideal estrogen. Such a compound would produce the beneficial effects of estrogen on bone, cardiovascular system, brain, and urogenital tissues without stimulating endometrial growth or producing effects on mammary tissue that might increase the risk of breast cancer. Several compounds are under investigation for such activities. The first noted is tibolone, which clearly produces bone effects at doses that do not appear to produce much endometrial stimulation. It is not at clear, however, whether tibolone can reduce the risk of ischemic heart disease. Since the effects of estrogen on the cardiovascular system probably include multiple effects on lipid metabolism and on the physiology of the circulatory system, further investigation of this compound would seem to be warranted, even though it does not produce an estrogen-like response in terms of circulating lipoproteins. The realization that tamoxifen exerted estrogen-like effects in some cell systems stimulated evaluation of its effects in women who were being given tamoxifen as adjuvant treatment for breast cancer (Cosman and Lindsay, 1999). Surprisingly these women were not losing bone when compared with postmenopausal controls. Formal controlled clinical trials confirmed that tamoxifen does appear to conserve bone mass and produce estrogen-like effects on lipoprotein metabolism (Cosman and Lindsay, 1999). Tamoxifen also stimulates the endometrium, albeit weakly, and endometrial cancer is a recognized risk of long-term tamoxifen treatment. Knowledge of these effects awakened interest in the pharmaceutical industry in compounds that might have tamoxifen-like effects on breast cancer but would produce estrogen-like effects on bone and cardiovascular diseases. Raloxifene, a benzothiaphene, is now available for prevention and treatment of osteoporosis. Raloxifene and tamoxifen produce similar estrogen-like effects on bone in ovariectomized rats (Black *et al.,* 1994). However, raloxifene appears to have little stimulatory effects on rat uterus. Raloxifene reduces bone remodeling and prevents bone loss in postmenopausal women. Raloxifene reduces the risk of vertebral fractures, but did not have a statistically significant effect on nonvertebral fractures (Ettinger *et al.,* 1999). The use of raloxifene in clinical practice is strongly influenced by its capacity to reduce the clinical appearance of ER-positive breast cancer over a 4-year period. Raloxifene also lowers circulating low-density lipoprotein similar to estrogen, although no increase in high-density lipoprotein was seen as is usually the case with oral estrogens (Draper *et al.,* 1996). Raloxifene increases the risk of deep vein thrombosis and pulmonary embolism to the same degree as HRT. Clearly compounds such as raloxifene will be powerful tools in the dissection of the mechanisms of estrogen action at the molecular level. The realization that different cells respond to estrogen-like molecules in different ways also raises the intriguing question of whether compounds can be found that reduce the

recruitment and activity of osteoclasts, like estrogen, and produce lesser effects on bone formation. Tamoxifen, for example, appears to have more potent effects on bone resorption *in vitro* than estrogens, perhaps by being a more potent stimulator of osteoclast apoptosis. Clomiphene also increases trabecular thickness in rats, an effect not usually associated with estrogens.

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CHAPTER 81

Vitamin D and Analogs

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Introduction

Vitamin D, its metabolites and analogs constitute a valuable group of compounds which can be used to modulate many aspects of osteoblast and osteoclast biology. The parent vitamin (or UV light which substitutes for any vitamin D pharmaceutical preparation as a source of the parent vitamin) has been used as a treatment for rickets and osteomalacia since its discovery in the 1920s. The first analog of vitamin D, dihydrotachysterol, was developed in the 1930s before the elucidation of the metabolism of vitamin D. In fact, it was not until the discovery of the principal metabolites: 25-hydroxyvitamin D_3 (25-OH-D_3) and 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$), in the early 1970s that further generations of vitamin D analogs were developed (DeLuca, 1988; Jones and Calverley, 1993). With the understanding of the molecular action of the hormonal form, 1α,25-$(OH)_2D_3$, has come an appreciation that it is not only a *calcemic agent,* regulating calcium and phosphate transport but also a *cell-differentiating agent,* promoting the terminal development of a number of cell types including the osteoclast, the enterocyte and keratinocyte (Miyaura *et al.,* 1981). Thus, pharmaceutical companies have striven hard in the 1980s and 1990s to separate these two properties and thereby develop synthetic vitamin D analogs with specialized "calcemic" and "noncalcemic" (cell-differentiating) uses (Calverley and Jones, 1992; Bouillon *et al.,* 1995). From this type of research has come several "low-calcemic" agents in recent years in the form of calcipotriol, OCT, 19-nor-1α,25-$(OH)_2D_2$, and 1α-OH-D_2, which have found widespread use in dermatology and hyperparathyroidism. Other analogs are under development for use in metabolic bone diseases, osteoporosis, and cancer (Jones *et al.,* 1998). This chapter will review the spectrum of compounds available, possible uses of these compounds and their potential mechanisms of action.

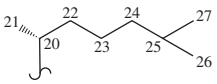
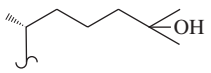
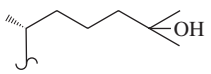
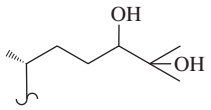
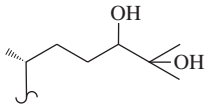
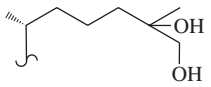
Pharmacologically Important Vitamin D Compounds

Vitamin D compounds can be subdivided into three major groups, listed in Tables I through III and described below.

Vitamin D and Its Natural Metabolites

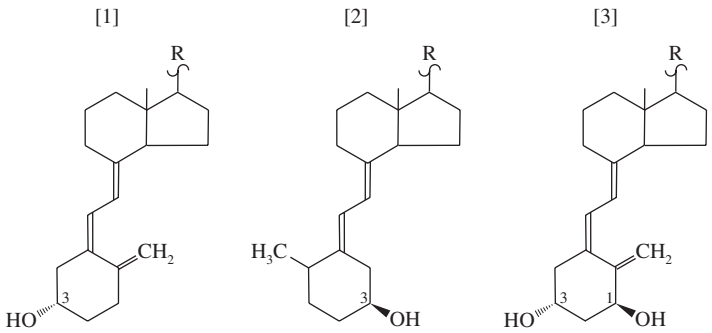
Table I lists some of the important metabolites of vitamin D_3. During the early 1970s most of these compounds were first isolated and identified by GC-MS and then their exact stereochemical structure determined (DeLuca, 1988). This led to chemical synthesis of the naturally occurring isomer and testing in various biological assays *in vitro* and *in vivo.* Currently, only the compounds representing the main pathway of vitamin D activation, namely vitamin D_3, 25-hydroxyvitamin D_3 (25-OH-D_3), and 1α,25-$(OH)_2D_3$, are synthesized for use as drugs. One exception to this is 24(*R*),25-$(OH)_2D_3$, which was synthesized in Israel by Teva Pharmaceuticals and sold as Secalciferol.

Table I Vitamin D and Its Natural Metabolites

Vitamin D metabolites [ring structure][a]	Side chain structure (R)	Site of synthesis	Relative VDR-binding affinity[b]	Relative DBP-binding affinity[c]	Reference
Vitamin D_3[1]		Skin	≤0.001	3,180	—
25-OH-D_3[1]		Liver	0.1	66,800	Blunt *et al.,* 1968
1,25-$(OH)_2D_3$[3]		Kidney	100	100	Fraser and Kodicek 1970; Holick *et al.,* 1971.
24(*R*),25-$(OH)_2D_3$[1]		Kidney	0.02	33,900	Holick *et al.,* 1972
1,24(*R*),25-$(OH)_3D_3$ [3]		Target tissues[d]	10	21	Holick *et al.,* 1973
25(*S*),26-$(OH)_2D_3$[1]		Liver?	0.02	26,800	Suda *et al.,* 1970

[a] Structure of the vitamin D nucleus (secosterol ring structure).
[b] Values reproduced from previously published data (Stern, 1981).
[c] Values reproduced from previously published data (Bishop *et al.,* 1994).
[d] Known target tissues included intestine, bone, kidney, skin, and the parathyroid gland.

Vitamin D Nucleus

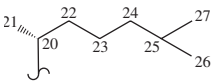
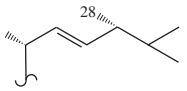
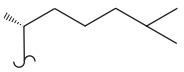
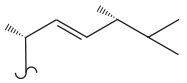


Vitamin D Prodrugs

Table II lists some of the important prodrugs of vitamin D. All of these compounds require a step (or more) of activation *in vivo* before they are biologically active. Included here is vitamin D_2 which is derived from the fungal sterol, ergosterol, by irradiation. Since vitamin D_2 is found rarely in nature and is hard to detect in humans eating nonfortified foods, we can consider it to be an artificial form of vitamin D or prodrug. Vitamin D_2 possesses two specific modifications of the side chain (see Table II) but is still able to undergo the same series of activation steps as vitamin D_3, giving rise to 25-OH-D_2, 1α,25-$(OH)_2D_2$, and 24,25-$(OH)_2D_2$. Two other prodrugs, 1α-OH-D_3 and 1α-OH-D_2, were synthesized in the early 1970s (Barton *et al.,* 1973; Paaren *et al.,* 1978) as alternative sources of 1α,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_2$, respectively, that in the process circumvent the renal 1α-hydroxylase enzyme, which was shown to be tightly regulated and prone to damage in renal disease.

The final compound in the list, dihydrotachysterol (DHT) has lived a complex history as a prodrug. Originally it was believed to be "active" when converted to 25-OH-DHT by virtue of an A-ring rotated 180° such that the 3β-hydroxyl function assumes a pseudo-1α-hydroxyl position (Jones *et al.,* 1988). The mechanism of action of DHT has become less clear with the description of the extrarenal metabolism of 25-OH-DHT to 1α,25-$(OH)_2$-DHT and 1β,25-$(OH)_2$-DHT, two further metabolites which have greater biological activity than either 25-OH-DHT or DHT itself (Qaw *et al.,* 1993).

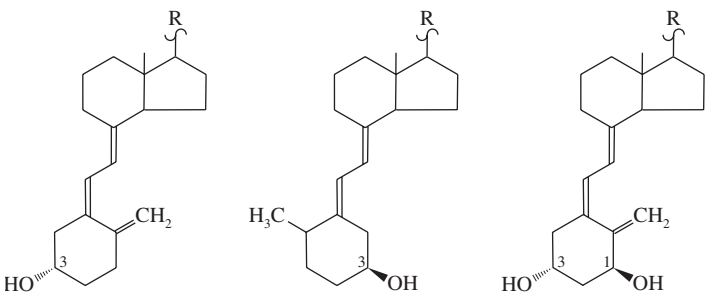
Table II Vitamin D Prodrugs

Vitamin D prodrugs [ring structure][a]	Side chain structure (R)	Company	Possible target diseases	Mode of delivery	Reference
1α-OH-D_3[3]		Leo	Osteoporosis	Systemic	Barton *et al.,* 1973
1α-OH-D_2[3]		Bone care	Hyperparathyroidism	Systemic	Paaren *et al.,* 1978
Dihydrotachysterol [2]		Duphar	Renal failure	Systemic	Jones *et al.,* 1988
Vitamin D_2[1]		Various	Rickets Osteomalacia	Systemic Systemic	Fraser *et al.,* 1973

[a] Structure of the vitamin D nucleus (secosterol ring structure).

Vitamin D Nucleus

[1] [2] [3]



Vitamin D Analogs

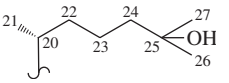
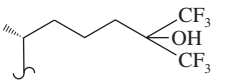
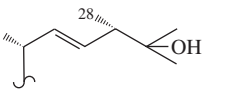
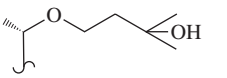
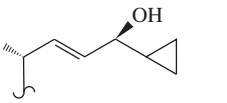
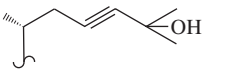
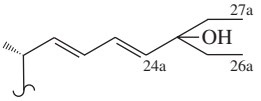
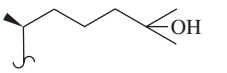
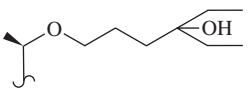
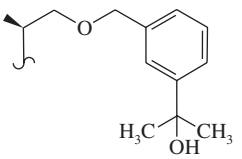
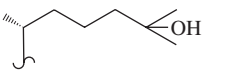
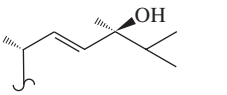
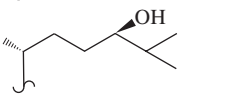
Table III lists some of the most promising vitamin D analogs of 1α,25-$(OH)_2D_3$ already approved by governmental agencies or currently under development by various industrial/or university research groups. Since the number of vitamin D analogs synthesized now lists in the hundreds, the table is provided mainly to give a flavor of the structures experimented with thus far, the worldwide nature of the companies involved and the broad spectrum of target diseases and uses.

The first generation of calcitriol analogs included molecules with fluorine atoms placed at metabolically vulnerable positions in the side chain and resulted in highly stable and potent "calcemic" agents such as 26,27-F_6-1α,25-$(OH)_2D_3$. More recently, attention has focused on features which make the molecule more susceptible to clearance, such as in calcipotriol (MC903), where a C22=C23 double bond, a 24-hydroxyl function, and a cyclopropane ring have been introduced into the side chain or in 22-oxacalcitriol (OCT) where the 22-carbon has been replaced with an oxygen atom. Both modifications have given rise to highly promising analogs (Kragballe, 1992; Abe-Hashimoto *et al.,* 1993).

The C-24 position is a favorite site for modification and numerous analogs contain 24-hydroxyl groups, e.g., 1α, 24(*S*)-$(OH)_2D_2$ and 1α,24(*R*)-$(OH)_2D_3$ (Strugnell *et al.,* 1995). Other analogs contain multiple changes in the side chain in combination including: unsaturation; 20-epimerisation, 22-oxa replacement; homologation in the side chain or terminal methyl groups. The resultant molecules such as EB1089 and KH1060 are attracting strong attention of researchers because of greatly increased potencies *in vitro* and are being pursued as possible anti-cancer and immunomodulatory compounds, respectively. KH1650 is a potential second-generation anti-psoriatic.

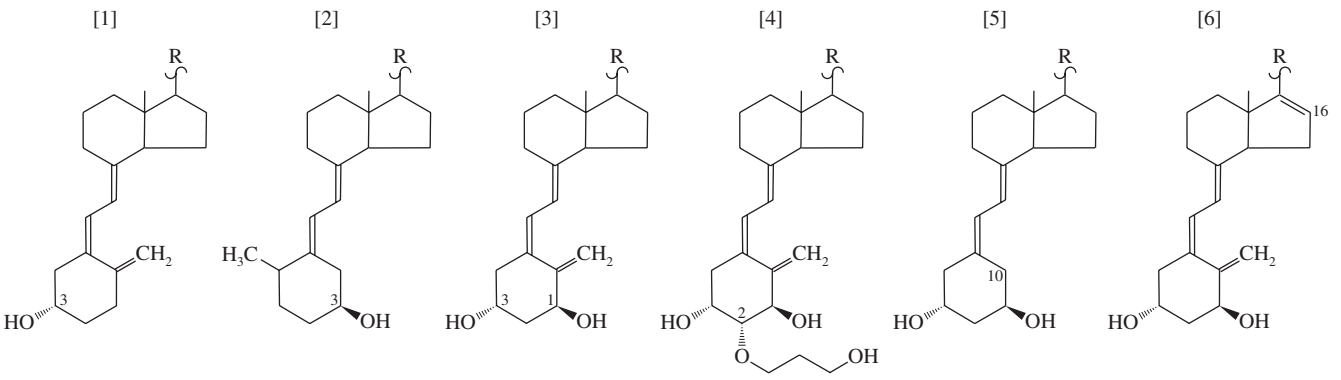
Few attempts have been made thus far to modify the nucleus of calcitriol. The Roche compound 1α,25-$(OH)_2$-16-ene-23-yne-D_3 touted as an anti-tumor compound *in vivo* possesses a D-ring double bond. Declercq and Bouillon have introduced a series of biologically active analogs without the C/D rings but with a rigid backbone to maintain the spatial arrangement of the A-ring hydroxyl groups and the side chain (Verstuyf *et al.,* 2000). Relatively recently, the A-ring substituted 2-hydroxypropoxy-derivative, ED71, has been tested as an anti-osteoporosis drug. The Abbott compound, 19-nor-1α,25-$(OH)_2D_2$, lacks a 19-methylene group and is styled upon the *in vivo* active metabolite, 1α,25-$(OH)_2$

Table III Analogs of 1,25-$(OH)_2D_3$

Vitamin D analog [ring structure][a]	Side chain structure (R)	Company	Possible target diseases	Mode of delivery	Reference
1α,25-$(OH)_2D_3$[3]		Roche Duphar	Hypocalcemia psoriasis	Systemic Topical	Baggiolini *et al.,* 1982
26,27-F_6-1α,25-$(OH)_2D_3$[3]		Sumitomo–Taisho	Osteoporosis hypoparathyroidism	Systemic Systemic	Kobayashi *et al.,* 1982
19-nor-1α,25-$(OH)_2D_3$[5]		Abbott	Hyperparathyroidism	Systemic	Perlman *et al.,* 1990
22-Oxacalcitriol (OCT)[3]		Chugai	Hyperparathyroidism psoriasis	Systemic Topical	Murayama *et al.,* 1986
Calcipotriol (MC903)[3]		Leo	Psorisis Cancer	Topical Topical	Calverley, 1987
1α,25-$(OH)_2$-16-ene-23-yne-D_3 (Ro 23–7553) [6]		Roche	Leukemia	Systemic	Baggiolini *et al.,* 1989
EB1089 [3]		Leo	Breast cancer	Systemic	Binderup *et al.,* 1991
20-epi-1α,25-$(OH)_2D_3$[3]		Leo	Immune Diseases	Systemic	Calverley *et al.,* 1991
KH1060[3]		Leo	Immune diseases	Systemic	Hansen *et al.,* 1991
KH1650[3]		Leo	Psoriasis	Topical	Jones *et al.,* 2000
ED71[4]		Chugai	Osteoporosis	Systemic	Nishii *et al.,* 1993
1α,24(*S*)-$(OH)_2D_2$[3]		Bone care	Psoriasis	Topical	Strugnell *et al.,* 1995a
1α,24(*R*)-$(OH)_2D_3$ (TV-02) [3]		Teijin	Psoriasis	Topical	Morisaki *et al.,* 1975

[a] Structure of the vitamin D nucleus (secosterol ring structure).

Vitamin D Nucleus



DHT_2, formed from dihydrotachysterol, which retains biological activity though the C-19 methylene is replaced by a C-19 methyl. Many other compounds have been developed with rigid or altered *cis*-triene structures (Okamura *et al.,* 1995) or modifications of the 1α- ,3β- , or 25-hydroxyl functions not for the purpose of developing active molecules for use as drugs but in order to allow us to establish minimal requirements for biological activity in structure/activity studies (Calverley and Jones,1992; Bouillon *et al.,* 1995). One series of compounds not depicted in Table III are the substituted biphenyls developed by Ligand, representing totally synthetic scaffolds selected by high-throughput screening, which show weak VDR-binding but good transactivation through VDRE-driven, vitamin D-dependent genes and produce hypercalcemia *in vivo* (Boehm *et al.,* 1999). Accordingly, this is the first class of vitamin D mimics that lack the conventional *cis*-triene secosteroid structure while maintaining the spatial separation of the A-ring and side-chain hydroxyl functions needed to bind to certain residues of the ligand-binding pocket of the VDR.

Clinical Applications of Vitamin D Compounds

The clinical usefulness of vitamin D analogs has been reviewed comprehensively by both Bikle (1992) and Bouillon *et al.* (1995) in overviews and also within this book. This chapter summarizes some of the highlights in this area.

Rickets and Osteomalacia

When the nutritional basis of rickets and osteomalacia became apparent in the first half of the 20th century, vitamin D (particularly vitamin D_2 because it was less expensive) became the treatment of choice for these diseases. Of course, prophylactic vitamin D in the form of supplements to milk, margarine, and bread have replaced much of the need for therapeutic vitamin D. In fact, since then rickets has become very uncommon in North America because vitamin D supplementation is required by law, whereas it was quite common before the practice of food fortification and it is still more prevalent in the world where food fortification is not permitted.

On the other hand, vitamin D deficiency is still quite prevalent in the elderly and usually is treated with modest doses of vitamin D (Chapuy *et al.,* 1992). In fact in recent years, several world, continent-wide, and national food agencies have put out new guidelines raising the recommendations for vitamin D intake for all age groups, but particularly for those in the postmenopausal category, to try to ensure adequate intakes irrespective of geographical, dietary, and sun exposure differences (US National Academy of Sciences Reference Intakes, 1997; FAO/WHO Nutritional Guidelines, 2000). However, the need for the use of expensive pharmaceutical preparations containing calcitriol or its analogs is usually not warranted.

Osteoporosis

While the etiology of this disease is complex and likely to be multifactorial (Seeman *et al.,* 1995; Nordin, 1997), there have been consistent claims that levels of 1α,25-$(OH)_2D_3$ are low in osteoporosis (Riggs and Melton,1992). In addition, the recent debate over VDR genotypes correlating with bone mineral density (Morrison *et al.,* 1994; Whitfield *et al.,* 2000) suggests some genetically inherited vitamin D-related basis exists for susceptibility to osteoporosis. As a consequence it is not surprising that clinical trials of 1α-OH-D_3 (Orimo *et al.,* 1987), 1α-OH-D_2 (Gallagher *et al.,* 1994), and 1α,25-$(OH)_2D_3$ (Gallagher *et al.,* 1989; Ott and Chestnut, 1989; Tilyard *et al.,* 1992) have been undertaken. Modest gains in bone mineral density and reductions in fracture rates are reported in many of these studies, and this subject has been reviewed recently by Seeman *et al.* (1995).

With the demonstration that ovariectomy results in enhanced interleukin-6 (1L-6) and interleukin-11 (1L-11)-mediated osteoclast recruitment and increased bone resorption has come a clearer understanding of the molecular processes underlying postmenopausal osteoporosis (Manolagas and Jilka, 1995). These theories focusing on osteoblast/osteoclast communication have been extended with the discovery of osteoprotegerin and its ligand (also known as RANKL) and how agents such as 1α,25-$(OH)_2D_3$ can influence osteoclastogenesis and bone resorption (Aubin and Bonnelye, 2000). Although 1α,25-$(OH)_2D_3$ treatment might be expected to exacerbate the excessive bone resorptive component of osteoporosis, the vitamin D hormone also raises plasma Ca^{2+} levels and stimulates synthesis of bone matrix formation in osteoblasts. In fact Raisz and coworkers (Hock *et al.,* 1986) have shown that pharmacological doses of 1α,25-$(OH)_2D_3$ administered to rats, in great excess over the doses used in osteoporosis, result in hypercalcemia and nephrocalcinosis which is accompanied by a hyperosteoid or undermineralized condition in the long bones. Thus, the use and effectiveness of vitamin D metabolites are controversial. Nevertheless, the experience seems to have been that beneficial effects can be observed and bone loss reduced, but at the expense of occasional hypercalcemia. In North America, where dietary Ca intakes and absorption are higher, this has led to intolerable side effects and the use of 1α,25-$(OH)_2D_3$ and 1α-OH-D_3 for the treatment of osteoporosis. As a result some pharmaceutical companies have sought to synthesize "milder" but "longer-lived" calcitriol analogs for use in osteoporosis. ED-71 represents such an analog which by virtue of an A-ring substituent at C-2 and tighter-binding affinity to DBP has a longer $t_{1/2}$ in the plasma (Nishii *et al.,* 1993). ED-71 has performed well at restoring bone mass without causing hypercalcemia in long-term studies involving ovariectomized rats (Okano *et al.,* 1991).

Renal Osteodystrophy

Renal failure is accompanied by hypocalcemia and secondary hyperparathyroidism, both of which are relieved by the administration of a 1α-hydroxylated vitamin D

preparations. Both oral 1α-OH-D_3 and 1α,25-$(OH)_2D_3$ raise plasma Ca^{2+} concentrations and, in addition, lower PTH levels partly by raising [Ca^{2+}] and shutting off PTH secretion and partly by direct action on the PTH gene promoter. Slatopolsky and colleagues (Delmez *et al.,* 1989) showed that intravenous infusion of "active" vitamin D preparations results in a more effective suppression of plasma PTH levels without such a profound increase in plasma [Ca^{2+}]. Subsequent work has employed "low-calcemic" vitamin D analogs such as OCT or 19-nor-1α,25-$(OH)_2D_2$ as substitutes for the more calcemic natural hormone (Brown *et al.,* 1989; Slatopolsky *et al.,* 1995). The FDA recently approved both oral and intravenous 1α-OH-D_2 (trade name Hectorol) for the treatment of secondary hyperparathyroidism. In clinical trials, 1α-OH-D_2 effectively suppressed PTH in renal failure patients with very low incidences of hypercalcemia and hyperphosphatemia (Frazao *et al.,* 2000).

Psoriasis and Cancer

The demonstration that 1α,25-$(OH)_2D_3$ is an anti-proliferative, pro-differentiating agent for certain cell types *in vivo* and many cell lines *in vitro* suggested that vitamin D analogs might offer some relief in hyperproliferative disorders such as psoriasis and cancer. Early psoriasis trials with 1α,25-$(OH)_2D_3$ were moderately successful but plagued with hypercalcemic side effects. Modifications to the protocol included:

(a) administration of the calcitriol overnight when intestinal concentrations of [Ca^{2+}] were low
(b) substitution of "low-calcemic" analogs for the calcitriol.

According to Holick (1995), oral calcitriol is an effective treatment for psoriasis when administered using an overnight protocol. However, by far the most popular treatment for psoriasis is the topical administration of the "low-calcemic" analog calcipotriol, formulated as an ointment (Kragballe, 1992). When given orally, calcipotriol is ineffective due to the fact that it is rapidly broken down (Binderup and Bramm, 1988). When given topically as an ointment, calcipotriol survives long enough to cause improvement in over 75% of patients (Kragballe *et al.,* 1991). Both 1α,25-$(OH)_2D_3$ and calcipotriol are effective in psoriasis because they block hyperproliferation of keratinocytes, increase differentiation of keratinocytes and help suppress local inflammatory factors through their immunomodulatory properties. Calcipotriol is now approved worldwide for use in psoriasis. The success of calcipotriol has spawned development of second-generation analogs.

Several vitamin D analogs have been tried with some degree of success in various types of cancer. In mice inoculated with fulminant leukemia, moderate leukemia, or slowly progressive leukemia, the Roche compound 1α,25-$(OH)_2$-16-ene-23-yne-D_3 administered at 1.6 μg/qod was significantly more effective than 0.1 μg/qod 1α,25-$(OH)_2D_3$ at increasing survival time even though the 1α,25-$(OH)_2D_3$-treated group developed mild hypercalcemia and the analog-treated animals remained normocalcemic (Zhou *et al.,* 1990). With the analog EB1089, the promising anti-proliferative effects seen *in vitro* and in the NMU-induced mammary-tumor model have been extended into the clinic (Colston *et al.,* 1992). Early trials in limited numbers of breast cancer patients have been followed up with more extensive ongoing Phase II and Phase III clinical trials in a number of different cancers (Gulliford *et al.,* 1998; Evans *et al.,* 2000). The results remain promising. Following similarly promising results in Phase I clinical trials, another analog 1α-OH-D_2, is currently undergoing Phase II trials for the treatment of advanced prostate cancer.

The principal problem in anti-cancer studies involving orally administered vitamin D compounds is hypercalcemia. Though the newer analogs appear to be less calcemic than calcitriol itself, they still retain some ability to raise serum calcium; they are not "noncalcemic" as is sometimes claimed. It remains uncertain whether we will ever develop a vitamin D compound devoid of calcemic activity while retaining sufficient anti-proliferative activity to be valuable in cancer.

Immunosuppression

The immunosuppressive properties of 1α,25-$(OH)_2D_3$ and its analogs have been the subject of two excellent reviews (Bouillon *et al.,* 1995; Van Etten, 2000). 1α,25-$(OH)_2D_3$ or its analogs have been shown to be of benefit in autoimmune diseases (Lemire and Clay, 1991), to suppress the onset of Type I diabetes in NOD mice (Mathieu *et al.,* 1995), and to work synergistically with cyclosporine to provide immunosuppression in transplantation medicine (Mathieu *et al.,* 1994a). This latter development has led to some optimism that coadministration of a vitamin D analog with cyclosporine can reduce the dosage of the latter drug and minimize the serious side effects associated with its use. Several studies (Mathieu *et al.,* 1995, 1994b; Veyron *et al.,* 1993) have focused on the immunosuppressive effects of Leo drugs, KH1060 and 20-epi-1α,25-$(OH)_2D_3$, both of which contain the 20-*S* side-chain configuration. Nevertheless, it remains unclear if drugs that show strong promise in certain immunological screening tests *in vitro* will prove to be effective immunomodulators *in vivo.*

Criteria That Influence Pharmacological Effects of Vitamin D Compounds

Activating Enzymes

It has been shown using *in vitro* models that some vitamin D compounds lacking 1α-hydroxylation (e.g., 24(*R*),25-$(OH)_2D_3$) are capable of interacting with the vitamin D receptors (VDRs) and transactivating reporter genes but this occurs only at high concentrations of ligand (Uchida *et al.,* 1994). It seems unlikely that these concentrations will be reached *in vivo* except in hypervitaminosis D. Most of the compounds described in Tables I and II lack vitamin D

biological activity *unless* they are activated *in vivo.* This is particularly the case for the parent vitamin D_3 itself, for its main circulating form 25-OH-D_3 or for any of the prodrugs listed in Table II. Vitamins D_2 and D_3 depend upon both the liver 25-hydroxylase and kidney 1α-hydroxylase enzyme systems in order to be activated, whereas most prodrugs require only a single step of activation. Indeed, the 1α-OH-D drugs were designed to overcome the tightly regulated 1α-hydroxylase step which is easily damaged in chronic renal failure. In essence, prodrugs depend on the weakly regulated 25-hydroxylase step in the liver for activation. In recent years, the cytochrome P450 thought to be responsible for 25-hydroxylation of vitamin D_3, CYP27A, has been cloned and shown to be a bifunctional polypeptide that executes both activation of vitamin D_3 and the 27-hydroxylation of cholesterol during bile acid biosynthesis (Okuda *et al.,* 1995). However, the CYP27A enzyme has a relatively low affinity for vitamin D, does not 25-hydroxylate vitamin D_2, and when mutated results in cerebrotendinous xanthomatosis not rickets. Consequently, another "physiologically relevant" 25-hydroxylase may exist. However, it is clear that CYP27A efficiently 25-hydroxylates 1α-OH-D_3 to give 1α,25-$(OH)_2D_3$ (Guo *et al.,* 1993); and is present in a variety of tissues as well as the liver (e.g., kidney and bone). In fact studies (Ichikawa *et al.,* 1995; Jones *et al.,* 1999a) suggest that cultured bone cells and even keratinocytes are able to synthesize 1α,25-$(OH)_2D_3$ from 1α-OH-D_3. If these findings can be extrapolated to the *in vivo* situation, the implications of this work are that vitamin D target cells may have some ability to synthesize the active form from a prodrug *without* the need for the hormone to enter the bloodstream.

The ability of extrarenal tissues to 1α-hydroxylate various 25-hydroxylated metabolites and analogs has always been a controversial story. However, it is widely believed that 1α-hydroxylase activity is associated with cells of the myelomonocytic lineage in certain granulomatous conditions (e.g., sarcoidosis) (Adams and Gacad, 1985). Thus 25-OH-D can be converted to 1α,25-$(OH)_2D_3$ in sarcoid patients, a step that is not subject to tight regulation by the renal 1α-hydroxylase enzyme and thus potentially likely to result in hypercalcemia. Exposure of such patients to sunlight or administration of 25-OH-D results in excessive levels of 1α,25-$(OH)_2D_3$. The recent cloning of the cytochrome P450 representing the1α-hydroxylase (officially named CYP27B) (St. Arnaud *et al.,* 1997; Takeyama *et al.,* 1997) has been followed by confirmation that the new cytochrome can be expressed extrarenally in skin and lung cancer cells (Fu *et al.,* 1997; Jones *et al.,* 1999b), but there is currently little information for why the tight regulation of the 1α-hydroxylase is lost in certain clinical situations such as sarcoidosis.

Most of the calcitriol analogs listed in Table III are thought to be active as such, not requiring any step of activation prior to their action on the transcriptional machinery or in nongenomic pathways. It remains a theoretical possibility though that the biological activity of one of these parent analogs could be altered by enzyme systems *in vivo,* either by the generation of a more potent metabolite or by giving rise to a less active but more long-lived catabolite.

Vitamin D-Binding Protein

The vitamin D-binding protein (DBP) serves several functions including providing transport for a lipid-soluble vitamin D analog. Most of the analogs of calcitriol, designed to date, contain modifications to the side chain and this is detrimental to binding to DBP. Several analogs, for example calcipotriol or OCT, have very weak affinities for DBP, reduced by two to three orders of magnitude relative to 1α,25-$(OH)_2D_3$. This property has important implications for metabolic clearance rates, delivery to target cells and tissue distribution (Bouillon *et al.,*1991; Kissmeyer *et al.,* 1995). Detailed studies with one analog, OCT, have shown it to bind primarily to β-lipoprotein and exhibit an abnormal tissue distribution *in vivo,* with abnormally high concentrations in the parathyroid gland (Tsugawa *et al.,* 1991). It was thus proposed that this unusual distribution may make OCT a useful systemically administered drug in the treatment of hyperparathyroidism.

Another vitamin D analog with a modified side chain is 20-epi-1α,25-$(OH)_2D_3$, where the 20-*S* configuration of the side chain is opposite to the normal 20-*R* configuration. The DBP binding affinity of this analog is virtually unmeasurable because it does not displace [^{3}H]25-OH-D_3 from the plasma binding protein (Dilworth *et al.,* 1994). Confirmation that this is indeed the case comes from GH-reporter gene transactivation assays where 20-epi-1α,25-$(OH)_2D_3$ transactivates equally well in COS cells incubated in the presence and absence of fetal calf serum (as a source of DBP). On the other hand, 1α,25-$(OH)_2D_3$-induced GH reporter gene expression is sensitive to DBP in the external growth medium, requiring 2-fold less hormone in the absence of DBP as in its presence (Dilworth *et al.,* 1994). It therefore appears that analogs which bind DBP less well than 1α,25-$(OH)_2D_3$ derive a target cell advantage over the natural hormone, *if they are able to find alternative plasma carrier proteins to transport them to their target cells.* However, these same alternative plasma carriers presumably result in changes in the tissue distribution and hepatic clearance of analogs over the natural metabolites of vitamin D. The recent development of a DBP-knockout mouse (Safadi *et al.,* 1999) permits the study of alternate vitamin D analog transport mechanisms in an *in vivo* setting.

Vitamin D Receptor/RXR/VDRE Interactions

Previous chapters in this book have established that 1α,25-$(OH)_2D_3$ is able to work through a VDR-mediated genomic mechanism to stimulate transcriptional activity at vitamin D-dependent genes. Considerable progress has been made recently toward delineating the precise conformational changes which take place when the natural ligand binds to the VDR (Wurtz *et al.,* 1997; Rochel *et al.,* 2001);

and the nature of the postligand binding transcriptional events which occur, particularly the nature of the coactivator proteins involved (Rachez and Freedman *et al.,* 2000; Kato *et al.,* 2000). These developments have improved our thinking about how and where analogs might act differently from $1\alpha,25\text{-}(OH)_2D_3$ in the transcriptional cascade. Whether $1\alpha,25\text{-}(OH)_2D_3$ works through other non-VDR-mediated mechanisms to produce physiologically relevant effects is a question that currently remains unproven but this question is also important to our understanding of the pharmacological effects of vitamin D analogs.

Much evidence exists to support the viewpoint that vitamin D analogs mimic $1\alpha,25\text{-}(OH)_2D_3$ and use a genomic mechanism. The first clue that vitamin D analogs can work through a VDR-mediated transcriptional mechanism came 15 years ago from the bone resorption studies reported by Stern (1981). Stern showed that there exists a strong correlation between chick intestinal VDR binding of an analog and its potency in a [^{45}Ca] rat bone resorption assay. This suggests that a vitamin D analog is only as good as its affinity for the VDR. Certainly there are some apparent exceptions to this general rule but in the view of this reviewer they are not discrepancies that cannot be explained by other considerations such as DBP-binding or pharmacokinetics. Preliminary results with the analogs KH1060, EB1089, and 20-epi-$1\alpha,25\text{-}(OH)_2D_3$ (Binderup *et al.,* 1991) suggested that they might be active in immunoregulatory roles at concentrations orders of magnitude below their affinities for the VDR (e.g., at as low as 10^{-15} M for KH1060, whereas it binds VDR at 10^{-11}M). More recently results (Yang and Freedman, 1999; Dilworth *et al.,* 1994, 1997) suggest that 20-epi compounds including KH1060 are consistently only one to two orders of magnitude more potent than $1\alpha,25\text{-}(OH)_2D_3$ in gene transactivation assays and in differentiation assays, a difference that could be explained by fine-tuning the transcriptional model of analog action (eg by including pharmacokinetic considerations) rather than discarding the genomic hypothesis altogether. The majority of researchers agree with this view and are searching for differences in the newly delineated transcriptional machinery which might explain qualitative and quantitative differences between $1\alpha,25\text{-}(OH)_2D_3$ and its analogs.

Over the past decade it has been clearly established that the liganded VDR functions transcriptionally as a vitamin D–VDR–RXR heterodimer (Macdonald *et al.,* 1993; reviewed in Haussler *et al.,* 1998) and not as a VDR–VDR homodimer (Carlberg, 1995). The role of the RXR ligand is still controversial, many studies suggesting pan RAR and RXR ligands such as 9-*cis* retinoic acid inhibit VDR–RXR heterodimer formation while other studies demonstrate synergistic effects of pure RXR ligands (so called rexinoids) and $1\alpha,25\text{-}(OH)_2D_3$ on VDR–RXR driven transcription at a CYP24–VDRE (Zou *et al.,* 1997). Whether vitamin D analogs might differ from $1\alpha,25\text{-}(OH)_2D_3$ and act transcriptionally through VDR–VDR homodimers or other VDR–nuclear transcription factor heterodimers are ideas that have been considered as theoretical possibilities, and in some cases even shown to occur weakly *in vitro,* but largely dismissed as occurring *in vivo.*

Adding to the complexity of the target cell action of $1\alpha,25\text{-}(OH)_2D_3$, and thus that of vitamin D analogs, is the type and context of the VDRE involved (Haussler *et al.,* 1998). One possibility is that vitamin D analogs could show selectivity for certain genes based upon the type of VDRE within their promoter. Morrison and Eisman (1991) showed that a noncalcemic analog such as calcipotriol is capable of transactivating a calcemic VDRE such as the human osteocalcin promoter–VDRE placed in front of the CAT reporter gene and stably transfected into ROS 17/2 cells. One interpretation of this experiment is that a noncalcemic analog with good VDR affinity is just as calcemic as $1\alpha,25\text{-}(OH)_2D_3$ if it can be delivered to the target cell. Another idea put forward by Morrison and Eisman (1991) is that noncalcemic analogs may be capable of stimulating both cell-differentiating and calcemic genes but that the former genes require only a short pulse of analog to effect a switch in the cell cycle, whereas the latter genes require a sustained concentration of the vitamin D ligand. The concentration of $1\alpha,25\text{-}(OH)_2D_3$ may be sustained *in vivo* by renal synthesis and some protection by DBP, whereas systemically administered noncalcemic analogs reach a high initial concentration but do not bind DBP and are rapidly metabolized and cleared. This hypothesis remains to be adequately tested.

Carlberg *et al.* (1994) has also tested the idea that other vitamin D analogs (EB1089 and KH1060) might favor one specific VDRE using the mouse osteopontin gene VDRE (DR3-type) and the same human osteocalcin VDRE that Morrison and Eisman used. Carlberg *et al.* (1994) found that $1\alpha,25\text{-}(OH)_2D_3$ and the two analogs are unable to differentiate between the two different types of VDRE. Though many VDREs have been postulated in the literature it seems that the direct repeat-3 spacer type (DR3) of VDRE seems to be the sequence which is gaining widespread acceptance as the most physiologically relevant (Haussler *et al.,* 1998; Jones *et al.,* 1998). Whether other more exotic DR4, DR6, or inverted palindrome (IP9) nucleotide sequences are recognized by the analog-VDR/RXR complex *in vivo* still remains unclear (Carlberg, 1995). Even with the DR-3 type of VDRE, the gene and cell context seems to be important in determining the transactivation produced by the vitamin D analog. The work of Williams' laboratory (Brown *et al.,* 1994; Williams *et al.,* 1995; Kane *et al.,* 1996) suggests that $1\alpha,25\text{-}(OH)_2D_3$, KH1060, and EB1089 show different patterns of gene activation in bone marrow, osteoblastic cells (ROS17/2, ROS25/1, and UMR106), and intestine (HT29 and CaCo-2) which appear to be gene and cell-specific. Part of the explanation for gene- and tissue-specific effects probably lies in the influence of neighboring response elements to the VDRE and the binding of tissue-specific transcription factors at these sites. The laboratories of Lian and Stein have elegantly demonstrated (Guo *et al.,* 1997) that the response to $1\alpha,25\text{-}(OH)_2D_3$–VDR–RXR complex to the gene promoter of the osteocalcin gene in osteoblasts depends upon occupancy of an adjoining YY1 binding site which allows

for temporal changes in responsiveness to $1\alpha,25\text{-}(OH)_2D_3$. Though this work explains why $1\alpha,25\text{-}(OH)_2D_3$ might have gene- and tissue-specific effects, it does not explain the analog-specific differences in Williams' work.

As alluded to above, the emergence of important new information about (a) the structure of the ligand-binding domain of the VDR and (b) coactivator characterization and involvement in the $1\alpha,25\text{-}(OH)_2D_3$–VDR–RXR transcriptional machinery have opened up additional possibilities about where vitamin D analogs might differ in their action from the natural ligand. Evidence suggests that the "Trap Door Hypothesis" for retinoid binding to RAR/RXR (Renaud *et al.,* 1995) also applies to $1\alpha,25\text{-}(OH)_2D_3$ binding to VDR. In this model, $1\alpha,25\text{-}(OH)_2D_3$ binding to a central binding pocket triggers a dramatic conformational change of helix 12, a domain close to the C-terminus of the VDR, such that it moves from a position on the exterior of the VDR to one within the interior of the receptor, thereby closing the access channel to the ligand binding pocket. In the process, amino acid residues of the AF-2 domain which are hidden in the unliganded VDR become exposed in the liganded VDR and are now available to interact with coactivator proteins. The recruitment of coactivators to the $1\alpha,25\text{-}(OH)_2D_3$–VDR–RXR subsequently lead to the recruitment of other transcription factors which result in chromatin remodeling and gene transcription.

In the execution of this work, members of Moras' laboratory have modeled the ligand-binding pocket of VDR and shown it to be able to accommodate with ease several other analogs depicted in Table III (Rochel *et al.,* 2001). Though many of the active vitamin D analogs, especially the 20-epi analogs, have bulky side chain substituents or radically different side chain orientations (Yamamoto *et al.,* 1999), the pocket appears to have a great reserve capacity for binding (Rochel *et al.,* 2001). As a result, from modeling alone it is difficult to forecast radical changes in VDR conformations as a result of binding to these different analogs. Nevertheless, there is some indirect evidence, most notably from experiments measuring susceptibility to protease digestion, that subtle differences do occur in VDR–RXR containing transcription complexes when different ligands are used (Peleg *et al.,* 1995; Carlberg *et al.,* 1995; *et al.,* Van den Bemd, 1996). Binding of 20-epi-analogs (e.g., MC1288 and KH1060) to the VDR results in increased resistance to protease digestion as compared to $1\alpha,25\text{-}(OH)_2D_3$, which has been interpreted as evidence for differences in accessibility of protease to cleavage sites (Peleg *et al.,* 1995). Interestingly, there appears to be a direct correlation between transactivation activity of an analog and the resistance of the VDR-transactivation complex to protease, a relationship that applies to different analogs and even to metabolites from a single analog (Peleg *et al.,* 1995; Liu *et al.,* 1997; Van den Bemd *et al.,* 2000). Because the rearrangement in helix 12 of the VDR brings about exposure of the AF-2 domain and this is critical to coactivator binding, it might be expected that subtle conformational differences in VDR observed for different vitamin D analogs might also be reflected in differences in coactivator recruitment.

Consequently, several groups have looked for qualitative differences in the pattern of coactivators recruited or quantitative differences in the strength of RXR heterodimerization or coactivator binding following ligand binding. Liu *et al.,* (2001) used a series of AF-2 domain mutants to reach the conclusion that the conformational changes occurring in the VDR upon hormone or 20-epi-$1\alpha,25\text{-}(OH)_2D_3$ binding have a bigger impact upon RXR-heterodimerization than upon coactivator recruitment. This is in complete contrast to the work of Freedman and coworkers, who have shown repeatedly (Cheskis *et al.,* 1995; Yang and Freedman, 1999) that analog binding (20-epi-$1\alpha,25\text{-}(OH)_2D_3$ or $1\alpha,25\text{-}(OH)_2$-16-ene, 23-yneD_3) to VDR results in no difference in RXR-hetero-dimerization as compared to that of $1\alpha,25\text{-}(OH)_2D_3$. Instead, Freedman's group reports that the ability of a various analogs to transactivate vitamin D-dependent genes or to stimulate differentiation of cells is best correlated to their ability to recruit the coactivator, DRIP-205, one of the many components of the DRIP complex isolated by Freedman's group (Rachez *et al.,* 1999). Among the other coactivators/transcription factors implicated in vitamin D analog action is TIF-2, which has been purported to have a particular propensity to interact with the analog OCT (Takeyama *et al.,* 1999). Thus, it appears that there is a fairly strong basis for the hypothesis that differences in the biopotency advantage of certain vitamin D analogs over $1\alpha,25\text{-}(OH)_2D_3$ is due in part to changes in the recruitment of dimerisation partner and/or coactivators but there is no consensus on which of these proteins is the important one.

Target Cell Catabolic Enzymes

In recent years, much evidence has accumulated to support the hypothesis that $1\alpha,25\text{-}(OH)_2D_3$ is subject to target-cell catabolism and side chain cleavage via a 24-oxidation pathway to calcitroic acid (Makin *et al.,* 1989). The cloning of CYP24, the cytochrome P450 involved, has confirmed that it is vitamin D-inducible since its gene promoter contains a VDRE, carries out multiple steps in side chain modification process, and is present in many (if not all) vitamin D-target cells (Akiyoshi-Shibata *et al.,* 1994). We have postulated that the purpose of this catabolic pathway is to desensitize the target cell to continuing hormonal stimulation by $1\alpha,25\text{-}(OH)_2D_3$ (Lohnes and Jones, 1992). Recently, support for this hypothesis has emerged when St. Arnaud's group engineered a CYP24-knockout mouse which exhibits a 50% lethality by weaning, death resulting from hypercalcemia, and nephrocalcinosis (St. Arnaud, 1999). Surviving mice show an inability to rapidly clear a bolus dose of $1\alpha,25\text{-}(OH)_2D_3$ from the bloodstream and tissues (Jones *et al.,* 2000a) and a metabolic bone disease reminiscent of the excessive osteoid bone pathology observed in rodents given excessive amounts of $1\alpha,25\text{-}(OH)_2D_3$ (Hock *et al.,* 1986). Recent work with this model has shown that the defect is probably due to excessive $1\alpha,25\text{-}(OH)_2D_3$ since crossing the CYP24-knockout mouse with the VDR-knockout mouse produces a phenotype without the bone defect

(St. Arnaud *et al.,* 2000). Given the apparent importance of CYP24 to 1α,25-$(OH)_2D_3$ pharmacokinetics, one must ask the question of whether vitamin D analogs might be subject to the same catabolic processes? If not, what other drug-catabolizing systems are present within vitamin D-target cells to inactivate the vitamin D analog?

Certainly there are vitamin D analogs such as calcipotriol, OCT, EB1089, and KH1060 which are metabolized by vitamin D-target cells to clearly defined and unique metabolites (Masuda *et al.,* 1994, 1996; Shankar *et al.,* 1997; Dilworth *et al.,* 1997), which resemble products of the 24-oxidation pathway for 1α,25-$(OH)_2D_3$or which are unique to the particular analog. Furthermore, some of these metabolites are products only of vitamin D target cells and are D-inducible, implying that CYP24 is involved in their formation but this has not been proven. However, in the case of several analogs blocked at C-24 and subject to metabolism elsewhere on the side chain, the direct involvement of CYP 24 is strongly implicated or proven. These include 23-hydroxylation of 26,27-hexafluro-1α,25-$(OH)_2D_3$ (Sasaki *et al.,* 1995); 26-hydroxylation of 24-difluro-1α, 25-$(OH)_2D_3$ (Miyamoto *et al.,* 1997); 26-hydroxylation of 1α, 25-$(OH)_2$-16ene-23yne-D_3 (Satchell and Norman, 1995); and 26- and 28-hydroxylation of 1α,25-$(OH)_2D_2$ (Rao *et al.,* 1999; Shankar *et al.,* 2001). Since many of these same products are observed *in vitro* and *in vivo* and since pharmacokinetic parameters often parallel target cell metabolic parameters (Kissmeyer *et al.,* 1995; Jones, 1997), one concludes that target cell metabolism of vitamin D analogs must contribute to the pharmacokinetics and biological activity observed *in vitro* and *in vivo.* In fact, there is little doubt that the poor performance of some promising vitamin D analogs during *in vivo* testing is due to their poor metabolic stability. Accordingly, greater attention to the metabolic potential of *in vitro* testing systems and/or greater use of defined target cell (and hepatic) metabolic systems is warranted.

One factor regarding target cell metabolism considered in recent years is the possibility that vitamin D analogs might be *activated* rather than *catabolized* by the same enzymes (Siu-Caldera *et al.,* 1999). While this is potentially more important for prodrugs (Table II), the generation of large numbers of metabolites from such analogs as KH 1060 (Dilworth *et al.,* 1997) or the formation of long-lived metabolites such as 26,27- hexafluro-1α,23,25-$(OH)_3D_3$ from 26,27-hexafluro-1α,25-$(OH)_2D_3$ (Sasaki *et al.,* 1995) complicates the picture. In most cases, however, this issue can be resolved on pharmacokinetic grounds.

Other Factors

Hepatic Clearance of Vitamin D Analogs

The poor DBP binding properties of many side-chain modified calcitriol analogs opens up the possibility of alternative plasma carriers and accelerated degradation. The liver plays a major role in such metabolic clearance and a small number of detailed studies performed to date have included *in vitro* incubation with liver preparations. Calcipotriol (Sorensen *et al.,* 1990), OCT (Masuda *et al.,* 1996), EB1089 (Kissmeyer *et al.,* 1997), and KH1060 (Rastrup-Andersen *et al.,* 1992) are all subject to metabolism by liver enzymes. These give rise to intermediate polarity molecules or truncated metabolites, which can be further glucuronidated and excreted in bile (e.g., OCT; Kobayashi *et al.,* 1991). Few, if any, studies have separately considered the *rate* of such metabolism relative to 1α,25-$(OH)_2D_3$. However, data are available comparing the *in vivo* rate of metabolic clearance of vitamin D analogs to 1α,25-$(OH)_2D_3$, though inevitably this probably measures a few *in vitro* parameters such as the rate of both hepatic and target cell metabolism in addition to the affinity of DBP binding within a single *in vivo* parameter. Thus, in lieu of detailed *in vitro* metabolic analyses, the $t_{1/2}$ of the vitamin D analog is a useful term for indicating the general survival of the vitamin D drug *in vivo.* Data for this parameter have recently been published for some of the most interesting analogs (Kissmeyer *et al.,* 1995).

Nongenomic Actions of Vitamin D Analogs

The nongenomic actions of 1α,25-$(OH)_2D_3$ have been reviewed elsewhere (Norman *et al.,* 1992; Bouillon *et al.,* 1995) and were described in detail in Chapter I.D.31 and will not be repeated here. One analog purported to discriminate between genomic and nongenomic actions is 1β,25-$(OH)_2D_3$, the epimeric form of 1α,25-$(OH)_2D_3$, which is an antagonist of nongenomic but not genomic actions (Bouillon *et al.,* 1995). The membrane VDR initially described by Nemere *et al.* (1994) and now identified as annexin II (Baran *et al.,* 2000) may be involved in mediating putative nongenomic effects. It will be interesting to see if an annexin-II knockout mouse will possess a distinct phenotype which will aid in delineating the nongenomic actions of vitamin D in the same way that the VDR-knockout mouse has aided our understanding of the genomic actions. At this point in time, little work has been performed on the specificity of the vitamin D binding site of this membrane VDR/annexin II and thus the possibility that the nongenomic actions/membrane VDR might explain vitamin D analog actions seems premature.

Proposed Molecular Mechanisms of Action of Vitamin D Compounds

Building upon the data acquired from a variety of *in vitro* tests performed over the past 10–15 years and described briefly in the previous section of this chapter, one is able to identify those criteria that are important to vitamin D analog action. This in turn allows us to put forward a model for how vitamin D analogs may work *in vivo.* This is depicted in Fig. 1. As a general model, it allows for consideration of both prodrugs (those requiring 25-hydroxylation by CYP27A or those requiring 1α-hydroxylation by the kidney or extrarenal 1α-hydroxylase) and 1α,25-$(OH)_2D_3$ analogs. This model features a conventional VDR-RXR heterodimer

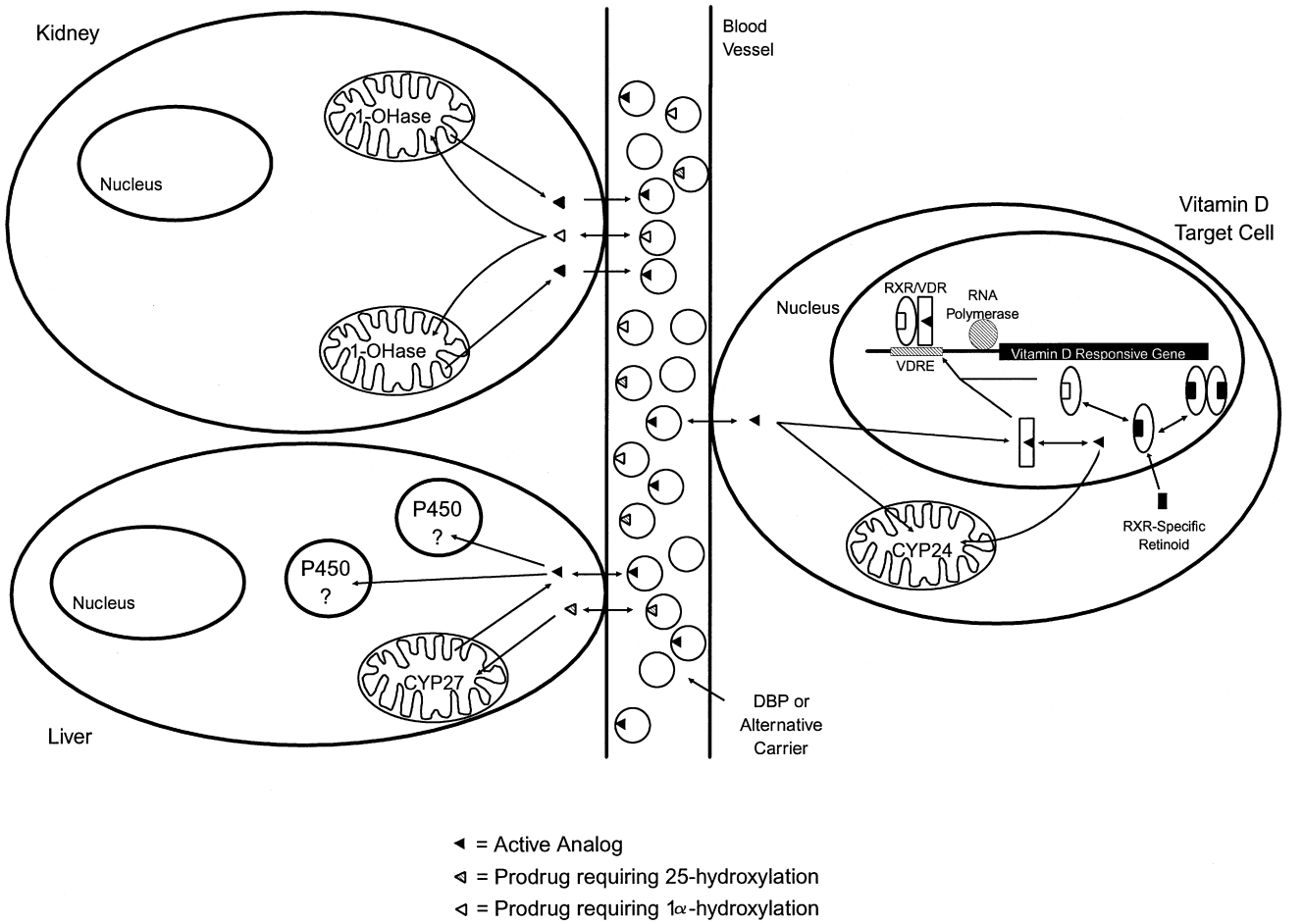


Figure 1 Proposed mechanism of action of vitamin D analogs. For discussion of the ideas contained within this figure please see text. The model incorporates plasma-binding proteins, which act as carriers of analogs; activating enzymes involved in activation of analog; target cell transcriptional machinery involved in biological actions of analogs; and target cell catabolic enzyme systems involved in degradation of the analog.

working through a DR-3 type VDRE in most genes. Crucial characteristics for each new analog (all measurable *in vitro*) are in our opinion:

(1) affinity for DBP,
(2) affinity for VDR,
(3) ability to recruit RXR and coactivators followed by transactivation of genes,
(4) rate of target cell metabolism (reflected partly in pharmacokinetic measurements),
(5) rate of hepatic clearance (reflected partly in pharmacokinetic measurements).

All parameters contribute significantly to the overall biological activity. Target cell distribution differences might be expected for those analogs that do not bind DBP and this model does not incorporate the differences observed at different genes within the same or different cells.

However, it might be useful to predict broad expectations for a new compound. Based upon this model one would predict that those analogs that have good VDR binding affinity but slow rates of target cell metabolism due to side chain blocks, such as strategically placed fluorine atoms or double bonds, might be more active than $1\alpha,25\text{-}(OH)_2D_3$ *in vitro* and perhaps also *in vivo.* Hexa-fluoro-$1\alpha,25\text{-}(OH)_2D_3$ and EB1089 are such compounds; looking alike *in vitro* and differing *in vivo* due to differences in DBP binding. On the other hand, a compound such as calcipotriol binds VDR well and is rapidly metabolized in both the liver and the vitamin D target cell and is moderately active *in vitro* or when applied to the skin topically but is inactive when administered orally and is forced to enter the bloodstream and pass the liver to get to its target site. It remains to be seen if this general model can be applied to all vitamin D analogs (e.g., KH1060, which has a very complex metabolic picture but very high biological activity *in vitro*) or must be adapted.

Future Prospects

A number of researchers remain optimistic that the unraveling of the genomic (or nongenomic) mechanism of action of $1\alpha,25\text{-}(OH)_2D_3$ will reveal new approaches by which the vitamin D signaling cascade can be exploited.

Certainly, the significant progress made in characterizing the coactivator proteins and the rest of the transcriptional apparatus will continue. One is able to predict fairly confidently from success in related steroid hormone fields that a fully functional vitamin D-dependent *in vitro* VDR–RXR transcriptional system will be reconstituted soon. Such a system devoid of the complications of metabolic enzymes will be the perfect model to test the transactivation activity of vitamin D analogs. Perhaps then it will be possible to dissect out the exact step(s) that give certain analogs a transcriptional advantage over $1\alpha,25\text{-}(OH)_2D_3$.

Modeling of the vitamin D binding pockets of VDR, DBP, and the three vitamin D-related cytochrome P450s will continue to be a major goal now that all these specific proteins have been cloned and overexpressed. While the full-length proteins are slightly beyond the current limits of NMR or X-ray crystallography, the ligand-binding domains or substrate binding pockets are not. It is also likely that technical problems with these procedures will be overcome shortly and the full-length proteins can be tackled. The initial work of the Moras group (Rochel *et al.,* 2001) on the ligand-binding domain of the VDR will be extended to new analogs and there will also be a growing focus on the other major proteins in the vitamin D signal transduction pathway.

The growing availability of recombinant cytochromes P450 will allow for modeling studies of the enzymes involved in vitamin D metabolism. The membrane-associated region of cytochromes P450 poses problems but enormous strides have been made based upon models built with X-ray data from soluble prokaryotic isoforms and with truncated mammalian isoforms such as CYP2C5 (Williams *et al.,* 2000). Access to full-length CYP24 and CYP27B will also permit a more efficient search for potential inhibitors. Such specific inhibitors of CYP24 and CYP27B (Schuster *et al.,* 2001; Muralidharan *et al.,* 1997) may be of value in blocking $1\alpha,25\text{-}(OH)_2D_3$ catabolism or synthesis in certain clinical conditions. Modeling of VDR and cytochromes P450 is expected to lead to more rational vitamin D analog design to take advantage of structural idiosyncrasies of all of these key proteins. Meanwhile, the not-so-rational synthesis of new analogs is likely to continue.

The list of applications for vitamin D compounds continues to increase (reviewed in Jones *et al.,* 1998). These applications have been further rationalized with the availability of VDR knockout mice to demonstrate vitamin D-dependent processes (Yoshizawa *et al.,* 1997; Li *et al.,* 1997). Elucidation of the mechanism by which $1\alpha,25\text{-}(OH)_2D_3$ and its analogs regulate the cell cycle and proliferation remains an important priority of the field. Current applications still fall mainly into calcium-related and cell-proliferative/differentiating ones. The goal of developing analogs that can completely separate the "calcemic" and "cell-differentiating" properties of $1\alpha,25\text{-}(OH)_2D_3$ has not yet been realized. However, some promising compounds have been synthesized and interesting idiosyncrasies of their biological actions have surfaced (e.g., tissue, cell, gene, and VDRE differences). It remains to be seen if these differences can be exploited. On the other hand, it must be stated that if vitamin D analogs work *only* through a VDR-mediated genomic mechanism, it is difficult to appreciate how the "calcemic" properties of $1\alpha,25\text{-}(OH)_2D_3$ can ever be fully resolved from the "cell-differentiating" properties given that pharmacokinetic differences have provided only a partial separation. On a more optimistic front, it can be stated that since the first edition of this book was published, several new vitamin D analogs (OCT, $1\alpha,24\text{-}(OH)_2D_3$, $1\alpha\text{-}OH\text{-}D_2$, and 19-nor-$1\alpha,25\text{-}(OH)_2D_2$) have received governmental approval to be used in the treatment of various clinical conditions worldwide. We must remain upbeat that more vitamin D analogs will be developed and important new applications of vitamin D, particularly in the area of bone biology, remain to be uncovered.

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CHAPTER 82

Molecular and Clinical Pharmacology of Calcitonin

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Historical Perspectives

Calcitonin was discovered as a hypocalcemic principle that was later confirmed to originate from the C cells of the thyroid gland (Copp and Cheney, 1962; Kumar *et al.,* 1963; Hirsch *et al.,* 1963). Subsequently, human calcitonin was purified and sequenced from extracts of medullary thyroid carcinoma (Neher *et al.,* 1968). It is a 32 amino-acid-long peptide with an N-terminal disulfide bridge and a C-terminal prolineamide residue. Calcitonin was found to be a potent inhibitor of bone resorption. Notably, the peptide was first shown to decrease calcium influx directly from isolated cat tibiae (MacIntyre *et al.,* 1967). Later histological and organ culture studies confirmed an effect of calcitonin on the osteoclast (reviewed in Zaidi *et al.,* 1991). Nevertheless, it remained unclear until the early 1980s whether calcitonin had a direct effect on osteoclastic receptors.

In 1983, Chambers and colleagues showed that osteoclasts were sensitive to femtomolar concentrations of salmon calcitonin (Chambers, 1982). Since then osteoclastic receptors for calcitonin have been cloned and sequenced. Systematic studies using deletion mutagenesis and chimeric receptors have clarified specific regions of the calcitonin receptor that are necessary for ligand binding and intracellular signaling through cyclic AMP and calcium. Because of its potent anti-resorptive effect and powerful analgesic action calcitonin has been used extensively for the treatment of Paget's bone disease, osteoporosis, and hypercalcemia of malignancy.

This chapter focuses on key aspects of the synthesis and structure of calcitonin, its mode of action at the cellular and molecular levels, and its clinical pharmacology.

Gene Structure

The calcitonin gene complex comprises two known genes, the α and β genes (reviewed in Zaidi *et al.,* 1991). In man, both genes are located on chromosome 11 between the catalase and parathyroid hormone genes (Kittur *et al.,* 1985). The α calcitonin gene has six exons; the first three exons are shared between calcitonin and the alternative splice product, calcitonin gene-related peptide (CGRP) (Fig. 1). The organization of the β gene is similar to that of the α gene, although the 3′ and 5′ non coding regions differ considerably (Alevizaki *et al.,* 1986). The β gene gives rise to β-CGRP, but not to a second calcitonin at least in man. The latter molecule was predicted but not proven.

The α gene is either spliced at exon 4 to yield calcitonin or at exon 6 to produce CGRP. The latter is a neuropeptide and potent vasodilator (Emson and Zaidi, 1989). Since the first three exons are spliced in either process, both calcitonin and CGRP have identical 5′-coding and noncoding regions. It is believed that splicing to calcitonin or CGRP involves recognition or nonrecognition, respectively, of exon 4 (Lou *et al.,* 1994; Zandberg *et al.,* 1995). Important regulatory elements in exon 4 have been identified; in cells producing

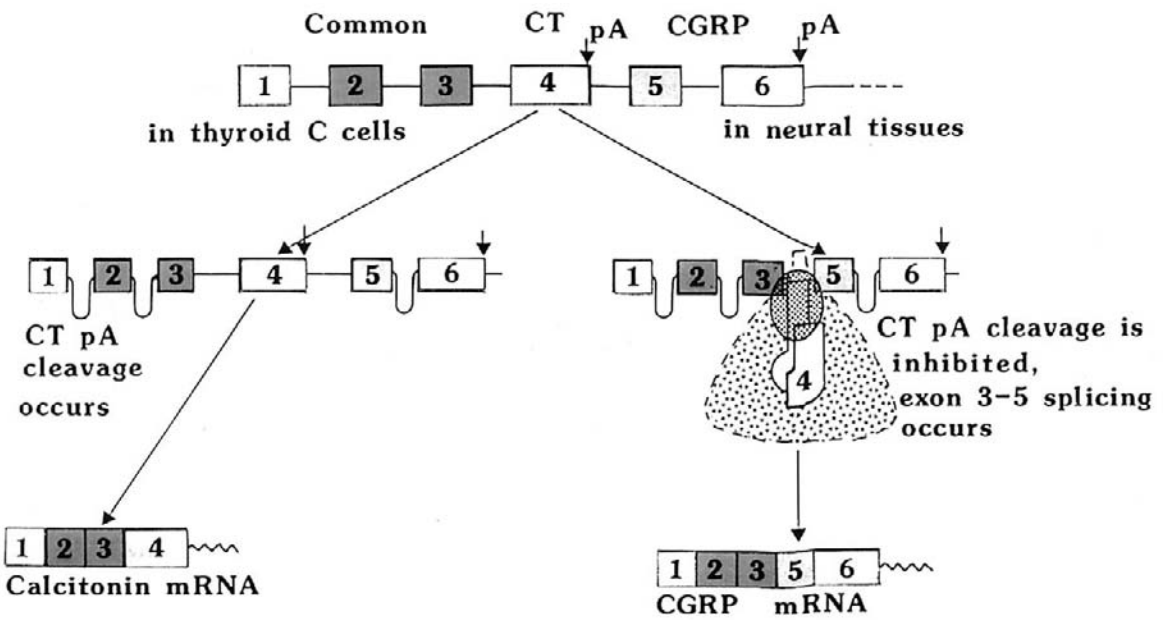

Figure 1 Alternative splicing of the α calcitonin (CT)/calcitonin gene-related peptide (CGRP) gene in a tissue-specific manner. For splicing to CGRP, the splice site in exon 4 is not recognized and transcription proceeds to exon 6 to produce CGRP.

calcitonin, these interact with cellular protein(s) facilitating exon 4 recognition (Cote *et al.,* 1992; van Oers *et al.,* 1994). It has also been shown that 3′ and 5′ coding sequences participate in recognition (Zandberg *et al.,* 1995). There is also an intron element that resides in intron 4 of the calcitonin gene (Lou *et al.,* 1996). The latter functions to enhance polyadenylation of an embedded alternative 3′-terminal exon and is potentially involved in tissue-specific regulation of calcitonin RNA processing (Lou and Gagel, 1998).

The regulation of tissue-specific calcitonin expression is being studied intensively. The cloning and sequencing of the calcitonin promoter has revealed a negative response element to vitamin D, a cAMP-response element (CRE), and a transcriptionally active octomer sequence (CRE-O) (Peleg *et al.,* 1993; Monla *et al.,* 1995). Following splicing to either calcitonin or CGRP mRNA, mature peptides are synthesized initially as large precursors that are cleaved intracellularly to release the active molecule. Post-translational processing also includes amidation at the C-terminus. It is interesting that procalcitonin has been used recently as a marker for severe bacterial infections (Hammer *et al.,* 1999).

Peptide Structure

Biologically active calcitonin comprises 32 amino acids. It has an N-terminal disulfide bridge between residues 1 and 7 in contrast to CGRP, in which the latter is positioned between residues 2 and 7 (Zaidi *et al.,* 1990a). There is also a proline amide at residue 32. The sequences of calcitonins from seven species are shown in Fig. 2. These display considerable variation in primary structure, varying from between a difference of 2 (between rat and human) to 19 (between human and ovine) amino acid substitutions. Eight residues (positions 1, 4, 5, 6, 7, 9, 28, and 32) are common to all species studied. Of these, six are clustered at the N-terminus, suggesting physiological constraints on this region of the molecule. The highly conserved regions at the two ends of the molecule also suggest that the termini are important for biological activity. The variable middle portion is thought to be involved in controlling the potency and duration of action of calcitonin. More recently, sardine calcitonin, currently the most potent calcitonin, has been sequenced (Suzuki *et al.,* 1994).

It has been suggested that the conformational flexibility of a given calcitonin molecule determines its biological potency (reviewed in Breimer *et al.,* 1988). A more flexible calcitonin potentially can attain a larger number of conformations at the receptor membrane complex. Molecular flexibility is dependent on the bulkiness of its side chains, glycine being the least bulky. As three glycine residues are conserved in salmon and eel calcitonin, both peptides possess a remarkably low potential for forming rigid helices. Although it appears that the valine residue at position 29 of eel calcitonin might induce conformational rigidity due to its bulky side chain, the effect is annulled by an alanine at position 29. The substitution of salmon calcitonin with amino acids containing less bulky side chains has been shown to produce more active analogs.

Kapurniotu and Taylor (1995) synthesized lactam bridge-containing derivatives of calcitonin and concluded that the lactam bridges are compatible with biological activity. They also show that the amphiphilic α helix and a type 1 β-turn (involving residues 17 to 20) may play an important role in receptor recognition by calcitonin. It has also been demonstrated through CD spectroscopy that human calcitonin forms a left-handed extended helix in solution (Siligardi

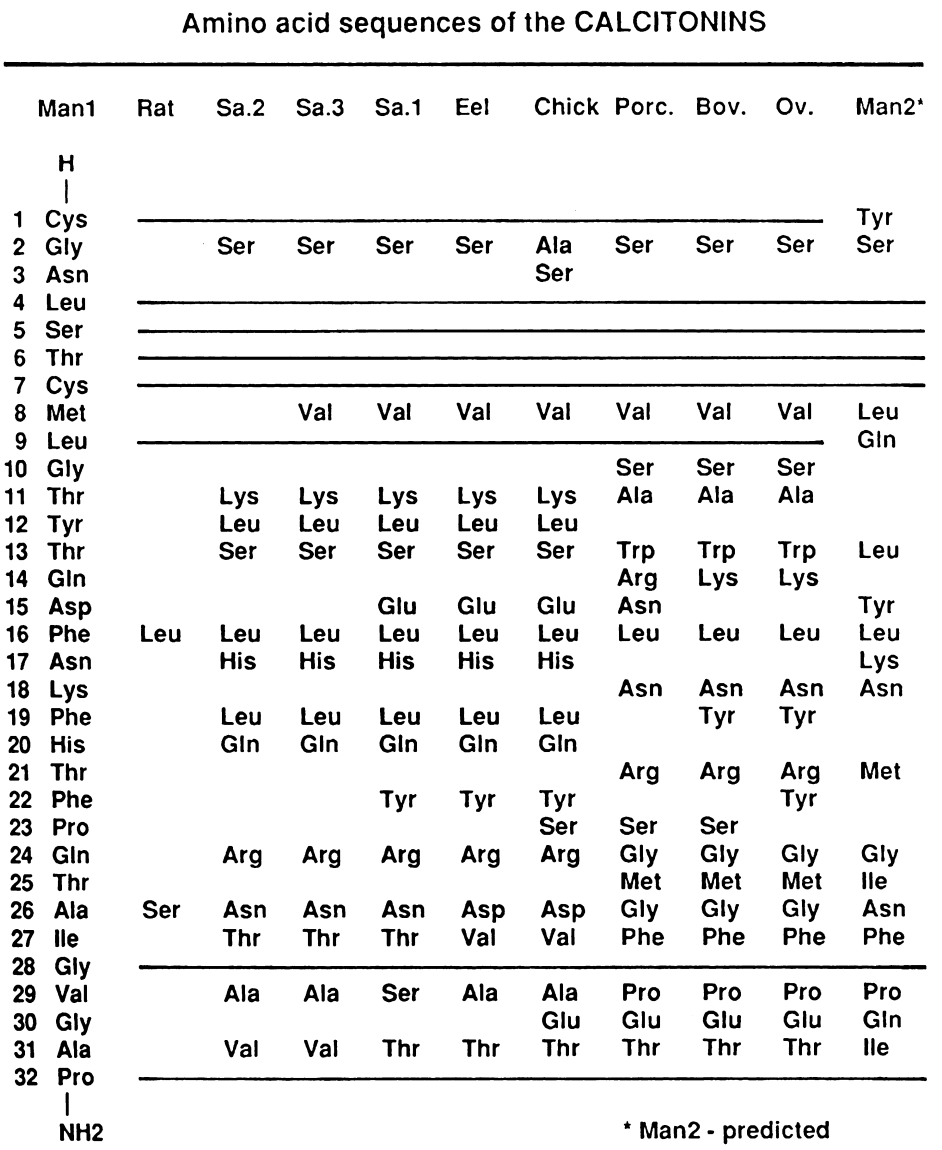

Figure 2 Structure of calcitonins from various species including human (Man1 and the predicted second human calcitonin, Man2), rat, salmon (Sa.1 to Sa. 3), eel, chicken (Chick), porcine (Porc.), bovine (Bov), and ovine (Ov) calcitonin. The lines indicate nonvariant residues.

et al., 1994). Another hypothesis arising from the 3-D structure determination of calcitonin is that human calcitonin has a hydrophobic face composed of one Met and four Leu residues that facilitate interaction with the receptor (Katahira *et al.,* 1995).

Modifications of the primary structure that cause a reduction in biological activity include deletion of the C-terminal proline amide, shortening of the C-terminal end, cleavage of the disulfide bond (except when it is replaced by a carba-type linkage as in asu$^{1-7}$-eel calcitonin), or oxidation of the methionine at position 8 (human calcitonin). Modifications that do not alter biological activity include oxidation of the methionine residue at position 25 in porcine, bovine, and ovine calcitonins. Modifications that tend to increase homology of a given calcitonin with salmon calcitonin generally increase biological activity. One recent revelation has been that human calcitonin fibrillates in solution due to the formation of hydrogen bonds (Kanaori and Nosaka, 1995). When fibrillation is avoided, potency can be enhanced (Cudd *et al.,* 1995). This new knowledge may have implications in the development of new analogs for human use.

There have been recent studies on the binding of calcitonin with its receptor—these highlight the secondary and tertiary structural requirements for binding. For example, with the parathyroid hormone (PTH) receptor, elegant photoaffinity cross-linking studies using benzolphenylalanine derivatives have identified two specific contact sites for ligand receptor interaction (Behar *et al.,* 1999). The entire bimolecular surface of the PTH–PTH1 receptor complex has now been mapped (Pischerio *et al.,* 2000). It

has been shown that there is spatial proximity between Lys^{27} of the hormone's principal binding domain and Leu^{261} of the first extracellular loop of the receptor (Greenberg *et al.,* 2000). Similar studies have also been initiated using a novel biologically active benzophenone-containing calcitonin analog for photolabelling the calcitonin receptor (Suva *et al.,* 1997). Such investigations should help us understand the precise biomolecular interaction between calcitonin and its receptor as a prelude to the future development of novel receptor-specific therapeutics.

Cellular Actions

Calcitonin inhibits basal and stimulated resorption of intact bone in organ culture (reviewed in Breimer *et al.,* 1988). In bone sections, calcitonin acts directly on the osteoclast to cause a rapid loss of the ruffled border (Holtrop *et al.,* 1974; Kallio *et al.,* 1972; Chambers and Magnus, 1982). When applied for a longer term, there is a reduction in the number of osteoclasts in bone.

When applied *in vitro* to isolated osteoclasts, femtomolar concentrations of calcitonin result in an acute cessation

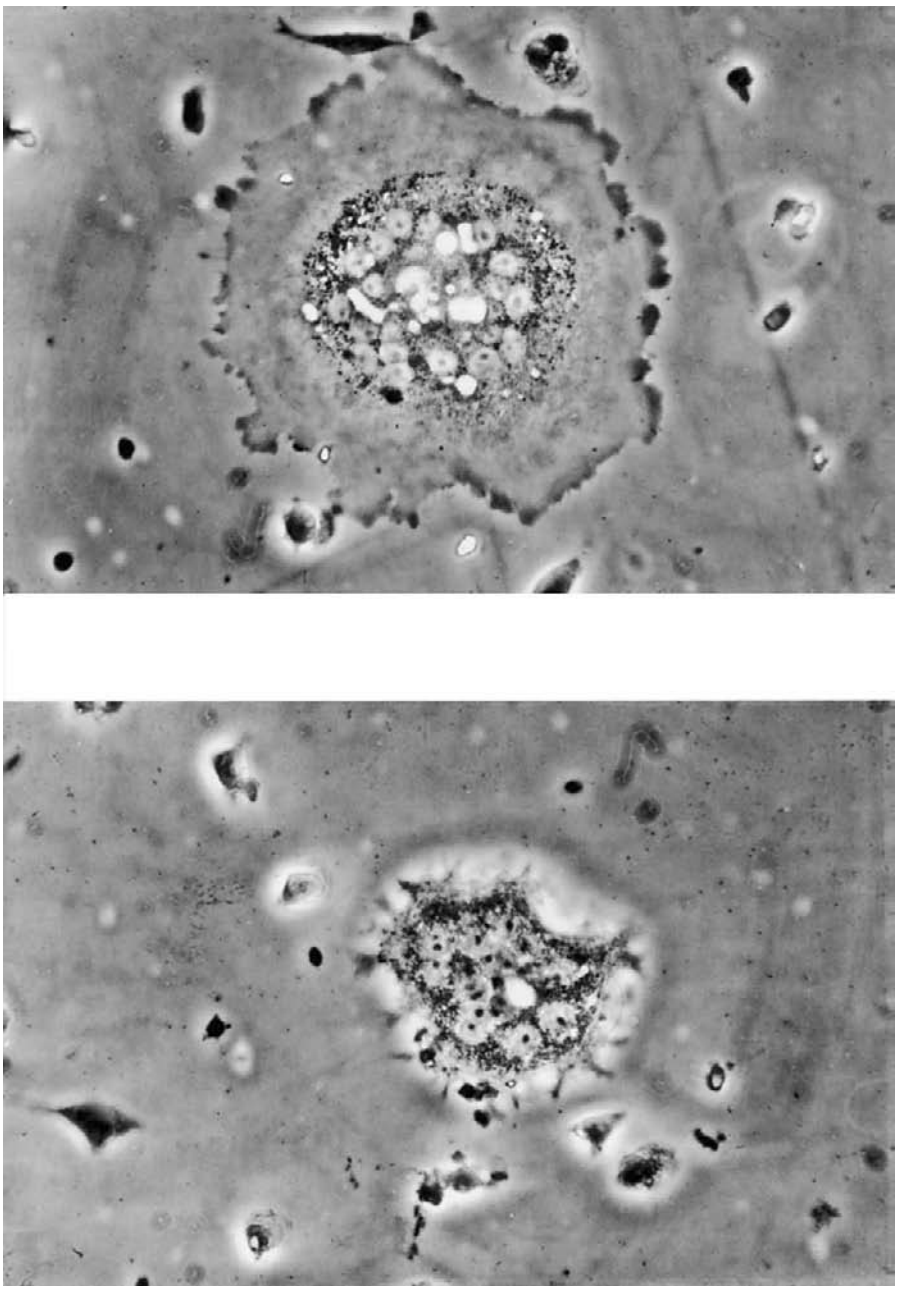

Figure 3 The effect of calcitonin on the morphology of isolated osteoclasts. Disaggregated osteoclasts with (bottom) or without (top) calcitonin treatment. Used with kind permission of Professor T. J. Chambers (London).

of cytoplasmic motility followed by gradual pseudopodial retraction (Chambers, 1982) (Fig. 3). The predictability of the response, its specificity for osteoclasts, and the near-physiological concentrations used clearly suggest that the phenomenon is of physiological significance. We provided the first quantitative estimates for calcitonin action on isolated osteoclasts using a novel method to quantitate osteoclast motility and shape change (Zaidi *et al.,* 1990a, 1992b). Calcitonin action was separable into more a rapid quiescence (Q) component with a $t_{1/2}$ of 15 min that was characterized by cessation of motility. The late retraction (R) component with a $t_{1/2}$ of 27 min was composed mainly of pseudopodial and margin retraction (Fig. 4). The latter, we believe, may reduce resorption by decreasing the area of contact of the osteoclast with the bone surface.

In addition, calcitonin inhibits osteoclast secretion. Notably, it reduces tartrate-resistant acid phosphatase (TRAP) secretion and Na^+-K^+-ATPase activity and alters the localization of carbonic anhydrase and thus acid secretion in osteoclasts (Chambers *et al.,* 1987; Moonga *et al.,* 1990; Akisaka and Gay, 1986). Additionally, calcitonin inhibits TRAP release and synthesis from osteoclasts (Yumita *et al.,* 1991).

These inhibitory effects of calcitonin on the osteoclast are translated into a marked inhibition of resorption that is reproducible in every osteoclast resorption assay studied. For example, calcitonin reduces the formation of osteoclasts in the classical pit forming assay (Chambers *et al.,* 1984; Zaidi *et al.,* 1987; Dempster *et al.,* 1987) (Fig. 5). This assay involves culturing osteoclasts onto devitalized bone or dentine substrate and quantifying either the number, area, or volume of bone resorbed. Calcitonins display a rank order of potency in inhibiting resorption with salmon calcitonin being the most potent and human the least potent (Fig. 5). These potency estimates correlate perfectly with the *in vivo* potency of the respective calcitonins (Zaidi *et al.,* 1988). CGRP and amylin both interact with the calcitonin receptor, but at 100- and 40-fold higher concentrations (Zaidi *et al.,* 1987, 1988, 1994; Datta *et al.,* 1989).

Calcitonin has surprising new actions. It releases osteoclasts from the inhibitory effects of a high extracellular Ca^{2+} (Zaidi *et al.,* 1996). Ca^{2+}-induced Ca^{2+} signaling was found to be markedly attenuated in the presence of femtomolar

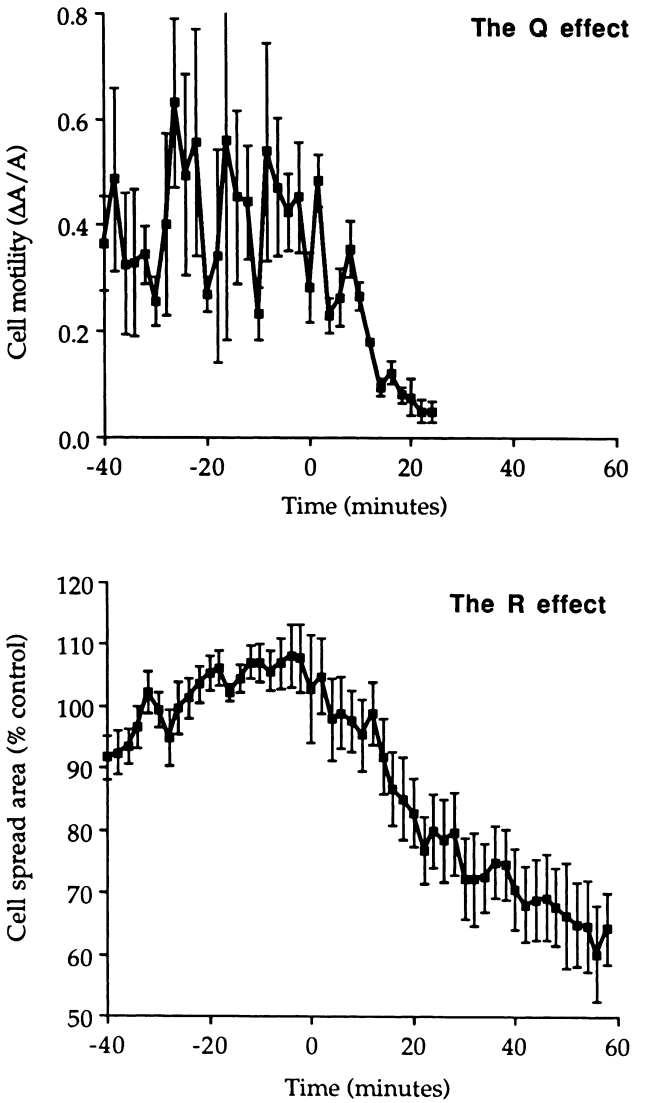

Figure 4 The Q (quiescence) and R (retraction) effects of calcitonin added at time 0. Cell motility was quantitated as the rate of change of shape area ($\triangle A$) per unit cell area. Used with permission from Zaidi *et al.* (1993).

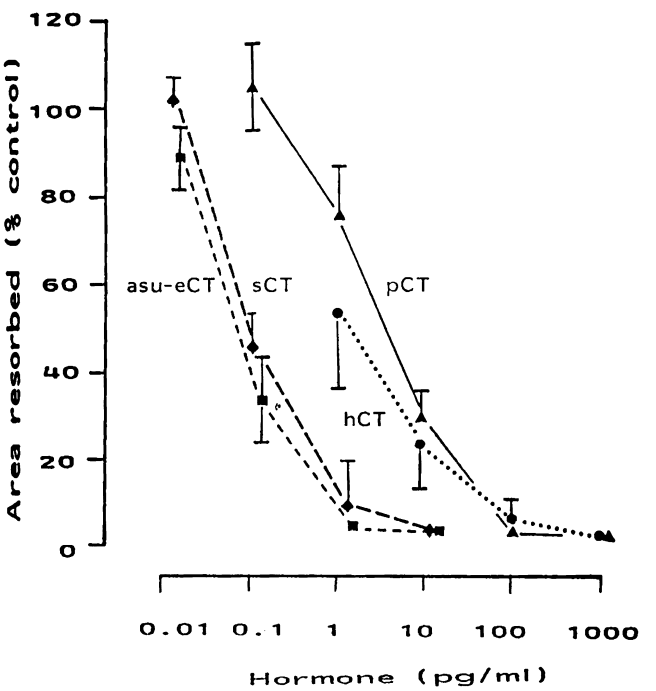

Figure 5 The effect of various therapeutically used calcitonins on bone resorption by isolated rat osteoclasts that were plated on devitalized cortical bone substrate. Area of resorption has been quantitated by scanning electron microscopy. Used with permission from Zaidi *et al.* (1987).

concentrations of the peptide. This apparent paradox to the known potent anti-resorptive action of calcitonin may explain the lack of any bone loss during the absence of calcitonin (such as after thyroidectomy) as well as the absence of osteopetrosis in the presence of high circulating calcitonin (as in medullary thyroid carcinoma). Second, calcitonin has been shown in some systems to interact with osteoblasts, although there is no convincing evidence for calcitonin receptors on any osteoblast-like cells. For example, eel calcitonin has recently been shown to have an anabolic effect on osteoblasts, specifically to enhance osteoinduction by BMP-2 (Furuichi *et al.,* 2000; Wallach *et al.,* 1999; Okubu *et al.,* 2000). It also increases the concentration of insulin-like growth factors in serum-free cultures of human osteoblast-like cells (Farley *et al.,* 2000). Calcitonin has also been shown to prevent osteoblast and osteocyte apoptosis (Plotkin *et al.,* 1999), but this action is controversial.

Another interesting cellular action of calcitonin had been the early demonstration of its effect on the growth of breast cancer cells. Calcitonin can induce growth retardation in calcitonin receptor-transfected HEK cells. New evidence indicates that the cyclin-dependent kinase inhibitor p21/WAF1/CIPI that causes cell cycle arrest in G1 and G2/M phases possesses calcitonin sensitivity (Evdokiou *et al.,* 2000). In fact, calcitonin affects gene transcription through the transcription factor, Sp1. It is also evident that the insert-negative (but not insert-positive) calcitonin receptors (see below) cause downstream activation of Erk1/2-MAP kinase and inhibition of cell growth (Raggatt *et al.,* 2000). More interesting is the observation that calcitonin suppresses expression of the growth factor, PTH-related peptide (PTHrP), in breast cancer cells (Rong *et al.,* 1999). Such emerging evidence for an effect of calcitonin on cell cycling molecules, growth factors, and cell growth itself revives a long forgotten area of calcitonin and cancer biology. Furthermore, calcitonin inhibits the osteoclastogenic effects of osteoprotegerin-ligand, an effect that may be relevant therapeutically (Mancini *et al.,* 2000).

Finally, calcitonin, at pharmacological concentrations, has for some years known to increase renal calcium and phosphate excretion and 1,25-dihydroxycholecalciferol production (Agus *et al.,* 1981). More recently, it has been shown to be a major regulator of the expression of the renal 25-hydroxyvitamin D_3 hydroxylase gene in normocalcemic rats (Shinki *et al.,* 1999).

The Calcitonin Receptor Family

Several calcitonin receptor subtypes have been cloned and sequenced (Goldring *et al.,* 1993). Essentially, these are membrane receptors with seven transmembrane domains and a long extracellular domain (reviewed in Zaidi *et al.,* 1994). They fall within the superfamily of PTH/PTHrP/calcitonin/adrenomedullin/secretin receptors. There is >30% homology between the human and porcine calcitonin and the opposum and rat PTH–PTHrP receptors. The human ovarian receptor and porcine renal receptor sequences are ~80% similar. The deduced peptide sequences exhibit multiple hydrophobic domains flanked by charged residues. There are six Cys residues in the extracellular loops and two N-linked glycosylation sites. Both structures appear well conserved and appear to be important requisites for ligand binding (Ho *et al.,* 1999). There are approximately 1 million calcitonin receptors per osteoclast (Nicholson *et al.,* 1986). All calcitonin receptors bind calcitonin with high affinity, and the alternative ligands, CGRP, amylin, and adrenomedullin, with low affinity. The ligand affinities of different receptors are reviewed in Zaidi *et al.,* 1994. (Fig. 6).

Several calcitonin receptor isoforms have been identified (Egerton *et al.,* 1995). These were demonstrated on the basis of pharmacological studies even prior to their molecular cloning (Alam *et al.,* 1993). Subtypes of the human calcitonin receptor arise from alternative splicing of the primary mRNA transcript of the human calcitonin receptor gene (Nakamura *et al.,* 1995; Moore *et al.,* 1995; Nusenszveig *et al.,* 1995). The gene consists of multiple exons separated by lengthy introns allowing for splicing (Yamin *et al.,* 1994).

The two most common human subtypes isolated from BIN-67 and T47D cells, respectively, differ in the sequence by a 16-amino-acid insert in the first putative intracellular domain (Force *et al.,* 1992; Gorn *et al.,* 1992) (Fig. 7). C1a, or the insert-positive form, is the most prevalent and is capable of transducing intracellular signals at least through G_s and G_q protein-coupled mechanisms (Su *et al.,* 1992; Nussenzveig *et al.,* 1994). The two rat isoforms, C1a and C1b, cloned from the osteoclast differ in the structure of their first extracellular loop (Houssami *et al.,* 1994; Ikegame *et al.,* 1995) (Fig. 7). Two further calcitonin receptors have been cloned from a hypothalamic cDNA library: these consist of 478 and 515 amino acids, respectively (Sexton *et al.,* 1993; Albrandt *et al.,* 1993; Wimalawansa, 1996). The 515-amino-acid-long receptor, has an inframe insert of 37 amino acids.

Little is known about the regulation of calcitonin receptor expression. New and important insights include an effect of calcitonin in inhibiting calcitonin receptor expression through a transcriptional mechanism (Inoue *et al.,* 1999). It is also known that glucocorticoids stimulate calcitonin receptor expression (Wada *et al.,* 1994). Another important observation is that calcitonin receptor expression in osteoclasts is downregulated during osteoclastogenesis (Takahashi *et al.,* 1995). A further more recent demonstration is that M-CSF and NFκB regulate the expression and function of the calcitonin receptor in human osteoclast-like cells (Samura *et al.,* 2000). A further interesting finding, albeit not unexpected, is that calcitonin receptors can be induced to appear in mammary tissue during pregnancy (Tverberg *et al.,* 2000). The physiological significance of this observation is unclear. Finally, the transgenic expression of calcitonin receptor in mice indicates its role in morphogenesis in general, and skeletal development in particular (Jagger *et al.,* 2000).

There are several reports of the cloning of calcitonin receptor-like receptors that bound primarily to CGRP and amylin.

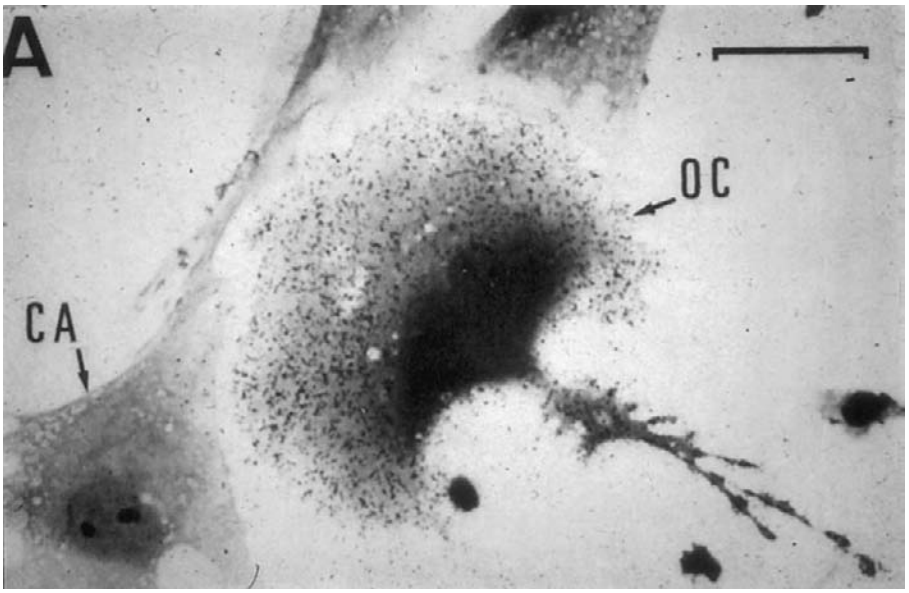

Figure 6 Autoradiograph of an osteoclast incubated with [^{125}I]salmon calcitonin showing a high calcitonin receptor number (1 million per cell). Used with permission from Nicholson *et al.* (1986).

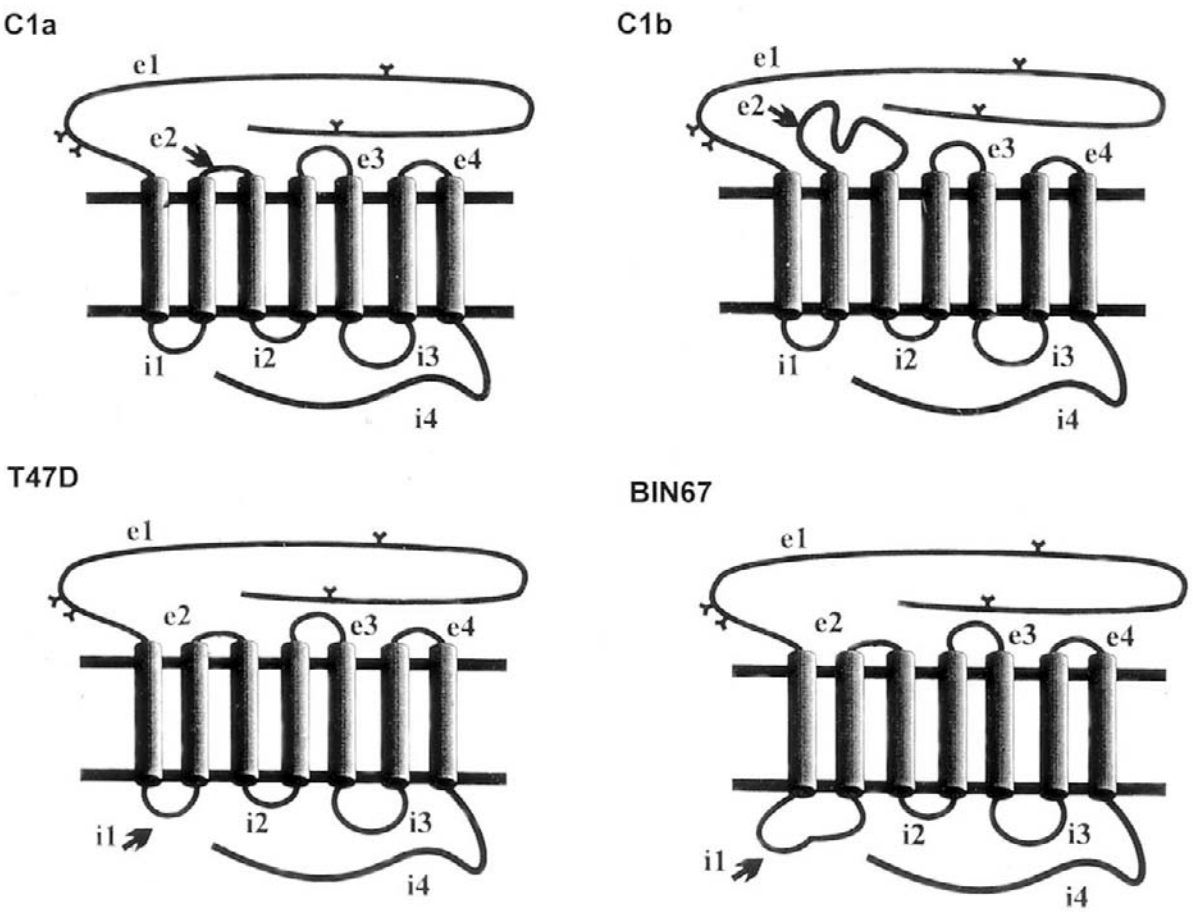

Figure 7 Receptor isoforms of the rat (top) and human (bottom) calcitonin receptors showing the extracellular (e1 to e4) and intracellular (i1 to i4) domains. In C1b, e2 contains an extra 37-amino-acid insert. In the human receptor from BIN-64 cells, i1 contains a 16-amino-acid-long insert that is not present in breast cancer cells, T47D. Used with permission from Martin *et al.* (1994).

The canine orphan receptor gene, RDC-1 gene, was tentatively assigned as being the CGRP-1 receptor (Kapas and Clark, 1995). More recently, Aiyar and coworkers (1996) also cloned and sequenced another CGRP-1 receptor. Like the calcitonin receptors, the CGRP receptors have seven transmembrane domains with up to three N-linked glycosylation sites and several disulfide bridges (reviewed in Wimalawansa, 1996). As with the adrenomedullin receptor, the canine CGRP receptor also has an unusual SIF-FLTCXS sequence, the significance of which is unclear. Finally, a 146-amino-acid linear peptide, the CGRP receptor component peptide, has been predicted from sequence analysis (Luebke *et al.,* 1996). This peptide is thought to bind to and stabilize the CGRP receptor–CGRP complex.

There was an initial suggestion that the osteoclast calcitonin receptor was the Ca^{2+}-sensing receptor on the osteoclast (Stroop *et al.,* 1993). This suggestion was later revoked and it was shown that calcitonin binding was in fact cation-sensitive (Stroop *et al.,* 1993; Stroop and Moore, 1995). It is now becoming clear that osteoclast Ca^{2+} sensing is a function of a uniquely expressed surface ryanodine receptor that is controlled by cyclic ADP-ribose generated from CD38, an ADP-ribosyl cyclase (Zaidi *et al.,* 1995, 1996; Sun *et al.,* 1999). It has also been shown that ryanodine receptors and CD38 are located in the nuclear membrane of bone cells, exemplifying a mechanism through which changes in cytosolic Ca^{2+}, for example triggered through calcitonin receptor activation, are transduced intranuclearly to affect gene transcription (Adebanjo *et al.,* 1999, 2000). Thus, it is likely that the calcitonin and Ca^{2+} receptor systems in the osteoclast interact at several levels of cellular organization. This is, however, by no means proven.

There has been significant new progress on understanding the biology of the calcitonin receptor. This has been pursued through the use of chimeric receptor constructs and site-directed mutagenesis. Orcell *et al.* (2000), by making chimeric receptors between the calcitonin receptor and the insulin-like growth factor receptor, have delineated the regions of the calcitonin receptor that are required for G_s-mediated signal transduction, most notably the third intracellular loop (Fig. 8). Deletion of 14 amino acids in the seventh transmembrane domain has been found not only to abrogate coupling to phospholipase C, but also to alter ligand binding (Shyu *et al.,* 1996). The first intracellular domain and N-terminus also exert a significant impact on both ligand binding and signal transduction (Stroop *et al.,* 1996). Stroop and colleagues have shown, again using a chimeric receptor between the calcitonin and glucagon receptors, that there are two dissociable binding sites for calcitonin (Stroop *et al.,* 1995). Interestingly, chimeric calcitonin-PTH receptors have been shown to be responsive to hybrid calcitonin-PTH ligands that do not activate either wild-type receptor. As indicated above, photoaffinity labeling has been used to understand bimolecular interactions between receptor and ligand. These strategies, taken together, are likely to unravel important tertiary structural prerequisites for the interaction between calcitonin and its receptor.

The possibility that allosteric activation of the calcitonin receptor using a calcitonin receptor mimic that does not occupy the ligand binding site, but instead sensitizes the receptor, is being explored. Preliminary data show that such lead compounds can be synthesized and used *in vivo.* Furthermore, drugs targeting one transmembrane domain over the other may abolish certain receptor functions while sparing others. Finally, because different receptor isoforms may

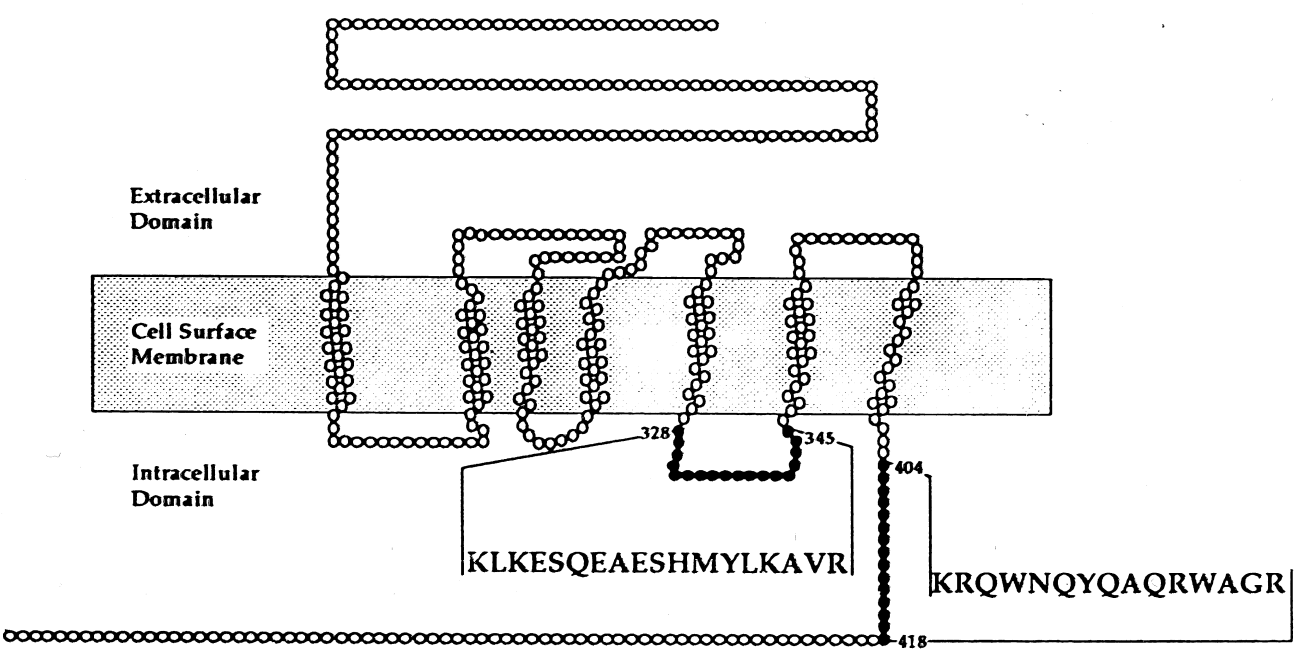

Figure 8 G protein-coupling sequences in the porcine calcitonin receptor. Modified from Orcell *et al.* (2000).

regulate different functions, drugs targeting one isoform over another may reduce side effects or preferentially target forms of osteoporosis that have high or low bone turnover rates. A comprehensive discussion of the structure and molecular biology of the calcitonin receptor is provided by Melson and Goldring in Chapter 34.

Molecular Actions

It has been shown in pharmacological studies with intact osteoclasts that distinct signal transduction pathways, possibly also involving separate calcitonin receptor subtypes, mediate the Q and R effects of calcitonin (Zaidi *et al.,* 1990a). A cholera toxin-sensitive G_s protein mediates the cAMP-dependent Q component (Fig. 9). A pertussis toxin-sensitive G_q protein mediates the R component via a rise in intracellular Ca^{2+}. The Q component is also activated by related peptides, including amylin and CGRP. In contrast, the R effect is highly specific for calcitonin (Alam *et al.,* 1991, 1992, 1993). We and others also provided direct evidence that calcitonin elevates intracellular Ca^{2+} in isolated osteoclasts (Moonga *et al.,* 1992; Teti *et al.,* 1995; Findlay *et al.,* 1995).

Different calcitonin receptor isoforms can couple to G_s, G_q, or G_i (Su *et al.,* 1992; Chabre *et al.,* 1992; Force *et al.,* 1992; Alam *et al.,* 1993; Ransjo *et al.,* 1994). This can also happen in a cell-cycle-dependent manner and may involve divergent structural requirements (Chakraborthy *et al.,* 1991; Houssami *et al.,* 1995). It has also been shown that receptor activity-modifying proteins (RAMPs) can form heterodimers with two G protein-coupled receptors to define ligand-specificity of a given receptor (Derst *et al.,* 2000). Several RAMPs have been cloned (Leuthauser *et al.,* 2000). RAMP-1 can interact with human calcitonin receptor C1b as well as the calcitonin receptor-like receptor. RAMP-3 can interact with the calcitonin receptor and make it amylin receptor-like (Armour *et al.,* 1999).

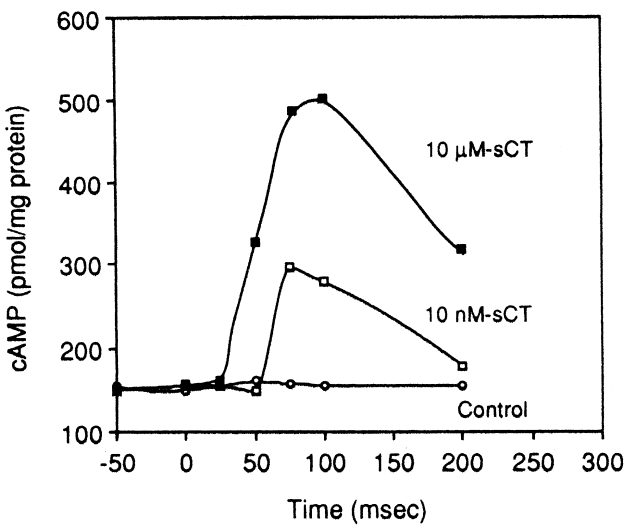

Figure 9 Ultrafast (millisecond) measurements of cyclic AMP in rabbit osteoclasts in response to salmon calcitonin (sCT). Used with permission from Zaidi *et al., Am. J. Physiol.* **271,** F637–F644.

Inhibiting or depleting protein kinase C does not affect the activation of adenylyl cyclase with the G_s-coupled response, indicating that this signal likely occurs through a protein kinase A pathway (Offermanns *et al.,* 1996). This pathway, occurring through the third intracellular loop and C-terminal tail of the calcitonin receptor (Orcell *et al.,* 2000), likely causes the observed cessation of motility or the Q effect. However, calcitonin receptor signaling coupled to G_i that would inactivate adenyl cyclase is suppressed in the presence of activated protein kinase C (Shyu *et al.,* 1999). Thus, protein kinase C activation may act as a positive regulator for signaling through the G_i-coupled pathway. Signaling through G_q activates both phospholipase C and protein kinase C in osteoclasts. This activation with resulting phosphorylation of Shc and ERK1/2 as well as the human enhancer of filamentation (HEF-1 or CasL) likely induces pseudopodial retraction or the R effect (Chen *et al.,* 1998). Finally, it has also been demonstrated that phospholipase D signaling can be triggered by calcitonin receptor activation (Naro *et al.,* 1998). The significance of this pathway is, however, unclear.

As part of the observed pseudopodial retraction, calcitonin also likely regulates adhesion mechanisms in osteoclasts (Fig. 10). Pyk2 is a major adhesion-dependent tyrosine kinase in osteoclasts that associates with c-Src via its SH2 domain (for a review of integrin signaling see Duong *et al.,* 2000). Engagement of the $\alpha_v\beta_3$ integrin, the major integrin present in osteoclasts, increases tyrosine phosphorylation of Pyk2 and leads to the formation of the sealing zone (Duong *et al.,* 2000). Hef-1 or CasL is a focal adhesion-associated, multiple-domain docking protein. It contains an SH3 domain that binds focal adhesion kinase (FAK) and Pyk2. Transduction from the calcitonin receptor that activates Hef-1 but not $p130^{cas}$ is dependent upon protein kinase C (and thus the G_q-coupled calcitonin receptor), but is independent of the G_s/cAMP/protein kinase A pathway (see Fig. 5) (Zhang *et al.,* 1999). Nevertheless, integrin engagement, actin cytoskeletal rearrangement, and c-*src* activation are required for calcitonin-induced tyrosine phosphorylation of paxillin and HEF1, but not for Erk1/2 phosphorylation (Zhang *et al.,* 2000). Activation of HEF-1 by calcitonin may therefore cause interactions with Pyk2 and other molecules that regulate osteoclast adhesion, although this remains to be established.

Calcitonin also has effects in other tissues that relate indirectly to bone remodeling. The enzyme 25-hydroxyvitamin D_3 1α-hydroxylase (CYP27B1) catalyzes the biosynthesis of 1α, 25-dihydroxyvitamin D_3 from 25-hydroxyvitamin D_3 in renal proximal tubules. Calcitonin has been shown to increase the mRNA expression of the CYP27B1 enzyme and thus could help regulate the production of active vitamin D_3 (Yoshida *et al.,* 1999). In addition, Baron's group have clearly demonstrated changes in localization of critical ion exchangers in renal tubular cells by calcitonin (Chakraborty *et al.,* 1994).

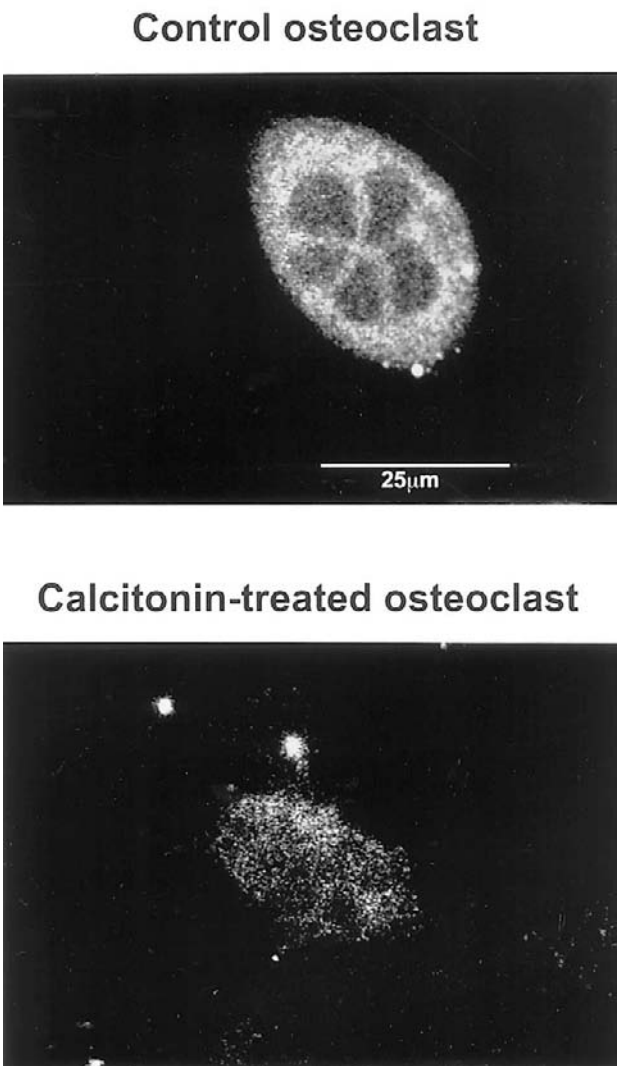

Figure 10 Calcitonin diminishes focal adhesion kinase (FAK) expression in isolated osteoclasts. Used with kind permission from Dr. H. K. Datta, Newcastle-upon-Tyne, England.

Clinical Pharmacology

Calcitonin's utility as a pharmaceutical agent is due to its inhibitory effect on osteoclastic bone resorption (review: Adebanjo *et al.,* 1998). Its efficacy has been demonstrated by the immediate decrease of urinary resorption markers that occurs within few a hours following the administration of pharmacological doses. Subcutaneous injection of 100 IU salmon calcitonin to osteoporotic subjects results in maximal inhibition of bone resorption within 8–16 hr (Gonzalez *et al.,* 1986). The higher the rate of bone turnover, the earlier and the more pronounced are the biochemical changes. Generally calcitonin's duration of action does not exceed 24 hr. For this reason, Paget's disease served as one of the first human models for testing the efficacy of calcitonin. After a subcutaneous or intramuscular injection, the small transient calcium lowering effect of calcitonin is accompanied by a transient rise of PTH which lasts for about 12 hr (Burckhardt *et al.,* 1973; Fournie *et al.,* 1977)

For achieving any drop in plasma calcium in healthy subjects, relatively high calcitonin doses are required (Buclin *et al.,* 1987; Thamsborg *et al.,* 1990). When calculated as area under the curve, the hypocalcemic response shows a significant dose relationship (Overgaard *et al.,* 1991), indicating that even relatively small doses might exert significant effects on bone metabolism.

After each dose of calcitonin, parathyroid hormone increases transiently, for less than 24 hr, in response to the transient fall in plasma calcium (Burckhardt *et al.,* 1973). Basal levels of plasma calcium and PTH are unchanged in the setting of longer periods of exposure. When unchallenged by concomitant oral calcium, this rise in PTH is associated with a nocturnal rise in bone resorption (Burckhardt *et al.,* 1988; Abbiati *et al.,* 1994; Vega *et al.,* 1989).

In addition to the injectable forms, salmon calcitonin has also been used as a nasal spray and rectal suppository (Kollerup *et al.,* 1994; Kraenzlin *et al.,* 1996). The latter two forms exhibit almost identical short-term effects in healthy subjects (Buclin *et al.,* 1987). Eel calcitonin has also been used effectively as a rectal suppository.

For practical clinical reasons, attempts have been made to establish bioequivalence for the nasal and injectable forms of salmon calcitonin. For clinical usage, 200 IU as nasal spray is deemed almost equivalent to 50 IU intramuscular. However, the data supporting its usage is fraught with discrepancies (Overgaard *et al.,* 1991). For example, nasal salmon calcitonin at 400 IU inhibits the increase in bone resorption caused by immobilization only partially, while 100 IU given intramuscularly exerts a total inhibition (Tsakalakos *et al.,* 1999; Van der Wiehl *et al.,* 1993). Furthermore, in Paget's disease, 50 IU subcutaneous salmon calcitonin and 400 IU nasal spray are equally effective as hypocalcemic agents (Vega *et al.,* 1989). Taken together, the data over the past 20 years show that calcitonin when administered subcutaneously is effective, while the nasal formulation is variable. For obtaining the same hypocalcemic effect with nasal calcitonin, up to 30 times higher dosages of salmon calcitonin must therefore be administered intranasally (O'Doherty *et al.,* 1990).

Given as a rectal suppository, salmon calcitonin shows the same acute biologic effects as when given as the nasal spray, although it provoked higher plasma levels of calcitonin (Buclin *et al.,* 1987). When compared with intramuscular administration, the dose equivalence of eel and salmon calcitonin was 50% (Fiore *et al.,* 1992; Pagani *et al.,* 1991). At high doses, rectal eel calcitonin provokes a transient hypocalcemia even in healthy subjects (Buclin *et al.,* 1987; Nuti *et al.,* 1992).

Oral preparations of analogs, such as ASC 710, have been tested in Paget's disease and have had the same effect to salmon calcitonin given by injection, but with an approximate dose equivalence of 100:1 (Devogelaer *et al.,* 1994).

Hypercalcemia

The usefulness of calcitonin as a therapy for acute hypercalcemia is covered elsewhere in this book (see Chapter 60).

Paget's Disease

As the first effective drugs for the treatment of Paget's disease, human and animal calcitonins (salmon, pork, eel) were unchallenged until the bisphosphonates became available. In Paget's disease, salmon calcitonin can be given over long periods at doses of 50–100 IU by subcutaneous or intramuscular injections three to seven times weekly; human calcitonin can be used at 100 IU (0.5 mg) per day. Treatment lowers biochemical markers of bone turnover by about 30–50%, relieves bone pain, and can lead to radiological healing of pagetic lesions (Murphy *et al.,* 1980). Even reversal of neurological deficits has been observed at times (Chen *et al.,* 1979), as has the stabilization of hearing loss, and improvement in abnormalities in cardiac output. However, the efficacy of the treatment is limited. First, bone turnover returns to normal only in mild cases. Second, the short duration of action of the drug leads invariably to a recurrence of disease manifestations after each interruption of therapy, heralded by a rise in markers of bone turnover, reappearance of hyperthermic bones, and hyperactive bones by bone scintigraphy. Unlike the potent bisphosphonates, with which one course of treatment can eliminate a whole generation of osteoclasts, calcitonin inhibits osteoclast function for several hours per day only, and decreases osteoclast number only when given over longer periods. The nasal spray formulation of calcitonin requires relatively high doses, such as 400 IU (Nagant *et al.,* 1987; Reginster *et al.,* 1992).

Treatment with calcitonin is limited further by two other effects. First, a plateau is reached in some patients, where no further improvement can be achieved (Singer *et al.,* 1990). A second limitation is due to the appearance of circulating antibodies against the animal calcitonins (Singer *et al.,* 1972). As explained later, these antibodies will neutralize the pharmacological effect of the drug in those few patients with very high titers. In these cases, switching to human calcitonin can reestablish responsiveness (Rojanasathit *et al.,* 1974). Antibody formation does not explain all cases of calcitonin resistance. Although transient loss of receptor sensitivity after calcitonin is known to occur, persistent lack of responsiveness to the usual therapeutic doses remains unexplained (Singer *et al.,* 1980).

Although largely replaced by the bisphosphonates in the treatment of Paget's disease, calcitonin represents an alternative when long-term safety considerations are raised as in younger populations (Tuysuz *et al.,* 1999).

Osteoporosis

Calcitonin is used widely as an antiresorptive agent in the treatment of osteoporosis. Some studies have demonstrated that it prevents bone loss, or even increases bone mass while lowering the incidence of vertebral fractures. Its effect on cortical bone is inconsistent in the forearm and absent in the femoral neck. Since calcitonin inhibits osteoclast activity transiently, markers of bone turnover are not as much reduced as with bisphosphonates. After cessation of treatment, bone markers return rather quickly to baseline levels (Kraenzlin *et al.,* 1996). The modest and transient effects of calcitonin on bone markers make them clinically impractical to use in the context of therapy.

In situations of accelerated bone turnover and bone loss, such as in acute immobilization, after fracture or after organ transplantation, nasal spray calcitonin was ineffective (Uebelhart *et al.,* 1999; Valimaki *et al.,* 1999), while 100 IU given by intramuscular injection had some benefit (Tsakalakos *et al.,* 1993). Vertebral bone loss due to corticosteroid therapy can be prevented by daily intramuscular injections and also by the nasal spray formulation (Adachi *et al.,* 1997). In perimenopausal or in early menopausal states, as well as later in the menopause, various regimens have been useful (Arnala *et al.,* 1996; Gennari *et al.,* 1992; Ellerington *et al.,* 1996; Overgaard *et al.,* 1994; Reginster *et al.,* 1995). Rectal administration is also effective (Kollerup *et al.,* 1994), but not practical for long-term use because of the frequency of local irritation.

The therapeutic endpoints of the treatment of osteoporosis are in increase in bone mass and a decrease in fracture incidence. 100 IU of injectable salmon calcitonin increases bone density, but only during the first 18 months of therapy. This dosage also increases cortical bone density (Crespo *et al.,* 1997). However, for reasons of compliance, long-term studies have been performed primarily with nasal spray calcitonin. With the nasal form, 100 IU of salmon calcitonin was insufficient (Adami *et al.,* 1995). However, when calcitonin was given together with calcium and vitamin D, the nasal formulation increased vertebral bone mineral density significantly in several studies (Overgaard *et al.,* 1995).

Calcitonin has reduced the incidence of vertebral fractures in several small studies (Rico *et al.,* 1995, Overgaard *et al.,* 1992), but significance could be achieved only when the results of several studies were pooled together (Burckhardt *et al.,* 1993; Kanis *et al.,* 1999). Again, nasal spray calcitonin was required for effectiveness to be demonstrated. The largest prospective clinical trial to determine the efficacy of calcitonin in postmenopausal osteoporosis, the PROOF study, was recently published by Chesnut *et al.,* (2000). The use of nasal calcitonin at only the 200-IU dose, and not at the 100- or 400-IU dose, was associated with a significant reduction in vertebral fracture incidence. There was a minimal change in bone mineral density and only a modest effect to reduce indices of bone resorption. It is not clear how the results of this study are best interpreted. The lack of a substantial effect on bone mass and on bone markers suggest that calcitonin might have reduced vertebral fracture incidence by skeletal effects that are not yet quantifiable by available measurements.

Calcitonin has also been used in other bone diseases, such as multiple myeloma, bone metastases, osteogenesis imperfecta, juvenile osteoporosis, osteopathy in thalassemia, reflex sympathetic osteodystrophy, spinal stenosis, rheumatoid arthritis, osteoporosis of biliary cirrhosis, and other disorders.

The Analgesic Effect of Calcitonin

In patients who have sustained an acute vertebral crush fracture, calcitonin has an apparent analgesic effect. Controlled, double-blinded studies have shown that calcitonin decreases pain and the use of analgesics, accelerates mobilization, and shortens length of hospitalization. This analgesic effect appears as early as the second day of treatment and cannot be readily explained by antiresorptive actions alone. Calcitonin was also efficient in treating pains due to bone metastases (Allan, 1993; Schiraldi *et al.,* 1987). Its effect on Paget's disease is related also to this analgesic property.

Binding sites for calcitonin in several areas of the brain might be related to its antinocioceptic action. Mechanisms include serotoninergic or adrenergic actions, modification of calcium fluxes in neuronal membranes, beta-endorphin production, cyclooxygenase inhibition, and histamine interference (Braga, 1994). The analgesic effects of calcitonin are likely to be multifactorial with evidence for involvement of beta endorphin not yet clear (Laurian *et al.,* 1986; Francescini *et al.,* 1989; Overgaard *et al.,* 1991; Vescovi *et al.,* 1990).

The analgesic effects have been seen via injection (Lyritis *et al.,* 1991), nasal spray (Pun *et al.,* 1989; Lyritis *et al.,* 1997), or rectal suppository (Lyritis *et al.,* 1999). The effectiveness seems to be limited to 2–4 weeks. In oncology, an analgesic effect was reached with very high doses (Schiraldi *et al.,* 1987).

Problems and Adverse Effects

There are several problems with calcitonin administered to humans via the nasal, oral, or rectal routes. Response variation is the main issue and this is shared with other large peptides, such as insulin or parathyroid hormone. Highly charged and folded peptides cannot effectively cross cell membranes (Zaidi *et al.,* 1992a). Another problem is that of rapid compartmentalization of absorbed peptide, particularly when the absorption is slow. It has been speculated that circulating monomeric calcitonin is in direct or indirect communication with several compartments (Zaidi *et al.,* 1991; MacIntyre *et al.,* 1990). The transfer coefficients into these compartments are unknown, although one compartment could possibly be the receptor itself (MacIntyre *et al.,* 1990; Hee *et al.,* 2000). A further complication is that calcitonin in the circulation binds to plasma proteins. This effect may be reversible, but again, the rates of association and dissociation are unknown. Thus, the measurement of plasma calcitonin levels at a certain time point may reflect the amount of biologically active calcitonin in one compartment, but not another.

A further problem with long-term treatment of humans with a nonhuman calcitonin (salmon, porcine, and eel) is the development of antibodies (Haddad and Caldwell, 1972; Plehwe *et al.,* 1977; Tagliaro *et al.,* 1995; Singer *et al.,* 1972, 1980). The incidence of antibody formation is directly proportional to the duration of treatment. Within 6 months of salmon calcitonin treatment, 40% of patients develop antibodies. The incidence increases by 10% with every additional 6 months of treatment (Muff *et al.,* 1991; Reginster *et al.,* 1993).

It has been demonstrated by various groups that low antibody titers do not attenuate the effect of the drug; that is, they do not decrease the reduction in bone turnover in Paget's disease (Plehwe *et al.,* 1977), do not diminish the transient hypocalcaemic response seen after each administration (Muff *et al.,* 1990; Hosking *et al.,* 1979), and do not reduce the efficacy of calcitonin in preventing a decline in bone mineral density (Tagliaro *et al.,* 1995; Reginster *et al.,* 1993). Calcitonin-induced cyclic AMP formation (in a human breast cancer cell line) also remains unaffected (Grauer *et al.,* 1990).

However, there is a linear correlation between antibody titer and the serum dilution required for half-maximal inhibition of cAMP production (Muff *et al.,* 1991). At high titers, therefore, antibodies do reduce the efficacy of the drug. This explains the resistance to salmon calcitonin first observed in Paget's patients (Woodhouse *et al.,* 1977; Singer *et al.,* 1980). Patients who become resistant to salmon calcitonin usually respond well when switched to the human peptide, as shown with patients with Paget's disease (Grauer *et al.,* 1990; Muff *et al.,* 1990; Altman *et al.,* 1987). Antibodies to human calcitonin are rare (Dietrich *et al.,* 1979). Only 1 out of 33 patients with osteoporosis treated with human calcitonin developed neutralizing antibodies, and the neutralizing effect was comparatively mild compared with the effects of antibodies against salmon calcitonin (Grauer *et al.,* 1993).

High antibody titers do not provide an explanation for all cases of calcitonin resistance. In fact, it has been shown that antibodies to eel calcitonin for example, do not impact on efficacy; instead the pharmacokinetics of the drug becomes altered (Tagliaro *et al.,* 1995). Another factor is the loss of responsiveness to calcitonin due to receptor downregulation. Calcitonin inhibits the synthesis of its own receptor (Lee *et al.,* 1995; Rakapoulos *et al.,* 1995; Liu *et al.,* 2000). Notably, calcitonin-dependent downregulation of the C1a osteoclastic receptor has been shown to involve a transcriptional mechanism (Inoue *et al.,* 1999). There is also significant evidence of homologous desensitization of the calcitonin receptor (Schneider *et al.,* 1993). Additionally, it has been shown that postmenopausal women treated with calcitonin had decreased calcitonin receptor mRNA levels compared to placebo-treated counterparts (Beaudreuil *et al.,* 2000). In these women, prevalence of calcitonin receptor-2 was inversely proportional to bone turnover rates (Beaudreuil *et al.,* 2000).

Current and Future Therapeutics

Several prospective controlled trials have also documented stabilization of, and in some cases modest short-term increases in, bone mineral density in osteoporotic patients treated for 5 years or less (Gruber *et al.,* 1984; Mazzuoli *et al.,* 1986; Reginster, 1993). Much of this benefit has been observed in trabecular bone; the benefit of calcitonin in reducing cortical bone loss has not been clearly demonstrated

(Reginster, 1993; Overgaard *et al.,* 1995). Similar effects have been demonstrated utilizing calcitonin as a preventive treatment for menopausal trabecular bone loss (Reginster, 1993; Mc Dermott *et al.,* 1987). Three studies directly evaluating the impact of calcitonin on fracture reduction have all shown significant fracture reduction. This benefit was observed both in postmenopausal and elderly women (Overgaard *et al.,* 1995; Kanis *et al.,* 1992; Rico, 1992). Long-term studies using calcitonin reveal a 1.2% increase in bone mineral density at the spine over 5 years (Chesnut, 2000).

Compromised trabecular microarchitecture is an important and independent causal factor in the pathogenesis of vertebral fractures in both men and women (Legrand *et al.,* 2000). It is now emerging that there is a temporal dissociation between reduction in fracture risk that can occur between 12 and 18 months, bone markers, and long-term effects on bone mineral density. Notably, a benefit in fracture risk occurs before a change in bone mineral density. Recent clinical trials with antiresorptives, such as calcium plus vitamin D, calcitonin, and raloxifene, indicate significant protection from fracture despite only modest increases in bone mineral density. This has led to the belief that anti-resorptives, such as calcitonin, by preventing osteoclastic resorption, conserve bone microarchitecture (Dempster, 2000).

It is not that calcitonin is an inactive drug (MacIntyre and Zaidi, 1990); rather the problem of efficacy arises almost purely from its bioavailability (Lee and Sinko, 2000). Several new approaches are currently being used to enhance calcitonin access to the osteoclast, its target cell. First, there is interest in the development of novel allosteric activators of the calcitonin receptor with a goal of making the receptor more sensitive to changes in circulating calcitonin (see above) (Chen *et al.,* 1999). This strategy has been achieved for the parathyroid cell Ca^{2+}-sensing receptor, another G protein-coupled seven-pass receptor, whereby an oral calcimimetic (or Ca^{2+} receptor agonist) is being evaluated for use in hyperparathyroidism (Silverberg *et al.,* 1997; Collins *et al.,* 1998; Goodman *et al.,* 2000). The latter drug shifts the PTH–serum calcium response curve to the right, and hence, by increasing the sensitivity of the Ca^{2+} sensing receptor to serum Ca^{2+}, decreases PTH secretion.

Second, there is the possibility of modulating the release of endogenous calcitonin itself by a drug that activates the C-cell Ca^{2+} sensing receptor, again through an allosteric mechanism, by making it more sensitive to circulating serum Ca^{2+}. However, this approach is unlikely to succeed, as the calcimimetic is likely also to depress PTH secretion through the parathyroid cell Ca^{2+} receptor.

Third, eminent is the development of an oral calcitonin that is coupled to a molecule that will enable absorption and prevent breakdown, but retain biological activity (Lee *et al.,* 2000; Dogru *et al.,* 2000). These compounds are also likely to be absorbed in a site-specific manner, for example, in the colon (Tozaki *et al.,* 2001). There is the likelihood of a polyethylene glycol-modified salmon calcitonin that has significant biological activity and prolonged plasma half-life (Yoo *et al.,* 2000). Pulmonary and transdermal routes are also under consideration (Chang *et al.,* 2000; Patton, 2000). Finally, there are now gene therapy vectors available that would allow osteoclast precursors to carry the calcitonin gene to specific bone sites of osteolysis. The next few years should therefore see the revival of calcitonin as an important therapeutic agent.

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CHAPTER 83

Growth Hormone and Insulin-like Growth Factor-I Treatment for Metabolic Bone Diseases

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Introduction

The growth hormone (GH)/insulin-like growth factor-I (IGF-I) regulatory system, which includes endocrine/paracrine/autocrine effects of IGF-I, is involved in determining bone size, length, density and architecture of the mature mammalian skeleton. As noted in an earlier chapter, skeletal IGF-I, in concert with other determinants, regulates osteoblast recruitment and differentiation. Although it is still not clear exactly how the effects of IGF-I partition in respect to skeletal regulation, it is likely that both types of action (i.e., endocrine and autocrine/paracrine) are essential for acquisition and maintenance of peak bone mass (see Fig. 1). Indeed, the importance of the endocrine aspects of IGF-I in respect to bone modeling and remodeling have recently been highlighted. For example, several studies have shown that serum levels of IGF-I correlate with bone mineral density (BMD), cross-sectional area of the femur, risk of future hip fracture, and a polymorphism in the IGF-I gene (Donahue and Rosen, 1998; Boonen *et al.,* 1997; Langlois *et al.,* 1998; Nicholas *et al.,* 1994; Sugimoto *et al.,* 1997; Bauer *et al.,* 1998; Rosen and Pollak, 1999; Garnero *et al.,* 2000; Kurland *et al.,* 1997). In mice, serum IGF-I cosegregates with the BMD phenotype, is directly correlated with femur length, and has been associated with a marked increase in bone formation indices (Rosen *et al.,* 2000). Moreover, changes in skeletal expression and content of IGF-I reflect serum levels of this peptide (Rosen *et al.,* 2000). Hence, circulating and tissue expression of IGF-I are important in maintenance of skeletal homeostasis, and thereby provides a significant rationale for considering the administration of either GH or IGF-I as a potential anabolic agent for the treatment of metabolic bone disorders associated with low bone formation. In this chapter, I will review the evidence for and against the use of these peptides in bone diseases such as osteoporosis.

Growth Hormone Therapy for Osteoporosis

Physiology of GH

Overview of GH Secretion

The regulation of growth hormone secretion from the pituitary is complex and involves the elaboration of discrete neurosecretory peptides from the hypothalamus. These include growth hormone-releasing hormone (GHRH) and other growth hormone-releasing peptides (GRPs). Somatostatin (SMS), a small but ubiquitous polypeptide, inhibits GH synthesis and secretion, thereby tightly controlling GH production. Both neuropeptides (GHRH and SMS) are synthesized in specialized hypothalamic neurons, released from axon terminals in the median eminence, and transported through the hypophyseal portal circulation into the anterior pituitary (Cronin and Thorner, 1989). GH secretion is pulsatile (due to episodic release of GHRH) and circadian, with the highest pulse amplitude occurring between 02:00 and 06:00 (Winer *et al.,* 1990; Ho and Weissberger, 1990). Puberty has a dramatic effect on the amplitude of GH pulses,

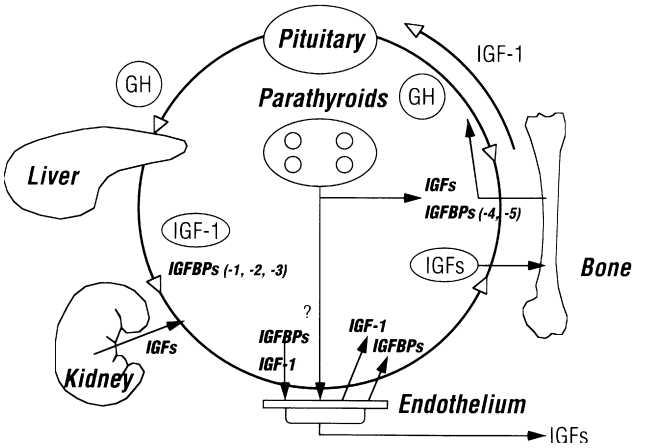

Figure 1 The circulating IGF regulatory system represents the sum of IGFs produced by the liver, kidney, and skeleton, as well as IGFBPs which come from various tissue sources and are regulated by a myriad of autocrine, paracrine, and endocrine factors. The endothelium serves as a site for passage of IGFs and IGFBPs to and from the circulation.

due to changes in the hypothalamic milieu as a result of rising sex steroid concentrations (Jannson *et al.,* 1985). Apart from tight neuroendocrine regulation of GH secretion by SMS and GHRH, there is a negative feedback loop on GHRH and GH by IGF-I.

Effects of Gonadal Status on GH/IGF-I

The pattern of GH secretion in animals and humans depends highly on age and sex (Cronin and Thorner, 1989; Winer *et al.,* 1990; Ho and Weissberger, 1990). Both factors strongly influence the frequency and amplitude of GH pulses, GH basal secretory rates, and the levels of serum IGF-I. Characteristic changes during puberty in rats parallel pubertal changes in humans (Jannson *et al.,* 1985). GH secretion in male and female rats is identical after birth but at puberty, a sexually differentiated pattern of secretion appears, with male rats displaying high-amplitude low-frequency pulses, and female rats displaying pulses of high frequency but low amplitude (Jannson *et al.,* 1985). This sexual dimorphism can be altered by manipulating the gonadal steroid environment, suggesting that sex steroids are important modulators of GH secretion.

In humans, sexual differences in GH secretion during puberty are less pronounced, even though administration of gonadal steroids to prepubertal children increases GH pulses and mimics the pubertal milieu of the hypothalamus. Various sampling techniques (profiles versus stimulatory tests) and assays with different sensitivities, have produced disparate findings. However, spontaneous and stimulated GH peaks in humans are enhanced during puberty. Matched for age and body mass index, young girls were found to have higher integrated GH (IGHC) levels than boys (Ho and Weissberger, 1990; Weissberger *et al.,* 1991). Other secretory characteristics, including pulse amplitude, frequency, and the fraction of GH secreted as pulses (FGHP), were similar in both sexes of the same age. In a preliminary study, black adolescents (males and females) had higher GH secretory rates than age-matched whites (Wright *et al.,* 1994). Higher GH secretion rates in adolescent blacks could lead to greater acquisition of bone mass.

Gonadal steroids affect GH secretion in perimenopausal women. IGHC, mean pulse amplitude of GH and FGHP are lower in older women than premenopausal women (Weissberger *et al.,* 1991). GH secretory indices in postmenopausal women correlate with serum estradiol, but not with total serum androgen levels. During menopause, GH secretion is reduced (Ho and Weissberger, 1990; Weissberger *et al.,* 1991). However, oral administration of estradiol (or conjugated equine estrogens) increases GH secretion as a result of reduced hepatic generation of IGF-I (Ho and Weissberger, 1990; Jannson *et al.,* 1985; Weissberger *et al.,* 1991; Wright *et al.,* 1994; Dawson-Hughes *et al.,* 1986). On the other hand, transdermal administration of 17-β estradiol, increases serum IGF-I concentration, suggesting that suppression of IGF-I by oral estrogens is due to "first-pass" hepatic effects (Weissberger *et al.,* 1991). Impaired IGF-I generation in the liver removes a key component of negative feedback on the hypothalamus, resulting in increased GH release (Bellantoni *et al.,* 1991).

Effects of Age on GH–IGF-I

The GH–IGF-I axis undergoes changes over a life span so that elders have lower spontaneous GH secretion rates and serum IGF-I levels than younger people (Rosen *et al.,* 1990). These age-related differences are a function of an altered hypothalamic–pituitary set point due in part to changes in lifestyle and nutrition (Ghigho *et al.,* 1993). The GH secretory response to common stimuli such as GHRH, clonidine, L-dopa, physostigmine, pyridostigmine, hypoglycemia, and met-enkephalin, but not arginine, are reduced by aging (Carpas *et al.,* 1993). Somatotrope responsiveness to GHRH and arginine does not vary with age, implying that the maximal secretory capacity of somatotropic cells is preserved in elderly people (Carpas *et al.,* 1993; Kelijman, 1991).

There is fairly strong evidence that GH and IGF-I concentrations decline with advanced age although several large cross-sectional studies have either failed to demonstrate an association between diminished serum IGF-I and age-related bone loss or a weak association between serum IGF-I and bone mineral density (Donahue and Rosen, 1998; Boonen *et al.,* 1997; Langlois *et al.,* 1998; Nicholas *et al.,* 1994; Bennett *et al.,* 1994). On the other hand, concentrations of IGF-I, IGF-II, and IGFBP-5 in femoral cortical and trabecular bone decline significantly with age (Donahue and Rosen, 1998; Boonen *et al.,* 1997; Langiois *et al.,* 1998; Nicholas *et al.,* 1994).

Mechanisms of GH Action on Bone

Growth hormone has direct and indirect effects on bone, depending on age and skeletal maturity. Indirectly, GH can enhance BMD through its effects on muscle mass and calcium transport in the gut. In addition, growth hormone can directly

stimulate bone remodeling and increase endochondral growth through its actions on the osteoblast. For example, in orchiectomized rats, GH stimulates bone formation and bone density (Prakasam *et al.,* 1999). Similarly, administration of GH and or IGF-I, without estradiol, can enhance bone formation and trabecular bone volume in ovariectomized rats (Verhaeghe *et al.,* 1996). Overall, GH is considered essential for both the growth and maintenance of skeletal mass. This is especially evident in growth hormone deficiency (GHD) states, whether it be in childhood or adulthood, in which there is reduced BMD. In one of the largest observational trials to date, KIMS, GHD was linked to a marked increase in fracture risk compared to that seen in age-matched normals (Rosen, 1997).

Substantial differences between the direct and indirect effects of GH on the osteoblast partially explain changes in skeletal responsiveness to GH and IGF-I. For example, exogenous GH stimulates longitudinal growth in normal rats, but rhIGF-I does not. Similarly, transgenic mice that overexpress GH grow to twice their normal size while transgenic IGF-I mice are not longer, and exogenous administration of IGF-I is far less efficient in stimulating long bone growth (Guler *et al.,* 1988). Thus, despite the fact that GH induces IGF-I production in the skeleton and elsewhere, treatments with GH and with IGF-I are not equivalent. In general, skeletal responses to GH and IGF-I depend on the species, the GH status of the animal, and the mode of administration. Even the systemic side effects of rhGH and rhIGF-I therapy may differ substantially.

Effects of GH on Bone Mineral Density and Bone Turnover

GH Treatment for GHD Children

Early clinical experiences with rhGH in GHD children provided investigators with a model for studying skeletal responsiveness to somatotropin. Intermittent (daily or three times weekly) injections of rhGH results in a prolonged and sustained GH profile with resultant catch-up growth evident during the first year of treatment (Rappaport and Czernichow, 1993). This increase in skeletal growth is accompanied by a rise in serum levels of type I procollagen peptide (Carey *et al.,* 1985). Although dosage schemes vary between the United States and Europe (0.1 mg/kg/tiw [US] to 0.7 U/kg/week [Europe]), there is a strong dose-related growth response to rhGH (DeMuinck Keizer-Schrama *et al.,* 1992).

The skeletal response to GH depends on several factors including: (i) GH secretory status, (ii) pretreatment IGF-I levels, (iii) pretreatment height velocity, and (iv) GH dosage. The rate of change in serum IGF-I (rather than the absolute level of IGF-I attained by GH treatment) is a relatively nonspecific predictor of growth as are procollagen I and osteocalcin concentrations (DeMuinck Keizer-Schrama *et al.,* 1992). Serum procollagen III levels do correlate with growth rates during GH treatment (Carey *et al.,* 1985).

Linear growth is a measurable response to exogenous GH, but changes in bone mineral density in children are more difficult to quantify. In some studies, bone mineral content is increased during GH treatment to a greater extent than expected for change in bone size (Inzucchi and Robbins, 1994). However, it should be remembered that short stature, which is the central phenotypic component of GHD in children, leads to an underestimation of true bone mineral density. In general, a 20-cm difference in body height is associated with an approximately 1 SD decline in BMD. However, this effect can be taken into account and an areal density corrected for size (BMAD) can be calculated for the femur or vertebrae. Similarly, pQCT and QCT measurements demonstrate that GHD children have low volumetric BMD, which responds to GH treatment. In one of the longest intervention trials to date, 26 GH-deficient children were given rhGH (0.6 IU/kg per week) for 12 months (Saggese *et al.,* 1993). Baseline radial bone mineral content (BMC) *Z* scores (corrected for their chronological, statural, and bone ages) were significantly reduced, as were serum osteocalcin and procollagen peptide levels. Treatment with rhGH six times per week increased BMC and normalized *Z* scores of the radius in nearly 50% of the subjects. Serum levels of procollagen peptide during the first week of treatment were positively related to growth velocity at 6 and 12 months and radial BMC at 12 months. Although there are striking differences between longitudinal growth in children and remodeling in adults, criteria that determine skeletal responsiveness in children may also be relevant for the effects of rhGH on the skeleton in older individuals.

GH Administration to Healthy Adults

The treatment of adult GHD is a relatively new option for patients with low BMD. Yet some of the same principles of treatment hold for adults as for children with GHD. Evidence from several longitudinal controlled trials provides insight into the expected responses of different adult populations to rhGH. These include: (i) healthy adults, (ii) GHD adults, (iii) elderly men and women with/without osteoporosis. Initial studies with rhGH in adults focused primarily on changes in body composition. Short-term treatment with rhGH leads to a decrease in adiposity and an increase in lean body mass (Crist *et al.,* 1991). There is also a marked shift in extracellular water (Holloway *et al.,* 1994). Detailed analysis of skeletal markers during GH treatment was first reported by Brixen *et al.* (1990). Twenty male volunteers (ages 22–31) were given a relatively large dose (0.1 IU/kg) of rhGH twice daily for 7 days. Serum osteocalcin increased after 2 days of treatment and remained elevated for 6 months. Bone alkaline phosphatase decreased initially (during the 7 days of GH treatment) but then increased slightly over 6 months (Brixen *et al.,* 1990). Serum calcium and phosphate increased but only during the 7-day treatment phase. Like bone formation indices, urinary markers of bone resorption (urinary Ca/Cr and hydroxyproline/creatinine) rose during treatment and remained elevated for up to 4 weeks after discontinuation of therapy.

Treatment with rhGH stimulates bone remodeling. More importantly, the anabolic effect on bone may persist well beyond discontinuation of growth hormone. Early (2-day)

and late (2-week) osteocalcin responses imply that GH can stimulate existing osteoblasts and enhance recruitment of new osteoblasts. Very recently, Biller *et al.* (2000) demonstrated that after discontinuation of rhGH in GHD, patients still acquired BMD at a significant rate. Still, it is uncertain if those effects are mediated through IGF-I. For example, Brixen *et al.* (1990) was unable to find a significant correlation between the rise in serum IGF-I and an increase in osteocalcin or bone alkaline phosphatase. The absence of a significant correlation between bone formation markers and serum IGF-I, however, may be due to the low skeletal specificity of serum IGF-I.

Skeletal resistance to GH has been considered a possible cause for postmenopausal osteoporosis. On the basis of one double-blinded rhGH trial in postmenopausal women, this is unlikely. Kassem *et al.* (1994) noted that administration of rhGH (0.2 IU/kg/day) for 3 days increased serum IGF-I, osteocalcin, and procollagen type I C-terminal propeptide (PICP) to the same extent in 15 women with severe postmenopausal osteoporosis as in 15 age-matched control women. Serum and urinary markers of bone resorption also did not differ between the two groups. *In vitro* studies of marrow stromal cells from osteoporotic women demonstrate full GH responsiveness (Kassem *et al.,* 1994). Therefore, it is unlikely that the osteoporotic skeleton is resistant to rhGH treatment.

GH Treatment for Growth Hormone-Deficient Adults

Generalized GH Actions during Treatment in Adult GH Deficiency Growth hormone deficiency can be documented by provocative stimuli (GHRH, insulin, glucagon) and serial GH measurements. The majority of adult patients treated with rhGH have either idiopathic GHD or a history of previous central nervous system (CNS)/pituitary–hypothalamic tumors. Early trials with rhGH replacement therapy examined changes in muscle mass, muscle strength, and body fat. Daily administration of subcutaneous rhGH to GHD patients produced a marked rise in serum IGF-I and an increase in muscle mass and basal metabolic rate (Jorgensen *et al.,* 1991). Some of those anabolic changes were noted soon after the initiation of rhGH. For example, mean nitrogen retention during the first 15 days of rhGH treatment was as much as 2.8 g per day (approximately 20 g of muscle mass) (Valk *et al.,* 1994). GH treatment can also increase the total cross-sectional area of thigh muscles and quadriceps as well as improve hip flexors and limb girdle strength (Jorgensen *et al.,* 1991; Cuneo *et al.,* 1991). At least one group has suggested that rhGH can increase the number of type II muscle fibers. Total fat mass, however, consistently decreases during rhGH treatment (Jorgensen *et al.,* 1989, 1991; Cuneo *et al.,* 1991). Based on these and other studies, the U.S. FDA approved the use of rhGH in patients with established growth hormone deficiency.

GH Effects on Biochemical Markers of Bone Turnover in GHD Several biochemical tests reflect the physiologic action of GH on the skeleton. Serum calcium, osteocalcin, and urinary hydroxyproline all increase, while PTH declines slightly during rhGH treatment (Van der Veen and Netelembos, 1990). Newer and more sensitive markers of bone turnover also reflect changes during rhGH treatment. Urinary deoxypyridinoline, and N-telopeptide increases threefold and the amino-terminal propeptide of type III procollagen doubles during 4 months of daily rhGH (Johansen *et al.,* 1990; Christiansen *et al.,* 1991). After cessation of rhGH treatment, deoxypyridinoline excretion decreases but type III procollagen levels remain higher than controls for several months (Jorgensen *et al.,* 1990). The balance between resorption and formation markers suggest that rhGH would modestly enhance trabecular BMD. It is conceivable that in the post-treatment period, the persistent increase in bone mineral density noted by Biller *et al.* (2000) is a function of enhanced bone formation at a time when bone resorption is declining. Further studies should clarify this potentially important effect on remodeling.

Effects of GH on Bone Density in GHD If prolonged growth hormone deficiency in adults results in profound changes in the musculoskeletal system, then GH replacement would be expected to enhance muscle performance and bone mass. However, skeletal responses to rhGH therapy are very dependent on several factors: (1) GH dose, (2) time of onset of GHD (i.e., childhood [lower BMD] vs adult), (3) age of treatment, (4) timing of response, (5) skeletal site measured, and (6) gender (women tend to have slightly less brisk of a BMD response than men) (Burman *et al.,* 1997). Notwithstanding, it does appear that muscle mass is significantly enhanced by rhGH treatment in GHD patients (Welle *et al.,* 1996). In general, doses of rhGH to adult GHD patients have tended to decrease with longer experience, such that the replacement dose recommended by the U.S. FDA is approximately 0.005 mg/kg/day. For example, in earlier studies with relatively higher daily doses of rhGH, 14 GHD adults given a nightly dose of 0.5 IU/kg/ week showed increases in exercise capacity, maximum oxygen consumption, and alkaline phosphatase even though quadriceps strength and spinal bone density did not change (Whitehead *et al.,* 1992). When 0.25 IU/kg/week of rhGH was administered to 12 GH-deficient adults for 1 year, there was a marked increase in trabecular bone density (measured by single- and dual-energy QCT of the spine) at 6 and 12 months. At 12 months, proximal and distal forearm BMC increased, mid-thigh muscle area was greater, and fat cross-sectional area decreased. Since the rise in spine BMD was noted with both single- and dual-energy CT measurements of the spine, this increase probably resulted from enhancement in bone mass not a reduction in marrow fat.

More recently, several groups have performed longer studies with rhGH for replacement in the GHD syndrome. Although changes in BMD are not significant at 12 months, by 24 and 36 months, BMD can increase by as much as 5–8% in the spine (Janssen *et al.,* 1998; Johannsson *et al.,* 1996; Baum *et al.,* 1996; Papadakis *et al.,* 1996; Rahim *et al.,* 1998). Moreover, some investigators have also reported

a concomitant increase in muscle strength after 2 years of treatment. However, it should be noted that the longer duration trials were not placebo-controlled for more than 18 months; those subjects followed out to 3 years had open label rhGH after either 6, 12, or 18 months of treatment. Notwithstanding that caveat, it appears that those individuals with earlier onset of GHD, as well as those with the lowest BMD, had the greatest likelihood of showing significant changes in bone mass with rhGH. Moreover, in those individuals receiving rhGH as replacement therapy, it appears that any judgement about efficacy in respect to BMD will require a follow-up BMD not earlier than 24 months. A relatively large randomized placebo-controlled trial of rhGH with BMD as a primary end point, and of 2 years duration, is currently entering its final stages and may provide our best indication yet about the magnitude of the anabolic effects of BMD on the skeleton. For now, it is apparent that rhGH treatment to adult GHD patients increases bone turnover, enhances BMD, and increases muscle mass and strength.

GH Administration to Elders

Generalized GH Effects Elderly people have lower GH secretory amplitudes and reduced serum levels of IGF-I and IGFBP-3 compared to younger adults (Finkelstein *et al.,* 1972; Lieberman *et al.,* 1994). Moreover, the pulse frequency for GH is lower in older people. Based on these data, it was assumed that skeletal responsiveness to GH in elders would be identical to that seen in GHD patients. In elderly men one group has reported a blunted serum IGF-I response to 0.1 mg/kg GH (36% lower) compared to that in younger men or adults with GHD (Finkelstein *et al.,* 1972; Lieberman *et al.,* 1994). However, Rosen *et al.* (1999) noted recently that generation of IGF-I after various doses of rhGH to frail elders was not associated with growth hormone resistance. Based on some recent data it appears that GH replacement for adult GHD or for pharmacological treatment of elders results in similar IGF-I responses.

Effects of GH on Bone Density and Bone Remodeling in Elders The most widely publicized growth hormone trial in elders involved 21 men over age 65 randomized to receive 0.03 mg/kg of rhGH three times per week (as a subcutaneous injection) or to no treatment whatsoever. Twelve men received rhGH while 9 men served as observational controls. The men were selected on the basis of a low serum IGF-I (<350 IU/liter) concentration (Rudman *et al.,* 1991). rhGH produced a threefold rise in circulating IGF-I, an increase in lean body mass (as measured by ^{40}K analysis), and a decline in total adipose mass. Bone density of the lumbar vertebrae (L_1–L_4) as measured by dual photon absorptiometry, increased 1.6% after 6 months in the treatment group while no change was noted in controls. Biochemical markers of bone turnover were not examined and no changes in bone density were detected in the mid- or distal radius or three areas of the hip. Furthermore, the spinal BMD changes at 6 months were not sustained at 1 year (Rudman *et al.,* 1991).

Marcus *et al.* (1990) studied the effects of rhGH in 16 men and women over age 60. Daily doses of rhGH (0.03, 0.06, or 0.12 mg/kg BW/day) were randomly assigned to each subject and administered once daily for 7 days. Serum IGF-I, osteocalcin, PTH, and calcitriol concentrations all increased during treatment. In this short-term study, there was also a significant rise in urinary hydroxyproline and urinary calcium excretion with a decline in urinary sodium. Holloway *et al.* (1994) conducted a longer randomized double-blinded placebo-controlled trial of daily rhGH for 1 year in 27 healthy elderly women, 8 of whom took a stable dose of estrogen throughout the study. Thirteen women completed 6 months of treatment and 14 women completed 6 months in the placebo group. Side effects prompted a 50% reduction in the original dose of rhGH (from 0.043 mg/kg BW or approximately 0.3 mg rhGH/kg/week to 0.02 mg/kg/day) and led to several dropouts in the treatment group. Fat mass and percentage body fat declined in the treatment group but there were no changes in bone density at the spine or hip at 6 or 12 months in other groups (Holloway *et al.,* 1994). Although BMD did not change, there were changes in some biochemical parameters. In particular, urinary markers of bone resorption (hydroxyproline and pyridinoline) increased after 6 months of rhGH treatment. The response of bone formation markers was more variable. Osteocalcin increased but type I procollagen peptide levels did not change. For women taking estrogen replacement therapy, indices of bone turnover (both formation and resorption) were blunted.

More recently, Rosen *et al.* (1999) reported that there is a dose-dependent decrease in bone mass after 1 year of rhGH in frail elderly men and women. This occurred despite striking increases in osteocalcin and serum IGF-I with the highest doses of rhGH (0.01 mg/kg/day). In part, the absence of a GH effect on BMD is not surprising since resorption is coupled to formation and GH activates the entire remodeling sequence. Indeed, in the same trial of 132 frail elderly subjects by Rosen and colleagues (1999), urinary N-telopeptide and osteocalcin both rose to the same extent, suggesting that total bone turnover, not just bone formation, was increased by rhGH therapy. Also, 1 year is too short a time to conclude definitively that rhGH would not increase bone mass. This is best exemplified by the rhGH trials in GHD. However, the relatively high incidence of side effects (weight gain, carpal tunnel syndrome, edema, glucose intolerance) in GH trials, especially in the frail elderly, is particularly troublesome. Ongoing rhGH studies in elders will examine factors such as the age of the person, presence of preexisting conditions, and IGF-I levels after GH to determine if these side effects can be predicted.

GH Treatment for Osteoporotic Patients

Short nonrandomized clinical trials with GH in osteoporosis were attempted well before GH replacement therapy was considered. As early as 1975, two patients with osteogenesis imperfecta and one patient with involutional osteoporosis were treated with GH (Kruse and Kuhlencordt, 1975).

Histomorphometric parameters of increased bone formation and resorption were noted. Subsequent studies employed GH with and without anti-resorptive agents. Aloia *et al.* (1976) administered between 2 and 6 U/day of GH for 12 months to eight patients with postmenopausal osteoporosis (the first 6 months of treatment featured low-dose GH; the last 6 months consisted of high-dose GH (6 U/day). Radial bone mineral content dropped slightly and histomorphometric parameters did not change during treatment. However, severity of back pain decreased considerably in several people (Aloia *et al.,* 1976). Daily GH injections (4 U/day) combined with alternating doses of calcitonin produced an increase in total body calcium (measured by neutron activation analysis) but a decline in radial bone mass after 16 months (Aloia *et al.,* 1977). In a separate trial, 14 postmenopausal women were given 2 months of GH and then 3 months of calcitonin in a modified form of coherence therapy (Aloia *et al.,* 1987). Total body calcium increased 2.3%/year and there were few side effects, but there were no changes in bone mineral density or histomorphometric indices. Dambacher *et al.* (1982) administered 16 U of rhGH every other day along with daily sodium fluoride to six women with postmenopausal osteoporosis. On histomorphometric analysis, there was a significant increase in the number of osteoblasts and osteoclasts but bone mass was unchanged.

Johannson *et al.* (1994) conducted a placebo-controlled double-blinded crossover trial of rhGH and IGF-I in 14 men with idiopathic osteoporosis. In this 7-day trial with rhGH (2 IU/m^2), procollagen peptide and osteocalcin levels increased after treatment as did urinary markers of bone resorption. The changes in osteocalcin were relatively small, however, and were not sustained after discontinuation of growth hormone treatment. There are no GH trials (past or present) which have examined spinal fractures as a therapeutic end point. Therefore, it is difficult to judge the potential efficacy of GH in the treatment of osteoporosis. However, GH stimulates bone remodeling activity, thereby leaving open the possibility that GH can be coupled to anti-resorptive agents. Holloway and colleagues (1997) tested this thesis in a 2-year randomized trial. In that study, rhGH and nasal calcitonin increased spine BMD by approximately 2%. This, however, was not much different than the use of CT alone, and certainly less than what has been seen in very large randomized trials with anti-resorptive agents. Once again, there were several side effects that produced limited enthusiasm for rhGH as a primary treatment for osteoporosis. Therefore, it is likely that GH may induce small but significant changes in bone mass which over an extended period could translate into fewer spine fractures. However, there are still no data to support that contention. In the meantime, several very small trials have looked at the effects of GH-releasing analogs on bone turnover and bone mass. Not unlike rhGH, however, these studies have been small, and the results somewhat conflicting. However, in contrast to rhGH, GH-releasing analogs are NOT associated with significant side effects. Hence, further trials are likely to begin with these analogs.

IGF-I for the Treatment of Osteoporosis

Introduction

In the late 1980s clinical trials with recombinant human IGF-I for diabetes mellitus were begun. The availability of this recombinant peptide and the absence of other treatments to stimulate bone formation accelerated animal and human studies of rhIGF-I in metabolic bone diseases. Theoretically, there are potential benefits for rhIGF-I compared to rhGH. These include: (i) more direct stimulation of bone formation, (ii) bypass of skeletal GH resistance, (iii) reduction in GH-induced side effects such as carpal tunnel and diabetes mellitus. There are, however, considerably fewer animal and human studies using rhIGF-I than rhGH. Therefore, these advantages have either yet to be fully realized or have not been validated. More importantly, we are still not certain how autocrine/paracrine/or endocrine IGF-I affects bone modeling and remodeling. For example, IGF-I stimulates bone formation by enhancing collagen biosynthesis in osteoblasts; however, the temporal sequence *in vivo* is not entirely clear. Moreover, there is now ample evidence that IGF-I can enhance recruitment of osteoclast precursors, and that IGF-I suppresses production of OPG, the dummy receptor for RANK ligand. These lines of evidence suggest that IGF-I may be important for both bone resorption and bone formation. Unfortunately, there are no long-term studies with rhIGF-I to confirm this putative role. Some newer studies, as noted below, are provocative and provide a framework for further investigation.

Animal Studies with rhIGF-I

rhIGF-I and Bone Growth

IGF-I is not a potent mitogen in most tissues and bone is no exception. There are high-affinity receptors for IGF-I on osteoblasts and IGF-I can stimulate preosteoblast replication and provoke resting cells to proceed through their growth cycles. IGF-I maintains the differentiated osteoblast phenotype, stimulates collagen synthesis, and prevents collagen degradation. It may also be critical in preventing apoptosis of osteoblasts. As noted above, IGF-I may also play a role in the recruitment of osteoclasts. Theoretically, therefore, despite its relatively weak mitogenic properties, IGF-I could have significant anabolic activity on the skeleton.

In hypophysectomized rats, growth can be fully restored by administration of either GH or IGF-I but not IGF-II (Schoenle *et al.,* 1982, 1985). A similar growth response occurs after rhIGF-I in streptozotocin-diabetics rats but not in sex linked dwarf-mutant chickens (Zapf *et al.,* 1989; Tixier-Bouchard *et al.,* 1990). In normal rats, rhIGF-I administered either systemically or locally (hindlimb infusions), does not stimulate longitudinal bone growth (Zapf *et al.,* 1990). In the spontaneously diabetic BB rat, rhIGF-I treatment does not result in changes in epiphyseal width, osteoblast surfaces, or osteocalcin concentration (Verhaege *et al.,* 1992).

The skeletal response to rhIGF-I is determined by the growth hormone/IGF-I status of the animal. For example, IGF-I does not increase bone formation in normal rats, whereas it stimulates bone growth and normalizes type I procollagen mRNA levels in hypophysectomized rats (Schmid *et al.,* 1989; Spencer *et al.,* 1991; Tobias *et al.,* 1992). Similarly, in the spontaneous mouse mutant (*lit/lit*), absence of GH releasing hormone receptors results in very low levels of IGF-I and skeletal dwarfism. IGF-I treatment restores growth and increases total body water but does not enhance bone mass in these mice (Donahue *et al.,* 1993). These findings are somewhat similar to the effects of GH on the skeleton in GH-deficient animals. However, rhIGF-I and rhGH differ in their actions on the circulatory IGF regulatory system. Growth hormone stimulates hepatic production of both IGF-I and IGFBP-3 while rhIGF-I administration increases the total circulating pool of IGF-I but suppresses hepatic production of IGFBP-3, primarily through feedback inhibition of GH secretion. It is conceivable that variations in IGF-I biological activity (between direct IGF-I administration and endogenously produced IGF-I as a result of GH treatment) may be due to the relative proportion of IGF-I bound to IGFBP-3.

rhIGF-I Effects on Bone Density in Animals with Altered Bone Turnover

Several experimental paradigms have been employed to study the effects of IGF-I on bone turnover in animals. These include: (i) oophorectomy, (ii) diabetes mellitus (spontaneous or induced), (iii) immobilization. In each situation, bone remodeling is markedly altered prior to IGF-I treatment in order to study growth factor actions on bone resorption and formation. These experimental models provide useful clinical information since IGF-I has been considered a potential therapeutic agent under conditions similar to those produced experimentally.

In oophorectomized rats, administration of rhIGF-I has variable effects on bone remodeling, bone mass, and bone strength. Kalu *et al.* (1991) reported partial restoration of trabecular bone volume after oophorectomy in adult rats treated with rhIGF-I. In older oophorectomized rats, rhIGF-I increased mid-shaft tibial BMD and enhanced periosteal bone apposition (Ammann *et al.,* 1993). Six weeks of rhIGF-I (delivery by mini-osmotic pump) to older rats caused a dose-dependent increase in bone density in the lumbar spine and proximal femur although bone strength and stiffness did not change. Mueller *et al.* (1994) reported that subcutaneous administration of rhIGF-I to adult oophorectomized rats stimulated bone formation as evidence by increased osteoid surfaces, osteoblast surfaces, and mineral apposition rates. At high doses of rhIGF-I, osteoclast surface and osteoclast number also increased. In contrast, Tobias *et al.* (1992) found that rhIGF-I (200 mg/kg) administered for 17 days to 15-week-old rats increased longitudinal and periosteal growth but suppressed trabecular bone formation in both oophorectomized and control rats. Bone resorption was also slightly suppressed during rhIGF-I treatment, although not to the extent that bone formation was inhibited.

Type I insulin-dependent diabetes mellitus (IDDM) is associated with decreased cortical bone mineral density (Santiago *et al.,* 1977). Although the pathophysiology of diabetic osteopenia remains unknown, it appears that the duration of diabetes, the extent of diabetic control, and the timing of disease onset are each associated with higher risks of low density (McNair, 1988; Heath *et al.,* 1980). Serum markers of bone formation are reduced in type I diabetics, suggesting a possible defect in osteolastic activity (Mazess, 1981). Serum IGF-I levels are either normal or low in type I diabetes mellitus, but often are reduced in patients with poor diabetic control. In these same people, serum IGFBP-1 levels are quite high. This has led investigators to believe that changes in the IGF regulatory system during poor metabolic control contribute to impaired growth.

Spontaneously diabetic BB rats exhibit osteopenia and therefore provide a useful model for studying the effects of IGF-I on bone remodeling. Even though bone formation is lower in BB than control rats (as measured by serum markers), administration of rhIGF-I does not increase bone epiphyseal width, osteoblast surfaces or serum osteocalcin (Heath *et al.,* 1980). Thus, despite evidence that circulating levels of IGF-I are reduced in some patients with type I IDDM, preliminary animal studies have failed to show that IGF-I administration can correct any inherent defect in bone formation.

Chronic immobilization inhibits bone formation and leads to significant bone loss. The pathophysiology of immobilization caused by bed rest, hindquarter elevation, or spaceflight is unknown, but the bone remodeling unit is uncoupled due to a transient decrease in bone formation and a marked rise in bone resorption (Tuukkanene *et al.,* 1991; Heaney, 1962). Some investigators have proposed that reduced bone formation during immobilization results from resistance to skeletal IGF-I. Immobilization in rats by the hindlimb elevation method causes cessation of bone growth (Bikle *et al.,* 1994). Paradoxically, mRNA levels for IGF-I and the type I IGF-I receptor are substantially increased in the proximal tibia and distal femur of hindlimb elevated rats (Bikle *et al.,* 1994). Infusion of rhIGF-I (200 mg/day) during hindlimb elevation does not reverse the cessation in linear growth induced by immobilization, even though growth and bone formation resume relatively soon after immobilization is stopped (Bikle *et al.,* 1994). This would suggest that there may be, at least transiently, resistance to IGF-I bioactivity.

Other investigators have reported contrasting results during hindlimb elevation. Machwater *et al.* (1994) continuously infused rhIGF-I (1.3–2.0 mg/kg/day) for 14 days to 5-week-old hindlimb elevated rats. The decline in bone mineral density of the proximal femur with unloading was blunted by infusions of IGF-I. At the tibial metaphysis of IGF-infused animals, bone formation rate and trabecular number were markedly increased. Marrow stromal cells from unloaded rats exhibit decreased proliferative characteristics, but addition

of IGF-I greatly increased alkaline phosphatase positive cell proliferation. rhIGF-I also enhanced serum alkaline phosphatase activity and osteocalcin levels in immobilized rats.

Alternate ways of exploiting the anabolic properties of IGF-I in bone have been proposed. IGF-I has been administered by intraarterial infusion or coupled to IGFBP-3. Infusion of rhIGF-I continuously into the arterial supply of the right hindlimb of ambulatory rats for 14 days leads to a 22% increase in cortical and trabecular bone formation in the infused limb (Spencer *et al.,* 1991). By histomorphometry, the number of osteoblasts (but not osteoclasts) increases. Using an alternative model, Bagi *et al.* (1994) administered rhIGF-I or a complex of IGF-I–IGFBP-3 to 16-week-old oophorectomized rats. The IGF-I/IGFBP-3 complex (7.5 mg/kg/day) increased bone formation more than did IGF-I alone, even though both treatments increased longitudinal bone growth. The highest doses of rhIGF-I and rhIGF-I-/IGFBP-3 enhanced trabecular thickness in the lumbar vertebrae and femoral epiphyses and increased bone resorption but only in the femoral metaphysis. A similar study contrasting IGF-I with IGF-I–GFBP-3 was performed in 22-week-old oophorectomized rats (Bromage *et al.,* 1993). Bone mineral density increased in both groups but fewer than 10% of the rats treated with IGF-I–IGFBP-3 complex developed hypoglycemia, compared to nearly 50% with rhIGF-I alone.

Human Studies with IGF-I

The potential utility of insulin-like growth factors in several disorders has led to trials with IGF-I in humans (see Table I). To understand how IGF-I affects bone remodeling, three groups of adults have been studied before and after rhIGF-I: normal postmenopausal women, GH-resistant children and adults, patients with age-related, idiopathic osteoporosis and anorexia.

IGF-I Administration to Normal Postmenopausal Women

There is one published study of bone markers that employed rhIGF-I to healthy young postmenopausal women. Doses of rhIGF-I from 30 to 180 mg/kg/day were administered daily by subcutaneous injection for 6 days to older postmenopausal women without fractures and normal bone density (Eberling *et al.,* 1993). Very significant dose-dependent increases in serum type I procollagen carboxyl-terminal propeptide (PICP), osteocalcin, and urinary deoxypyridinoline were reported. Although the rise in PICP was greater than the increase in collagen breakdown (measured by deoxypyridinoline), it is uncertain whether this meant that formation was stimulated more than resorption. For the two highest doses of rhIGF-I (120 and 180 mg/kg/day), orthostasis, weight gain, edema, tachycardia, and parotid discomfort were noted. At lower doses (30 and 60 mg/kg/day) fewer side effects were reported, but less discrete changes in PICP were noted. As noted below, high- and low-dose rhIGF-I was also administered for 28 days to elderly postmenopausal women and the results are somewhat different in that bone formation was stimulated by lower doses of rhIGF-I.

Table I Studies Reporting on Effects of Recombinant Human IGF-I on Bone Metabolism in Adult Humans

Study	Patients	Age (yrs)	Dose (μg/kg/d)	Duration	Formation	Resorption	Comment
Johansson *et al.,* 1992	1 man; idiopathic osteoporosis	59	80 × 2	7 d	↑	↑	Sustained increase in markers of bone formation four weeks post-treatment.
Ebeling *et al.,* 1993	18 women; healthy & postmenopausal	66 ± 7	30, 60, 120, 180	6 d	↑	↑	The lowest dose enhanced bone formation alone.
Rubin *et al.,* 1994	1 woman; postmenopausal & Werner syndrome	43	30–75	6 months	↑	↑	BMD increased by 3% in the lumbar spine. The patient was also treated with estrogen.
Grinspoon *et al.,* 1995	14 women; young & healthy	22 ± 4	100 × 2	6 d	↑	↔	Treatment during fasting and after 4 d of fasting.
Johansson *et al.,* 1995	24 men; idiopathic osteoporosis	46 ± 10	20, 40, 80	6 weeks	↑	↑	The lowest dose enhanced bone formation alone.
Johansson *et al.,* 1996	12 men; idiopathic osteoporosis	44 ± 8	80	7 d	↑	↑	Comparison to effects of GH; qualitatively no difference.

Note. Arrows denote changes in bone formation and bone resorption, estimated by biochemical markers of bone metabolism in serum and urine. BMD = bone mineral density, GH = growth hormone.

IGF-I ADMINISTRATION TO GH-RESISTANT SHORT STATURE

One potential indication for IGF-I might be in the GH-resistant short stature syndrome (Laron dwarf). By the end of the past century, Sweden and several other European countries had approved rhIGF-I for that purpose. Patients with the Laron dwarf syndrome lack functional growth hormone receptors and thus do not respond to GH; their IGF-I levels are very low, growth is slow, and circulating GH levels are high (due to lack of negative feedback on GH by IGF-I) (Bondy, 1994). Underwood treated one such boy (age 9) with 2 weeks of continuous intravenous rhIGF-I (Bondy, 1994). Urinary calcium excretion increased while urinary phosphate and sodium decreased. After a 2-week continuous infusion of rhIGF-I, the patient was treated with twice daily sc rhIGF-I (120 mg/kg) for 2 years. Growth occurred at a rate of 10 cm/year, compared to 5 cm for the 3 years prior to treatment. Subsequently, Underwood and colleagues have treated eight patients in this manner without hypoglycemia while Laron and his group have treated five children (Bondy, 1994; Laron and Kupper-Auerbach, 1992). More recently, a child with an IGF-I deletion mutation in exon 5 has been reported. This patient had very short stature, mental retardation and other abnormalities along with very low levels of circulating IGF-I (Woods *et al.,* 1996). RhIGF-I treatment led to a marked increase in linear growth and a huge increase in spinal bone mass. However, when corrected for changes in size of the bone, the incremental changes in volumetric bone mass were much less impressive (Camacho-Hubner *et al.,* 1999). Hypoglycemia is avoided in many cases by having children eat 3 to 4 hr after their IGF-I injection, although several children had selective growth of adenoidal tissue.

Two unique aspects about these IGF-I data challenge previous concepts about the role of GH in skeletal homeostatsis. First, IGF-I can act as a classical endocrine hormone stimulating longitudinal growth independent of GH; second, GH may not be absolutely essential for statural growth; i.e., the stimulatory effect of GH on chondrocytes which permits skeletal responsiveness to IGF-I may not be as critical as once perceived. However, caution must be undertaken in examining the effects of rhIGF-I on bone mass in children since most of the changes in the skeleton relate to linear growth and periosteal enhancement, both of which can contribute to two-dimensional changes in BMD as measured by DXA, but lesser changes when corrected for size (Bachrach *et al.,* 1998).

IGF-I ADMINISTRATION IN IDIOPATHIC AND AGE-RELATED OSTEOPOROSIS

Idiopathic osteoporosis in men in an ill defined syndrome of low bone mass and spinal fractures without associated hypogonadism. By histomorphometry, these men often have low bone turnover, suggesting a possible defect in bone formation. Several groups of investigators has suggested that this syndrome is related to low serum IGF-I levels (Ljunghall *et al.,* 1992). Since the therapeutic options in males with osteoporosis are somewhat limited and treatment for low bone turnover states, in general, is frustrating, the therapeutic potential for anabolic agents like IGF-I in this condition should be quite high. In one male with idiopathic osteoporosis and low serum IGF-I, Johansson *et al.* (1992) administered subcutaneous rhIGF-I (160 mg/kg/day) for 7 days. Bone alkaline phosphatase, osteocalcin, and the carboxyterminal peptide of procollagen type I all increased more than 40% over baseline. However, urinary calcium/creatinine and hydroxyproline excretion rose during treatment. In a recent trial rhIGF-I (at doses of 80 mg/kg/day) and rhGH (2 IU/m^2/day) in 12 men, serum osteocalcin, serum procollagen peptide, and urinary deoxypyridinoline excretion all increased following 7 days of rhIGF-I treatment (Ljunghall *et al.,* 1992). Although there were slight differences in the response of certain biochemical markers to IGF-I and GH, both forms of therapy produced significant increases in bone resorption.

Clinical trials provide evidence that IGF-I acts by increasing the birth rate of remodeling osteons, thereby promoting bone resorption and formation. Yet, it is conceivable that low doses of rhIGF-I ($<$30 mg/kg/day) may differentially stimulate bone formation. In one trial of 16 healthy elderly women, 60 μg/kg/day (high dose) and 15 mg/kg/day (low dose) of rhIGF-I were tested for 28 days (Ghiron *et al.,* 1995). The high-dose rhIGF-I increased markers of bone resorption and formation. However, low doses of rhIGF-I caused increases in serum osteocalcin and type I procollagen carboxyterminal peptide, but had no effect on total pyridinoline excretion (Ghiron *et al.,* 1995). These data would support the thesis that low doses of rhIGF-I may directly increase osteoblastic function with only a minimal increase in bone resorption. However, further studies will be needed to assess the future therapeutic role of low doses rhIGF-I in osteoporosis.

Recently novel approaches to enhancing IGF-I action in bone have been proposed. One strategy is to administer a bone-specific agent which stimulates bone mass such as parathyroid hormone (PTH). Intermittent hPTH increases trabecular bone by stimulating osteoblasts to synthesize IGF-I and other growth factors (Donahue and Rosen, 1998). Another strategy is to administer IGF-I along with an IGF binding protein. Bagi *et al.* (1994) previously reported that IGF-I/IGFBP-3 complex could enhance bone mass in the metaphysis and epiphysis of rats (see Fig. 2). One very small randomized trial utilized subcutaneous infusions of IGF-I/IGFBP-3 in 24 older women with hip fractures. Bone loss in the contralateral hip was reduced considerably after 6 months (i.e. from 6 to 1.5%) in those subjects who were given the complex vs those receiving saline (Geusens *et al.,* 1998). Accompanying that change in BMD, there was also an increase in grip strength in those that received the active agent, while no significant side effects were reported. Larger clinical trials are likely to commence utilizing this or other combinations linking IGF-I to one or more IGFBPs, especially if bone is considered the principle target.

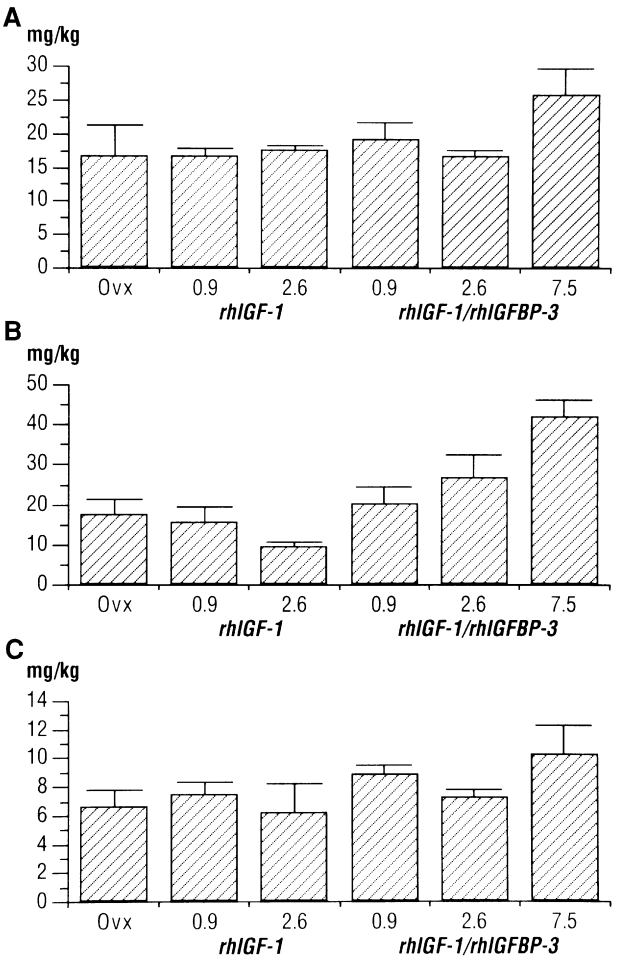

Figure 2 Dose-related effects of rhIGF-I and rhIGF-I/IGFBP-3 complex on bone formation rate in metaphyses (A), epiphyses (B), and lumbar vertebral bodies (C) after 8 weeks of treatment. Each column shows the mean ± SEM. The 7.5 mg/kg/day dose was significantly greater than OVX at $p < 0.05$ for the metaphysis and epiphysis. [Reproduced by permission of the publisher.]

IGF-I IN ANOREXIA NERVOSA

Osteopenia frequently accompanies anorexia nervosa (AN). The reduced bone mass in AN is characterized by reduced bone formation (Soyka *et al.,* 1999). Serum IGF-I levels are reduced by 50% despite a marked increase in the frequency of GH pulsatility. Recently, Soyka and colleagues (1999) reported that serum IGF-I concentrations accounted for nearly 70% of the variance in both osteocalcin and bone-specific alkaline phosphatase and, that bone resorption indices were comparable in both AN and age-matched controls. Grinspoon *et al.* (1996) administered 100 μg/kg, 30 μg/kg, or placebo of rhIGF-I to 23 women with AN and low spine BMD. After 6 days, one marker of bone formation increased without any change in bone resorption. These data were used to support a much larger, currently ongoing trial of 30 μg/kg of rhIGF-I in more than 70 AN patients. Results from that RPCT will help to determine whether this treatment is feasible on a larger scale. Clinical and pharmacological interest in IGF-I has been tempered by the fact that high normal levels of serum IGF-I have been linked to a greater risk of certain malignancies (Rosen and Pollak, 1999). If IGF-1 is ultimately to become a useful therapeutic agent, such concerns will have to be carefully addressed and critically evaluated.

Summary

Several lines of evidence suggest that administration of recombinant growth factors, such as IGF-I or GH, may be anabolic for the skeletal remodeling unit. First, both GH and IGF-I stimulate osteoblastic differentiation. Second, in animal models, GH and IGF-I enhance longitudinal growth, bone formation, and bone mineral density. Third, in GHD children and adults with an impaired GH-IGF-I axis, rhGH and rhIGF-I both increase trabecular and cortical BMD. However, evidence from randomized placebo-controlled trials that rhGH or rhIGF-I can differentially stimulate bone formation for a sustained period in older non-growth-hormone-deficient adults is lacking, in part because previous studies have been of short duration, and with small numbers of subjects. Therefore, favorable skeletal responses in properly controlled clinical trials will be required before rhGH or rhIGF-I can be recommended for the treatment of postmenopausal osteoporosis. In the meantime, in selected catabolic conditions such as anorexia nervosa , the use of these growth factors in trials will continue, with an eye toward the future application of these agents in metabolic bone diseases.

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CHAPTER 84

Anabolic Steroid Effects on Bone in Women

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Introduction

Anabolic steroids are androgenic compounds that produce both anabolic effects and promote virilization in men and women. Anabolic steroids modulate a variety of body functions, e.g., muscle mass and strength, and have been used to treat many conditions, including anemia, protein deficiency, psychiatric disorders, reproductive dysfunctions, and others (Caltin, 1995; Ehrmann *et al.,* 1995; Hiipakka and Liao, 1995; Parker, 1995). Their beneficial effect in anemia results from stimulation of erythropoiesis via increased erythropoietin production in the kidney and hemoglobin synthesis in erythroblasts. Their marked effect on muscle mass and strength has led to abuse of these compounds in athletes. Their diverse physiological effects makes it hard to separate between primary and secondary actions. For example, the proposed beneficial effects of anabolic steroids on bone may result either from direct effects on bone cells, or from effects on muscle or on calcium metabolism, via reduction in calcium excretion in the kidney and increased calcium absorption in the gastrointestinal tract (Caltin, 1995; Ehrmann *et al.,* 1995; Hiipakka and Liao, 1995; Parker, 1995). Since testosterone is rapidly metabolized, some of its effects could be due to aromatase-mediated conversion to estrogen or to other metabolites. Various anabolic steroids with improved resistance to metabolism have also been developed (Rasmusson and Tonney, 1994). Androgenic compounds rendered resistant to gastrointestinal and liver metabolism by containing an alkyl group at the C17α position, such as stanozolol, are orally active (see Table I), whereas anabolic steroids with increased lipophilicity due to esterification at the C17β position, such as nandrolone decanoate or testosterone esters such as testosterone enanthate, are given parenterally (Caltin, 1995; Melchert and Welder, 1995). Recently, transdermal delivery of androgens via skin patches was developed. The mode of administration of various anabolic steroids is shown in Table I.

This chapter deals specifically with effects of androgenic compounds on bone in women, in the context of osteoporosis. A separate chapter by Wiren and Orwoll (Chapter 43) covers the role of androgens in skeletal development and in shaping and maintaining the skeleton and the effect of androgens on bone cells. Questions that are addressed in this chapter, not all of which have been satisfactorily answered at this time, include: (1) In females, do androgens play a role primarily during bone development or do they play a role in mature bones as well? (2) Do androgens, like estrogens, mainly prevent bone resorption or do they stimulate bone formation? (3) Do they play a role in the pathophysiology of postmenopausal osteoporosis? (4) Do they act directly on bone tissue or indirectly via other organs? (5) What is their target cell and mechanism of action? This review will attempt to summarize the current literature on the subject and point to areas that are under investigation.

Androgen Levels in Women

Androgenic steroids are produced in women by the ovaries, the adrenal glands, and the adipose tissue. The natural androgens produced in the ovary are testosterone and 5α-dehydrotestosterone (DHT). Both have high affinity for the androgen receptor (AR), although DHT has a slower

Table I Androgenic Anabolic Steroids and Their Route of Administration

Parenteral administration	Oral administration
Nandrolone decanoate	Methyltestosterone
Testosterone cypionate	Methanadrostenlonone
Testosterone enanthate	Stanozolol
Testosterone propionate	Fluoxymesterone
Boldenone	Oxandrolone
	Norethandrolone
	Danazol

dissociation rate. The average normal plasma testosterone level in women is 1.3 n*M* (normal range 0.69–2.6 n*M;* Ehrmann *et al.,* 1995; Parker, 1995). The serum testosterone concentration falls after menopause to an average level of 0.7 n*M* (normal range 0.53–1.06 n*M,* Ehrmann *et al.,* 1995; Jassal *et al.,* 1995; Morales *et al.,* 1994; Ohta *et al.,* 1993; Parker, 1995). The serum DHT level in postmenopausal women is slightly lower, around 0.32 n*M* (Morales *et al.,* 1994). Androgens produced by the adrenal gland are androstenedione, dehydroepiandrosterone (DHEA), and its metabolite DHEA-sulfate (DHEAS). These androgens, which are present in young women at a concentration of 1.9 to 6.9 n*M,* 1.52 to 15.2 n*M,* and 1.28 to 5.2 μM, respectively, decrease significantly with age to an average concentration of 1.3 n*M* for androstenedione, 7.19 n*M* for DHEA, and 1.78 μM for DHEA-S (Morales *et al.,* 1994; Ehrmann *et al.,* 1995; Parker, 1995). In the circulation, androgens are bound to albumin, a high-capacity, low-affinity carrier, and to the more specific steroid hormone binding β-globulin (SHBG), a high-affinity, low-capacity carrier. Binding to serum proteins serves as a reservoir for steroid hormones and influences the availability of free (active) steroid hormones (Ehrmann *et al.,* 1995; Moore and Bulbrook, 1988; Parker, 1995). Sex steroids were reported to modulate the level of SHBG—estrogen and progesterone increasing it, and androgens decreasing it (Darney, 1995), thus regulating free steroid levels in that manner, as illustrated by the observation that bone mineral density (BMD) correlates better with free rather than with total androgen levels (Longcope *et al.,* 1984, 1986; Acar *et al.,* 1998).

Postmenopausal women maintain significant androgen levels (Moore and Bulbrook, 1988; Parker, 1995), derived from androgens produced by the adrenals and fat tissue, which serve as precursors for the synthesis of testosterone, as well as estrogen. Natural androgens can be converted to estrogens by the enzyme aromatase, also shown to be present in osteoblast-like cells, which could explain some of the effects of androgens on bone (Bruch *et al.,* 1992; Nawata *et al.,* 1995; Roapena *et al.,* 1994; Schwelikert *et al.,* 1995; Tanaka *et al.,* 1993). Other metabolic pathways for androgens include the convergence of androstenedione and testosterone to more potent androgens by 5α-reductase and 17β-hydroxysteroid dehydrogenase, which have also been found in bone cells (Bruch *et al.,* 1992).

The Role of Androgens in the Female Skeleton

It is well established that androgens play an important role in bone metabolism in men, which parallels the role of estrogens in women (Seeman, 1995, and Chapter 43). Elderly men experience a decline in bone density that parallels the decline in serum bioavailable testosterone with aging. Castration or treatment with AR antagonists for prostate cancer causes osteoporosis in men similar to that produced by estrogen deficiency in women. Part of the mechanism also appears similar in that androgen treatment reduces bone turnover and bone resorption (Chapter 43). The role of androgens and anabolic steroids in bone metabolism in women is less clear.

Overall, most of the evidence suggests that androgens and anabolic steroids have beneficial effects on bone in women, although reports vary regarding the extent of these effects. Following are some of the observations that support this conclusion.

The androgen insensitivity syndrome is caused by mutations in the AR, resulting in a partial or complete female phenotype in individuals who are genetically males. The external genitalia are usually female, but there is no uterus and instead of ovaries there are internal testes (Hiipakka and Liao, 1995). Testosterone is aromatized to estrogen, and the body build and bone types are female. It was recently reported that a 17-year-old individual with this phenotype had very low BMD (about 4 SD below the age-matched female average), and BMD did not increase during 4 years of treatment with estrogen and progestin, suggesting that androgens may play a role in reaching peak bone mass (Munoz- Torres *et al.,* 1995).

Genetic support for the role of AR on bone mass was demonstrated in women with androgen insensitivity syndrome (AIS) caused by androgen receptor abnormalities, rendering them partially or completely refractory to androgen. A study of 22 women with complete AIS shows that the average lumbar spine BMD *z* score of women with complete AIS was significantly reduced at -1.08 ($P = 0.0003$). Furthermore, 6 of these women had sustained cortical bone fractures, of whom three reported multiple (>3) fractures (Marcus *et al.,* 2000). The human AR is polymorphic at the polyglutamine (poly Q) expansion of the N-terminal domain. The length of this poly Q repeat correlates with the expression and activity of AR (short poly Q, higher AR expression/activity). A recent study in eugonadal men showed that longer AR poly Q tracts (i.e., lower AR activity) were significantly more common in osteoporotic men than in controls. In addition, in the Michigan Women's Health Study, women possessing AR alleles with longer poly Q tracts concomitantly had BMDs in the lowest quintile for the population studied (Sowers *et al.,* 1999). These results point to an important role for AR in the regulation of bone mass.

Further support for the role of androgens in determining BMD in the premenopausal period comes from the fact that women with hyperandrogenic amenorrhea have relatively high BMD, and BMD decreases following treatment with the antiandrogens spironolactone and lynestrenal (Prezelj and Kocijancic, 1993, 1994). In peri- and postmenopausal women, BMD was shown to correlate positively with androgen levels (Longcope *et al.,* 1984, 1986). A role for androgens in postmenopausal skeletal health was also strongly suggested by the fact that in a 16-year prospective follow-up study of postmenopausal women, low testosterone, rather than estrogen levels, showed a correlation with height loss, a measure of osteoporotic vertebral fractures (Fig. 1) (Jassal *et al.,* 1995). SHBG, mentioned above as an important determinant of bioavailable testosterone, was also found to have a negative correlation with vertebral fractures in elderly women (Longcope *et al.,* 1984, 1986; Moore and Bulbrook, 1988; Moore *et al.,* 1991).

Furthermore, DHEA-S levels were shown to correlate with BMD in the lumbar region, the femoral neck, and the radius midshaft (Deutsch *et al.,* 1987; Rozenberg *et al.,* 1990; Szathmari *et al.,* 1994). DHEA and DHEA-S levels decrease markedly with age, and their decrease parallels reduction both in lean body mass and in bone mass (Buchanan *et al.,* 1988a,b; Davidson *et al.,* 1983; Haffner and Bauer, 1992; Nordin *et al.,* 1985; Ohta *et al.,* 1993). These declines also correlate with the decline in growth hormone and insulin-like growth factor (IGF)-I, which play a role in bone metabolism, but it is not known if these correlations point to any causal relationships. Administration of adrenal androgens to elderly patients improves their physical and psychological well-being, but effects on BMD were not measured in that study (Morales *et al.,* 1994).

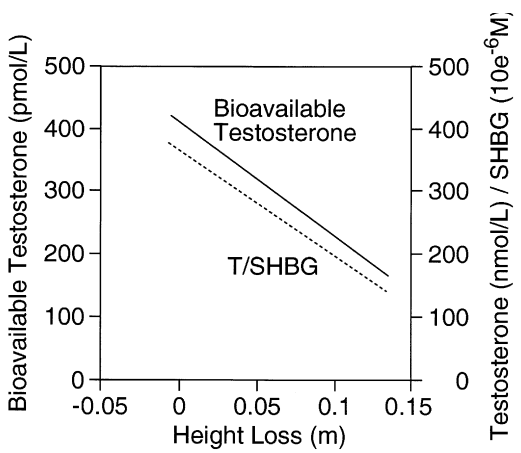

Figure 1 The multiply adjusted association of bioavailable testosterone and testosterone/SHBG with height loss. Height and plasma for sex hormone assays of 170 postmenopausal women with a mean age of 64.5 years were taken, and 16 years later, at a mean age of 80.7 years, height was remeasured. [Reprinted from *Journal of Bone and Mineral Research* 10 (4), Jassal, S. K., Barrett, C. E., and Edelstein, S. L. (1995) Low bioavailable testosterone levels predict future height loss in postmenopausal women, pp. 650–454, with kind permission from Blackwell Science Inc., Cambridge, MA]

Treatment Effects

Anabolic steroids have been used for many years in the treatment of osteoporosis. However, their use is limited due to virilization and adverse effects on serum lipids. Anabolic steroids such as methandrostenolone or stanozolol were shown to increase bone mass in postmenopausal women (Chesnut *et al.,* 1977, 1983). The largest treatment experience to date is with nandrolone decanoate, which was reported to increase BMD and bone mineral content (BMC) in several studies (Need *et al.,* 1986, 1987a,b). In one study, BMC fell after cessation of therapy, further supporting a role for nandrolone decanoate in increasing BMC, but in another study, BMC was maintained (Hassager *et al.,* 1989; Need *et al.,* 1989a,b). Another anabolic steroid, stanozolol, was shown by histomorphometric analysis of transiliac bone biopsies to increase bone formation rates significantly without changing the osteoclast surface. The major bone-forming activity was observed on endocortical surfaces (Beneton *et al.,* 1991).

Positive effects of androgens in the treatment of postmenopausal osteoporosis were also demonstrated in studies with combined testosterone and estrogen treatment (Davis *et al.,* 1995; Raisz *et al.,* 1996; Watts *et al.,* 1995). In a 2-year study (Davis *et al.,* 1995), combined treatment increased significantly the rate and extent of a rise in lumbar and hip BMD relative to treatment with estrogen alone (Fig. 2). Similar observations were made in a separate study in which post-surgical menopause patients, average age 45 years old, were treated for 2 years with either esterified estrogen alone or in combination with methyltestosterone. In this study, combined treatment increased lumbar BMD by 3.4% relative to baseline, whereas estrogen alone increased it only by about 0.5%. No significant changes in radius, femoral neck, Ward's triangle, or greater trochanter BMD were observed in either group (Watts *et al.,* 1995). Furthermore, methyltestosterone did not counter the positive effects of estrogen on lipids in that study.

Similarly, it was reported in a 2-year study in surgically menopausal women that the combined treatment of esterified estrogen plus methyltestorone increased lumbar spine and hip BMD significantly more than estrogen alone (Barrett-Connor *et al.,* 1999). Interestingly, estrogen in combination with the androgenic progestin norethisterone increased bone density more than with medroxyprogesterone (Hosking *et al.,* 1998).

Another agent with androgenic activity reported to produce beneficial effects on bone is danazol, which also has anti- estrogenic activity and is used in the treatment of endometriosis (Bergquist, 1990; Dawood *et al.,* 1995; Fukushima *et al.,* 1993; Rock *et al.,* 1993). This condition, which affects about 14% of women, is caused by excessive estrogen and is usually treated by suppressing ovarian function with GnRH agonists, which cause estrogen deficiency and bone loss. Danazol, which in addition to being an anti-estrogen has progesterone and androgenic activity, increased in 12 months thoracic and lumbar BMD by up to

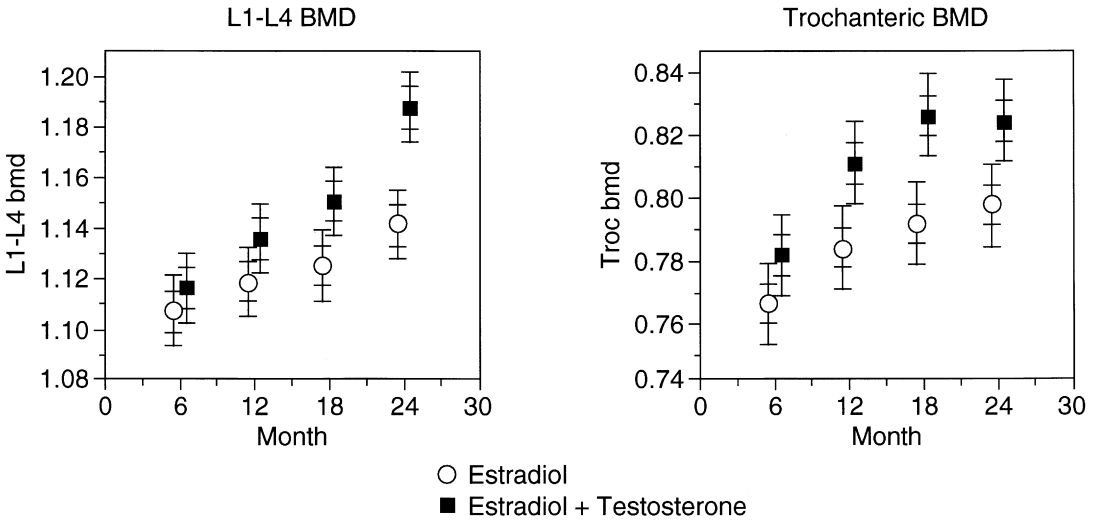

Figure 2 The effects of hormonal implants on BMD (g/cm^2): lumbar spine (L1 – L4) and femoral trochanter (Troc), estradiol, and estradiol plus testosterone. Error bars represent SEM. Inner error bars are used to compare means between times for the same treatment. The comparison between the groups is made with outer error bars. The means are significantly different by a P value of at least 0.05. [Reprinted from *Maturitas,* **21**(3), Davis, S. R., McCloud, P., Strauss, B. J., and Burger, H. (1995) Testosterone enhances estradiol's effects on postmenopausal bone density and sexuality, pp. 227 – 236, with kind permission from Elsevier Science Ireland Ltd., County Clare, Ireland.]

8%, suggesting stimulation of bone formation. Similar observations were made in a number of other recent studies. Measurement of biochemical markers in patients treated with danazol or the GnRH agonist nafarelin showed a 50% increase in hydroxyproline excretion, consistent with higher bone resorption caused by estrogen suppression. However, serum osteocalcin and alkaline phosphatase, biochemical indicators of bone formation, increased by 88–120 and 34–40%, respectively, consistent with increased bone formation. Both markers remained elevated 3 months after treatment with nafarelin, and osteocalcin levels remained elevated in patients treated with danazol (Dawood, 1994; Dawood *et al.,* 1995; Ylikorkala *et al.,* 1990).

To summarize this section, the data available so far suggest that androgens may play a positive role in determining the bone mass in women, both before and after menopause. The limited information available on the effects of androgen treatment suggests that it increases bone mass, in excess of the effects of estrogen alone, possibly via stimulation of bone formation.

Effects of Androgens on Bone in Animal Studies

In male animals, orchiectomy causes changes similar to those produced by ovariectomy in females—increased bone turnover and bone loss, which are reversed by androgen administration. In this section, we shall focus specifically on a small number of studies that examined the effects of androgens on female animals.

The role of endogenous androgens in the control of bone mass in female rats was suggested by the use of the nonsteroidal, anti-androgen flutamide, which antagonizes the action of androgens produced by the gonads and adrenal glands. In 3-month-old female Wistar rats, 4-week treatment with flutamide at 10 mg/kg/day decreased total body calcium and had little effect on urinary hydroxyproline or urinary ^{45}Ca excretion, suggesting suppression of bone formation rather than increased resorption (Goulding and Gold, 1993). In another study, it was shown that suppression of trabecular bone formation and reduction in trabecular thickness caused by flutamide in normal rats was not observed in OVX rats, suggesting that flutamide acted as an antagonist to androgens produced by the ovaries (Gallagher *et al.,* 1995). This study showed that the reduction in testosterone levels following OVX was associated with a decrease in bone formation rates and in trabecular thickness in the proximal tibia, even when bone resorption was inhibited by 3-amino-1-hydroxypropylidene-1-bisphosphonate, consistent with androgen effects on bone formation.

In another study rats were ovariectomized (OVX) at 13 weeks and DHT was administered starting at 90 days, when bone turnover had reached a steady state. Treatment with 0.01, 0.1, or 1.0 mg/kg/day for 60 days partially restored cancellous bone volume and trabecular thickness (Tobias *et al.,* 1994). DHT also increased cortical bone volume and endocortical and periosteal bone formation rates. Bone resorption parameters were not altered, suggesting a stimulatory effect of DHT on bone formation. These observations are consistent with those in another study in which DHT was administered to 12-month-old SD rats. Six months of treatment with 5 mg/kg/day restored the cortical (femur) and trabecular (vertebrae) bone mineral density measured by DXA (Vanin *et al.,* 1995).

The synthetic anabolic steroid nandrolone decanoate was also shown to increase bone volume and bone strength in the

Table II Mechanical, Structural, and Geometric Properties and Density of the Tibiae of Normal and Androgen-Treated Primates

	Normal	Androstenedione	Testosterone
Elastic modulus E (MPa)	8418 ± 1606	8353 ± 1436	8041 ± 1163
Shear modulus G (MPa)	2807 ± 510	2825 ± 407	2876 ± 368
Maximum shear strain γ_{max} (%)	1.86 ± 0.38	1.96 ± 0.26	2.15 ± 0.33
Energy U (J/cm^3)	0.2427 ± 0.0830*	0.3063 ± 0.0792	0.352 ± 0.0773*
Maximum shear stress τ_{max} (MPa)	46 ± 8.7*†	54.9 ± 11†	63.9 ± 12.4*
Torsional rigidity D (N cm^2 rad)	10557.2 ± 2361.4*	9367.2 ± 922.9†	12989.3 ± 2164.7*†
Bending stiffness K (N/mm)	348.2 ± 55.7*	321.6 ± 49.1†	401.1 ± 53.5*†
Midshaft external diameter d_e (mm)	7.89 ± 0.71	7.54 ± 0.52*	8.22 ± 0.38*
Midshaft internal diameter d_i (mm)	3.54 ± 0.69	3.55 ± 0.56	3.49 ± 0.66
Polar moment of inertia J (mm^4)	379.1 ± 129.8	307.4 ± 89.2*	435.3 ± 78.9*
Cross-sectional area A (mm^2)	39.1 ± 6.8	34.6 ± 4.4*	43.2 ± 4.1*
Density ρ (g/cm^3)	1.64 ± 0.096*†	1.729 ± 0.095†	1.781 ± 0.052*

Note. Three groups of 12 adult female cynomologus monkeys (mean age 7.4 years) were treated continuously subcutaneously with either control, androstenedione + estrone, or testosterone for 24 months. Reprinted with kind permission of the publisher from: *The effects of androgens on the mechanical properties of primate bone. Kasra, M., and Grynpas, M. D, Bone* **17,** 265 – 270. Copyright 1995 by Elsevier Science, Inc.

*†In each row, numbers with any of these symbols in common are significantly different ($P < 0.05$).

cortical bone of rats, along with an increase in the bone formation marker osteocalcin (Aerssens *et al.,* 1993). Another synthetic steroid anti-androgen, TZP-4238, which acts as an antagonist in the gonad (Murakoshi *et al.,* 1993), was reported to increase BMC, bone volume, and strength in ovariectomized and neurectomized rats (Fuse *et al.,* 1997), raising the possibility of tissue-specific androgen action.

The stimulatory effect of androgens on bone density and mechanical strength of long bones was also demonstrated recently in a long-term study on nonhuman female primates (Kasra and Grynpas, 1995). Adult, 4 – 11-year-old female cynomologus monkeys, dosed for 2 years with testosterone, had an increase in bone density, torsional rigidity, and bending stiffness of the tibia, as well as increased density and strength of the trabecular bone in the femoral head (see Table II).

To summarize this section, the small number of studies conducted in animal models suggest that androgens act on the female skeleton independently of estrogens to increase bone mass and strength, possibly via stimulation of bone formation.

Nuclear Receptors

Androgen action is mediated by the AR, a member of the nuclear receptor (NR) superfamily (Evans, 1988). NRs mediate the action of a large number of hormones that control bone metabolism, including estrogens, glucocorticoids, retinoids, thyroid hormones, and vitamin D. Recent progress in this field has identified several new ligands of NRs as putative regulatory molecules, which participate in the physiological regulation of important metabolic processes. These include prostaglandin J_2 as the ligand for peroxisome proliferator activator receptor (PPAR)γ, fatty acids for PPARα, oxysteroles for liver X receptor (LXR), bile acids for farnesoid X receptor/bile acid receptor (FXR/BAR), steroids and xenochemicals for steroid and xenobiotic receptor/pregnenolone X receptor (SXR/ PXR), and androstane for the constitutive androstane receptor (CAR) (Blumberg and Evans, 1998). PPARγ stimulation promotes the differentiation of adipocytes, which share the same precursor cells as osteoblasts (Forman *et al.,* 1995; Kliewer *et al.,* 1995). Eicosapentanoic acid (EPA), a potent activator of PPARs, was recently reported to prevent the reduction in bone density and bone strength caused by a low calcium diet in ovariectomized rats (Sakaguchi *et al.,* 1994). Recent reports suggest a role for other NRs in bone development or metabolism, e.g., the orphan nuclear receptors RAR-related orphan receptor (ROR)α (Meyer *et al.,* 2000) and the estrogen-related receptor α 1 (ERR1).

The classical model for activation of NR receptors, illustrated by the glucocorticoid receptor (GR), involves the following steps. Upon binding of the cognate hormone, the receptor undergoes a conformational change, dissociates from heat shock proteins to which it was initially bound in the cytoplasm, and translocates to the nucleus, where it interacts with target genes via specific steroid response elements (SRE) and activates transcription (Evans, 1988; Tsai and O'Malley, 1994). This initial relatively simple model has evolved since initially proposed. For example, many NRs were shown to be localized in the nucleus in the absence of hormone, bound to DNA in an active state. Thus, the ligands would have to enter the nucleus and activate the NRs. In addition, some receptors,

such as COUP-TF, are constitutively active in the nucleus in the absence of ligand, while ligand mediated inactivation/transcriptional repression was observed in other cases, such as for CARβ (Forman *et al.,* 1998). The NRs were shown to bind to DNA as dimers, either homodimers for the progesterone receptor (PR), AR, estrogen receptors (ER), and GR or as heterodimers with the retinoid X receptor (RXR) for the thyroid receptor, retinoid A receptors (RAR), vitamin D receptor (VDR), PPARs, LXR, FXR, PXR/SXR and CAR (Mangelsdorf and Evans, 1995; Repa and Mangelsdorf, 1999). There are three RXR receptors, which increases the combinatorial receptor–receptor interaction possibilities and thus the possible outcome of hormone action. ER was also reported to bind to DNA as a monomer, and NRs can not only stimulate but also suppress transcription.

All NRs have two conserved domains: a carboxy terminal region responsible for ligand binding, and a centrally located and highly conserved DNA binding domain. In addition, there are two nonconserved domains, the highly variable amino terminal region and the region that links the DNA binding to the ligand binding domain. Additional regions responsible for important receptor functions include two domains involved in cell-type-specific transcriptional transactivation, one in the variable amino terminal region, activation function-1 (AF-1), and one in the ligand binding region, AF-2, which acts as a ligand-dependent transactivation domain (Beato and Klug, 2000; Mangelsdorf, 1995).

In addition, transactivation is also influenced by regions in the carboxyl end of the ligand and in the DNA binding domains, that interact with nuclear receptor corepressor proteins (Chen and Evans, 1995; Horlein *et al.,* 1995; Kurokawa *et al.,* 1995). Sequences important for nuclear translocation are adjacent to and within the DNA binding domain. The ligand binding domain also contains the region important for the binding of heat shock proteins. Both the DNA and the ligand binding domains contain the amino acid residues important for receptor dimerization (Evans, 1988; Tsai and O'Malley, 1994).

Many NRs are expressed as proteins with variable amino terminal regions as a result of cell-type-specific alternative splicing or multiple promoter usage. The different isoforms can exhibit cell- and promoter-dependent transactivation. For example, the progesterone receptor has two isoforms, PR-A and PR-B, PR-A being 164 amino acids shorter than PR-B, which exhibits different effects on transcription.

Nuclear receptors (NRs) can either stimulate or suppress the transcription of genes depending on their cellular background and promoter context (Klinge, 2000; Xu *et al.,* 1999). In the case in which these NRs directly interact with their response elements upon ligand binding, stimulation of transcription occurs via a cooperative interaction of these NRs with coactivators, namely p160 coactivators (SRC-1/NCoA1, GRIP1/TIF2/SRC-2/NcoA-2, AIB1/ACTR/RAC3/ SRC-3), via LxxLL motifs (NR boxes) present in these coactivators. These coactivators recruit a large enzymatic complex involving CBP/p300 and P/CAF that leads to chromatin remodeling via histone acetyl transferase (HAT) activity present in these molecules. Ligand-bound NRs also interact with TRAP220/DRIP205 in a NR box dependent manner and recruit a TRAP/DRIP coactivator complex that interacts with the basal transcriptional machinery and stimulate transcription via a chromatin-dependent manner (Freedman, 1999). Conversely, in the absence of ligands, these NRs interact with corepressors such as N-CoR and SMART and recruit corepressor complexes resulting in transcriptional repression via histone deacetylase (HDAC) activity (Klinge, 2000). Similar negative or positive regulation occurs when NRs control transcription via protein–protein interaction with other transcription factor complexes such as Fos/Jun, NF-κB, and other transcription factor families.

Another level of complexity for NR action was revealed by studies with the ligand RU486. Normally, RU486 is a potent antagonist of both the PR and the GR. However, given together with 8BrcAMP to cells transfected with a progesterone-responsive promoter, RU486 behaves as an agonist and stimulates the transcription mediated by PR (Beck *et al.,* 1993a,b).

Tissue-specific effects of a ligand for NR are illustrated by raloxifene. Raloxifene is an anti-estrogen that has partial agonistic activity. In the uterus, it is an anti-estrogen and does not exhibit uterotrophic activity, whereas in bone it acts as an agonist and like estrogen, prevents ovariectomy-induced bone loss (Beck *et al.,* 1993a,b). Tissue-selective effects of AR ligands are illustrated by the fact that DHT produces gene activation in all androgen-responsive tissues, while testosterone cannot substitute for DHT in specific genital tissues (Hiipakka and Liao, 1995).

The complexity of steroid hormone action is further amplified by crosstalk between signaling pathways. For example, growth factors, such as EGF, and neurotransmitters, e.g., dopamine, can activate NRs by a ligand-independent mechanism (Power *et al.,* 1992). A pathway for receptor activation in the absence of cognate ligand was demonstrated for COUP-TF, PR, and ER. In this pathway, activation of G protein-coupled D1 dopamine receptor by dopamine initiates the signal transduction pathway that leads to NR activation, presumably by induction of a cascade of protein kinase activities that results in receptor phosphorylation and nuclear translocation of the activated receptor (Power *et al.,* 1991, 1992).

Furthermore, steroid hormones can produce effects via nongenomic signaling. For example, androgens can increase cellular calcium through activation of G-proteins, and progesterone metabolites can influence the activity of the brain GABA receptor (Callachan *et al.,* 1987; Lieberherr and Grosse, 1994; Maggi and Perez, 1984). Moreover, the metabolic interconversion of hormones mentioned above, testosterone to DHT, *trans*-retinoic acid to retinoic acid, T4 to T3, further increases the complexity of hormone action via NRs. This complexity can generate a diversity of responses that can be tissue-, promoter-, and ligand-specific and could depend on other signals impinging on the cell at the time.

The Androgen Receptor

The human AR is a protein that contains 918–919 amino acids and is transcribed from a major 10-kb and a minor 7-kb mRNA transcript (Chang *et al.,* 1988; Lubahn *et al.,* 1988; Tilley *et al.,* 1989; Trapman *et al.,* 1988). In mammals, only one AR has been identified, located on the X chromosome, while a second AR that binds to 11-keto testosterone as its preferred ligand has recently been identified in fish (Ikeuchi *et al.,* 1999). The amino terminal region contains a stretch of about 20 glutamine residues, which in humans exhibit polymorphism, ranging between 17 and 26 glutamine residues. Longer stretches were found to be associated with X- linked spinal and bulbar muscular atrophy (La-Spada *et al.,* 1991). The single AR gene identified so far implies that the AR protein should mediate the genomic actions of testosterone, DHT, and possibly the adrenal androgens, either directly or after conversion to testosterone. Both testosterone and DHT bind tightly to AR with K_ds of 1 and 0.1 nM, respectively (Rasmusson and Tonney, 1994). Naturally occurring mutations have been identified, both in the DNA and in the ligand binding domains of AR. These mutations cause androgen insensitivity resulting in different degrees of abnormality in male sexual development (Yarbrough *et al.,* 1990; Quigley *et al.,* 1995). An XY genotype with a complete lack of androgen responsiveness will develop as a female phenotype with female-type bones, as mentioned above. Aberrations in male sex organ development are also found in individuals with mutations in the 5α-reductase gene, who exhibit partial development of some male organs, such as epididymis and vas deferens, but not of the urogenital tract, the prostate, and the external genitalia, indicating that T cannot substitute for DHT as an activator of AR in genital development (Hiipakka and Liao, 1995). This ligand specificity for certain tissues raises the possibility that androgenic compounds with AR agonistic activity could have specificity for certain tissues, such as bone, while lacking activity in other tissues, such as those responsible for virilization.

The ligand-bound AR recognizes a palindromic androgen response element (ARE) as a homodimer and, like other NRs, activates transcription by recruiting large coactivator complexes. The prototypical androgen response element (ARE) was identified functionally in the promoter regions of androgen-responsive genes present in prostate, such as probasin or prostate-specific antigen (Rennie *et al.,* 1993; Brinkman *et al.,* 1999). The optimal ARE was later characterized by gel shift assays using random oligonucleotides as "RGAWCA NNN TGTTCT" (Schoenmakers *et al.,* 2000). Transcriptional activities of AR have been mapped to both N-terminal (NTD)and C-terminal (LBD) domains as AF1 and AF2. A unique feature of transactivation by AR is the functional interaction between NTD (AF1) and LBD (AF2) that is essential for transcriptional activity (Ikonen *et al.,* 1997). Unlike other NRs where AF2 activity is predominant, AR possesses a potent AF1 activity and weak intrinsic AF2 activity. The ligand-dependent activation of AF2 activity, evaluated in the context of Gal4DBD-AR-LBD fusion protein, depends on the recruitment of the NTD which is further potentiated by p160 coactivators and CBP/p300 (Brinkman *et al.,* 1999). In contrast to TR or RAR that interact with p160 coactivators via the AF2 domain alone, in a NR box dependent manner, AR also interacts with the C-terminal domain of p160 coactivators via the AF1 domain that interacts with the C-terminal domain of p160 coactivators via the AF1 domain. Mutational analysis of p160 coactivators documented the predominant role of AF1 interaction with p160 coactivators in AR-mediated transcriptional activation. Upon association with AR, activation domain 1 (AD1) of p160 coactivators interacts with CBP/p300 that remodel histones (Ma *et al.,* 1999). In addition to HAT activity present in CBP/p300 and P/CAF, a recent report documented the role of arginine methyl-transferases, CARM1 (coactivator-associated arginine methyltransferase) and PRMT1 (protein arginine N methyltransferase), that interact with AD2 of p160 coactivators to potentiate AR-mediated transcription by methylating histones (Chen *et al.,* 1999; Koh *et al.,* 2001).

The biological relevance of the interaction of AR NTD/LBD domains has been documented in mutations that disrupt the NTD/LBD interaction, causing marked reduction in transcriptional activity and androgen insensitivity syndrome (He *et al.,* 1999). Interestingly, a recent report documented the role of FXXLF and WXXLF motifs present in NTD for its association with LBD, suggesting that NTD interacts with the hydrophobic surface of LBD in a manner analogous to the interaction of AF2 with p160 coactovators (He *et al.,* 1999).

In addition to p160 coactivators, a number of coactivators are found to interact with AR. The LBD (AF2) of AR interacts with ARA54, a RING finger B box family protein (Kang *et al.,* 1999), ARA55/Hic-5 with a LIM motif ARA70, RIP140 (Ikonen *et al.,* 1997), and Tip60 (Brady *et al.,* 1999). The AR-NTD(AF1) interacts with ARA24/RAN (Hsiao *et al.,* 1999), a nuclear small G-protein, Cyclin E (Yamamoto *et al.,* 2000), and TFIIF (McEwan and Gustafsson *et al.,* 1997) and also with ANPK, a nuclear protein kinase (Moilanen *et al.,* 1998); ARIP3, a STAT binding protein (Moilanen *et al.,* 1999); PDEF, an ETS transcription factor (Oettgen *et al.,* 2000); SNURF, a ring finger protein (Poukka *et al.,* 2000); and ARIP4, a nuclear ATPase (Janne *et al.,* 2000). Like p160 coactivators, ARA160 (Hsiao and Chang, 2000) and FHL2, a LIM only protein (Muller *et al.,* 2000), were shown to interact with multiple regions of AR.

Negative transcriptional control by AR was reported for the expression of the maspin gene in prostate cells (Zhang *et al.,* 1997). It was shown that the maspin promoter contains an HRE (hormone response element) site that mediates the negative regulation of the maspin gene by AR. Transcriptional regulation, negative or positive by NRs can also occur via protein–protein interaction with other components of the transcription machinery, including AP-1, SP1, C/EBP, or Nf-KB, which in turn act on their respective DNA binding sites (Diamond *et al.,* 1990; Jonat *et al.,*

1990; Mukaida *et al.,* 1994; Perkins *et al.,* 1993; Ray and Prefontaine, 1994; Ray *et al.,* 1995; Stein and Baldwin, 1993; Stein *et al.,* 1993a,b). Such interactions can explain, for example, the effects of GR on the action of interleukins IL-6 and IL-8 (Diamond *et al.,* 1990; Jonat *et al.,* 1990; Mukaida *et al.,* 1994; Perkins *et al.,* 1993; Ray and Prefontaine, 1994; Ray *et al.,* 1995; Stein and Baldwin, 1993; Stein *et al.,* 1993a,b). Glucocorticoids were also shown to induce the expression of inhibitory protein-κB (I-κB), a nuclear factor that antagonizes the activity of the nuclear transcription factor NF-κB (Auphan *et al.,* 1995; Scheinman *et al.,* 1995).

The participation of AR in negative control of transcription via protein–protein interaction was demonstrated for the transcriptional repression of the matrix metaloproteinase-1 gene (MMP-1) in prostate cells (Schneikert *et al.,* 1996). In this case, the inhibition of transcription by AR was dependent on the presence of the Ets transcription factor binding site and the interaction of the Ets protein with the amino terminus region of AR. Above examples of negative and positive regulation of transcription by AR indicate that the role of AR in the control of bone metabolism may be the result of transactivation, transrepression or a combination of both activities on different target genes.

AR was also shown to modulate transcription from the promoter of the low affinity neurotropin receptor gene without directly interacting with a specific DNA element (Kalio *et al.,* 1994). This type of receptor transcription factor interaction could be responsible for AR or ER suppression of transcription from the promoter of the IL-6 gene, that lacks a typical ER or AR SRE (Bellido *et al.,* 1995).

Androgen Mode of Action on the Skeleton

The actual mechanism for androgen effects on bone is still under investigation. Improved methodology for the detection of AR led to the demonstration of both AR mRNA and protein expression in osteoblasts, supporting the hypothesis that androgens act directly on bone cells. AR mRNA was found in normal human osteoblasts as well as in human osteosarcoma TE85 cells (Benz *et al.,* 1991; Colvard *et al.,* 1989a,b; Liesegang *et al.,* 1994). Specific binding of androgens to nuclear proteins was documented in human osteoblasts in human osteosarcoma SaOS, US02, and TE85 cells, as well as in rat UMR106 cells and in mouse MC3T3-E1 osteoblastic cells (Benz *et al.,* 1991; Colvard *et al.,* 1989a,b; Orwoll *et al.,* 1991). The affinity of DHT to AR in human osteoblasts, isolated from both men and women was similar to that in classical androgen-responsive tissues (kDa between 0.43 to 1 nM). The number of AR per cell in human osteoblasts varied between 514 and 2800, sufficient for producing a physiological response. ARs were also visualized by immunocytochemistry in mouse osteoclast-like cells generated in culture, suggesting potential androgen effects on osteoclasts (Mizuno *et al.,* 1994). Other recent studies, however, failed to detect estrogen, testosterone, and their respective receptors in bone, although endogenous 1α,25-dihydroxyvitamin D_3 and progesterone and their receptors were localized at the ultrastructural level (Boivin *et al.,* 1994). Thus, the presence, abundance, and activity of ARs in bone cells and the type of bone cells that contain ARs require further investigation.

The addition of androgen to osteoblast cultures has generated various physiological responses. It was observed that androgens stimulate the proliferation of various osteoblast-like cells in culture and upregulate the expression of several mRNAs or proteins (Kasperk *et al.,* 1989, 1990; Gay *et al.,* 1992; Masuyama *et al.,* 1992; Takeuchi *et al.,* 1994). Testosterone increases production of type I collagen. TGFβ1, c-*fos,* and c-*jun* in HOS, TE85, and SAOS-2 human osteosarcoma cells (Takeuchi *et al.,* 1994). DHT upregulated the expression of alkaline phosphatase and TGFβ2 in TE89 and SAOS-2 human osteosarcoma cells (Takeuchi *et al.,* 1994). DHT upregulated the expression of alkaline phosphatase and TGFβ2 in TE89 and SAOS-2 human osteosarcoma cells (Kasperk *et al.,* 1989, 1990). Moreover, it was shown that DHT treatment increases the steady-state level of the AR mRNA in the human osteosarcoma TE85 cells, in contrast to reducing AR mRNA levels in prostate cancer cells (Takeuchi *et al.,* 1994). This modulation of AR mRNA levels by DHT treatment may contribute to the inconsistent observations on androgen effects in osteoblastic cells. The adrenal androgens DHEA and DHEA-S were shown to suppress c-*fos* expression at physiological concentrations in normal human osteoblasts and to increase TGFβ activity without altering the steady-state level of TGFβ_1 mRNA (Kasperk *et al.,* 1989, 1990). It was shown in human osteosarcoma TE85 cells that norethindrone pretreatment synergistically potentiates the effects of fluoride, an anabolic agent that stimulates bone formation in osteoporotic women (Takada *et al.,* 1995). In this context, similar pretreatment with either progesterone or DHT did not produce similar synergistic effects, suggesting a potentially unique property of this anabolic steroid. Some of the androgen effects observed in the various studies of osteoblasts in culture may be mediated by nontranscriptional effects. It was shown that osteoblast treatment with nanomolar concentrations of testosterone induces a rapid increase in intracellular calcium concentration, inositol 1,4,5-triphosphate, and diacylglycerol formation via G-protein-coupled pathways, consistent with so-called nongenomic effects of testosterone (Lieberherr and Grosse, 1994). The molecular basis of these effects require further study.

To summarize this section, androgens act via the AR, a nuclear receptor that has not been as extensively studied as the estrogen and progesterone receptors. AR is expressed in osteoblastic cells, and androgens at physiological concentrations produce effects on these cells in culture. However, further studies are needed to establish the target cells of androgens *in vivo* and relate the *in vitro* effects to their anabolic action on bone.

Conclusions

The evidence available to date suggests that androgens have a beneficial effect on the female skeleton and may increase bone mass both before and after menopause. The therapeutic use of anabolic steroids, most frequently in older women and reported mostly in uncontrolled studies, supports these conclusions. The use of anabolic steroids in postmenopausal osteoporosis is limited by side effects such as virilization and elevation of plasma lipids. Most studies were conducted with compounds developed for use in males, chosen on the basis of androgenic or anti-androgenic activities in rats. The human receptor was cloned later than that of other steroid hormones (1988) and the mode of action of androgens and anabolic steroids on the skeleton is still under investigation. Only one AR gene has been identified, and there are at least two isoforms produced from this gene. AR expression was documented in human and rodent osteoblastic cells, however, the mode of androgen action on bone *in vivo* remains to be elucidated. The tools are currently available to develop selective androgenic and anti-androgenic compounds and to study their effects in bone and their mode of action.

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CHAPTER 85

Estrogen Effects on Bone in the Male Skeleton

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Over the past decade, we have seen fundamental changes in the landscape that used to describe osteoporosis as a rather exclusive disease of postmenopausal women. We now appreciate the fact that osteoporosis affects men as well as women (Orwoll, 1998; Bilezikian, 1999). Moreover, osteoporosis is being recognized increasingly among elderly men as a medical, social, and economic burden (Orwoll, 1995a). As much as 20% of the total incidence of osteoporosis in the United States may be in men, with proportionate costs attributed to this incidence figure in the male (Looker *et al.,* 1997). These observations have led, in turn, to efforts to understand better the physiology of the male skeleton, in terms of both protective factors and factors that place the male skeleton at risk. The purpose of this chapter is to review this information in the context of the developing and adult male skeleton and the role of estrogens.

Cartoons that describe the change in bone mass with time have tended to emphasize the role of aging per se because it is associated with a rather constant rate of decline in bone density from the middle years onward (Fig. 1). The downslope of this curve is more telling in women because they are exposed to an additional insult, besides age, namely the abrupt cessation of ovarian estrogen production at the time of the menopause. The accelerated bone loss characteristic of the early postmenopausal period is believed to place women at greater risk for osteoporosis than men are because men, under normal circumstances, do not experience a menopause-equivalent during their middle years.

Such observations help to explain differences between men and women in their differential susceptibility to osteoporosis. However, equally important is the upswing in the curve describing acquisition of bone mass during the youthful years of skeletal accrual. Factors responsible for the establishment of peak bone mass are also key aspects to any discussion of the osteoporotic process. For example, the attainment of peak bone mass helps to define the reserve that is called upon during the period of age-related bone loss. Someone who has achieved optimal peak bone mass is likely to be relatively more protected from losing so much bone mass that places him/her at risk for osteoporotic fracture. In contrast, someone whose peak bone mass is suboptimal may show evidence for fracture risk earlier in the aging process. While a number of factors help to account for the degree to which an individual achieves optimal peak bone mass (e.g., genetics, nutrition, environmental, exercise, calcium intake), this discussion will deal only with the role of the sex steroids, with particular emphasis on estrogens.

Estrogen deficiency states such as delayed puberty, amenorrhea of any cause, and, perhaps, the use of progestational agents for contraception, can lead to suboptimal acquisition of peak bone mass. In the male, similarly, androgens are thought to be critical to the establishment of peak bone mass. Finkelstein has shown, for example, that delayed puberty in otherwise normal boys, is associated with reduced bone density for as long as 10 years thereafter as compared to boys who entered puberty "on time" (Finkelstein *et al.,* 1992, 1996). Although these results have been disputed by Bertelloni *et al.* (1998), who argue that volumetric bone density is not reduced among boys with delayed puberty, the point is made, nevertheless, that areal density might suffer in growing boys who are not exposed to androgens at a critical time in their skeletal development.

In sex steroid-sufficient, growing boys and girls, a 7–10% difference in the achievement of peak bone mass is believed

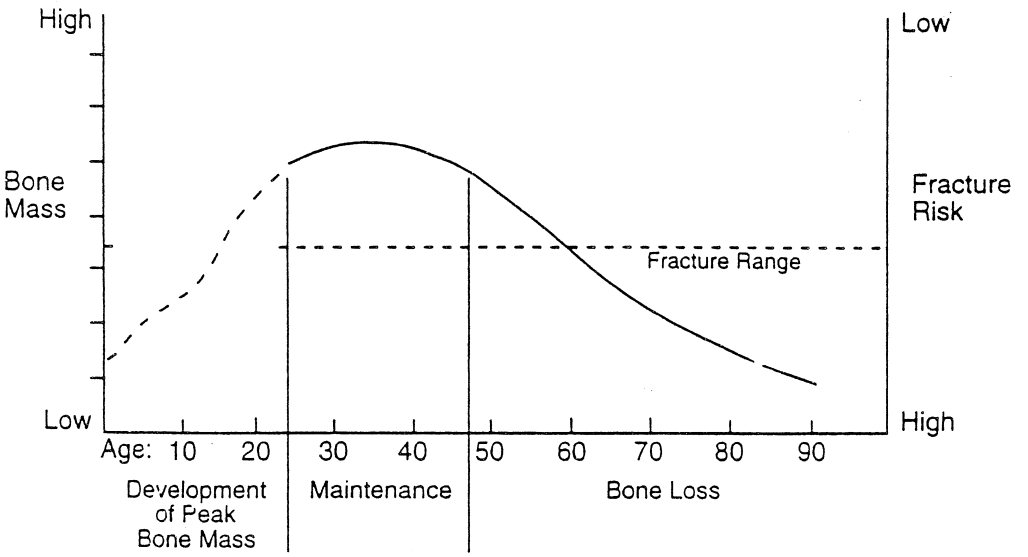

Figure 1 The change in bone mass as a function of the aging process. This schema illustrates the importance of establishing optimal peak bone mass in the chronology of events that take place thereafter. Adapted from Wasnich *et al.* (1991).

to be a key protective factor in the male. Thus, not only do men not normally experience an abrupt cessation of androgen production in their middle years, but they also achieve a greater reservoir of bone due to the attainment of greater peak bone mass in youth. Recent observations have helped to clarify these differences in the achievement of peak bone mass as a function of androgens and estrogens. When true bone density is measured, there is relatively little difference between the growing male and female skeleton throughout childhood (Gilsanz *et al.,* 1994). The difference appears to be bone size, a parameter that is compatible with a specific androgen effect (Beck *et al.,* 1992).

Seeman has emphasized the point that androgens have region-specific actions on bone growth and remodeling so as to lead to larger bones (Orwoll, 1995b; Duan *et al.,* 1999; E. Seeman, personal communication). Bones that are exposed to androgen display greater periosteal apposition, which, in turn, leads to larger cross-sectional diameter. Under the influence of androgens, therefore, bones are bigger, but not necessarily more "dense." In the aging male skeleton, similarly, the male demonstrates greater periosteal apposition than the aging female skeleton. For a given reduction in cortical bone mass, occurring presumably to the same extent among the genders when due only to aging, the male is conferred a relative mechanical advantage by increased bone diameter. This effect may well be a specific feature of androgens.

While it may have seemed clear that differences between the growing female and male skeleton as well as the skeleton in decline could be attributable satisfactorily, if not completely, to the sex-specific steroids (estrogens in women; androgens in men), recent observations have shattered this simple concept. It is no longer straightforward. The new observations underscore the normal biosynthetic pathway by which estrogens are made. C-18 estrogens are derived from androgens via the activity of aromatase, a cytochrome P450 product of the CYP 19 gene (Bulun *et al.,* 1993). The obligate precursors are the C-19 androgenic steroids. The human P450 aromatase enzyme is found in many tissues such as placenta, ovary, testis, brain, and adipocyte. Regulation of aromatase activity occurs through tissue-specific promoters and alternative splicing mechanisms (Simpson *et al.,* 1997). The translated gene product, however, is the same among all tissues that harbor an active aromatase gene.

With this background, one can quite reasonably revisit the classical observation assigning differences among male and female skeletal growth to androgens or estrogens, respectively. It is possible that the growing male skeleton is also dependent upon estrogens and that some skeletal events previously noted to be androgenic may actually be due, at least in part, to estrogens. Such thinking, which seemed rather heretical a short 5–7 years ago, has received seminal support by two fundamental experiments of nature. In two rare syndromes, the estrogen receptor or the aromatase gene has been "knocked out" by a point mutation, rendering subjects either estrogen resistant (receptor defect; Smith *et al.,* 1994) or estrogen deficient (aromatase defect; Morishima *et al.,* 1995; Carani *et al.,* 1997). A key role of estrogens in male skeletal development has emerged from insights gleaned from the study of these unusual subjects.

Alpha-Estrogen Receptor Deficiency in the Male

In 1994, Smith *et al.* described a 28-year-old man with a disruptive homozygous mutation in exon 2 of the alpha-estrogen receptor gene. The gene defect was inherited as an autosomal recessive from his consanguineous parents who were heterozygotes for the gene defect. A cytosine to

thymidine transition at codon 157 resulted in a stop codon and an alpha estrogen receptor that is severely truncated and cannot bind estrogen. He had no detectable response to administration of large doses of exogenous estrogen (transdermal ethinyl estradiol), achieving serum concentrations of estradiol 10-fold higher than the typical male, 270 pg/ml (nl, 10–50). His own baseline estradiol (119 pg/ml) and estrone (145 pg/ml) levels were markedly above normal. Although bound and free testosterone as well as dihydrotestosterone concentrations were normal, LH (37 mIU/ml) and FSH (33 mIU/ml) levels were in the mildy castrate range. He was extremely tall (204 cm) without acromegalic features and still growing with open epiphyses (bone age, 15 years) and genu valgum. The growth curve did not show a pubertal growth spurt although his puberty in all other respects was normal. His body proportions were eunuchoid. Bone density of the lumbar spine as measured by dual energy X-ray densitometry was 0.745 g/cm^2, corresponding to 2 standard deviations below average for a 15-year-old boy.

Aromatase Deficiency in the Male

In three male subjects, each products of consanguineous marriages, aromatase deficiency has been described (Morishima *et al.,* 1995; Carani *et al.,* 1997; Deladoey *et al.,* 1999). In each case a point mutation in the aromatase gene at exon IX (Morishima *et al.,* 1995; Carani *et al.,* 1997) or at exon V (Deladoey *et al.,* 1999) was associated with a completely inactive gene product. The report of Morishima *et al.* (1995) describes a 24-year-old man with a single base change at base pair 1123 (C to T) with an amino acid substitution at R375, adjacent to the heme binding site. Similarly, the report of Carani *et al.* (1997) describes a 31-year-old man with a single base pair change at position 1094 (G to A) with the amino acid substitution at R365. In the report of Deladoey *et al.* (1999) of a newborn male, the defect was discovered to be in exon V, causing a frame shift mutation with an ensuing premature stop codon. The truncated gene product is completely devoid of aromatase activity.

The two adult men were very tall and still growing with unfused epiphyses, a bone age of 15, eunuchoid features (upper segment/lower segment = 0.85–0.88), and genu valgum. The patient of Morishima *et al.* had markedly elevated androgen levels with the complete biochemical profile shown in Tables I and II. The growth curve of that patient, similar to that of the man with the alpha-estrogen receptor defect, did not give evidence for a pubertal growth spurt although in all other respects puberty was not delayed (Fig. 2). Both patients had reduced bone mass. The bone mineral density of the

Table I Biochemical Parameters in a Man with Aromatase Deficiency

Calcium	9.9	8.7–10.7 (mg/dl)
Phosphorus	3.3	2.5–4.5 (mg/dl)
PTH	26	10–65 (pg/ml)
25-Hydroxyvitamin D	36	9–52 (ng/ml)
1,25-Dihydroxyvitamin D	55	15–60 (pg/ml)
Alkaline Phosphatase	241	39–117 (IU/L)
Urinary calcium	185	150–300 (mg/24 hr)
Deoxypyridinoline	25.3	4–19 nmol/nmol Cr

Note. Adapted from Morishima *et al.* (1995).

Table II Sex Steroids and Gonadotropin Concentrations in a Man with Aromatase Deficiency

Estradiol	<7	10–50 pg/ml
Estrone	<7	10–50 pg/ml
Testosterone	2015	200–1200 ng/dl
5-α dihydrotestosterone	125	30–85 ng/dl
FSH	28.3	5–9.9 mIU/ml
LH	26.1	2.0–9.9 mIU/ml

Note. Adapted from Simpson *et al.* (1997).

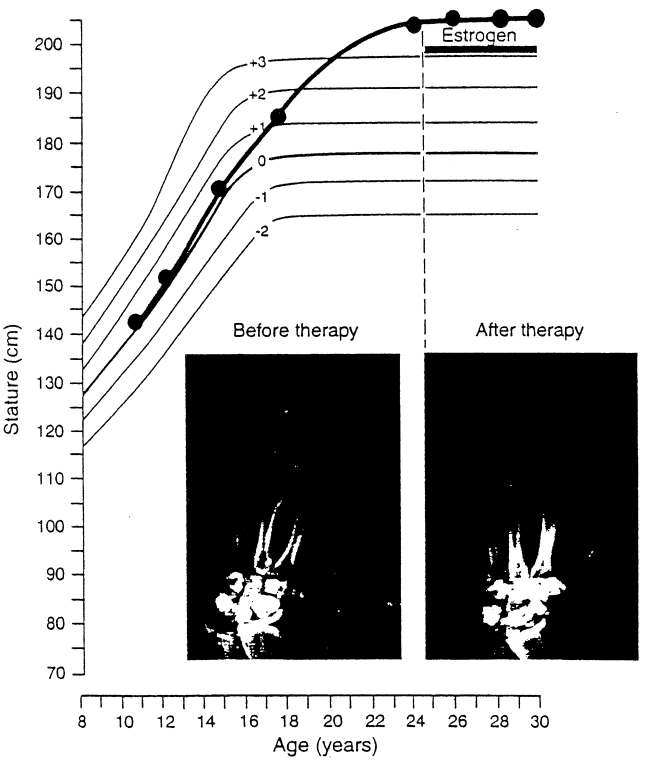

Figure 2 Growth curve and bone age before and after 5 years of estrogen therapy in a man with aromatase deficiency. After conjugated estrogen therapy was begun (bar), linear growth ceased immediately. Height has remained at 204 cm since therapy whereas previously it was showing continued increases. All epiphyses were closed within 6 months (insets). The curves with + and − numbers represent the mean and standard deviations for normal young men.

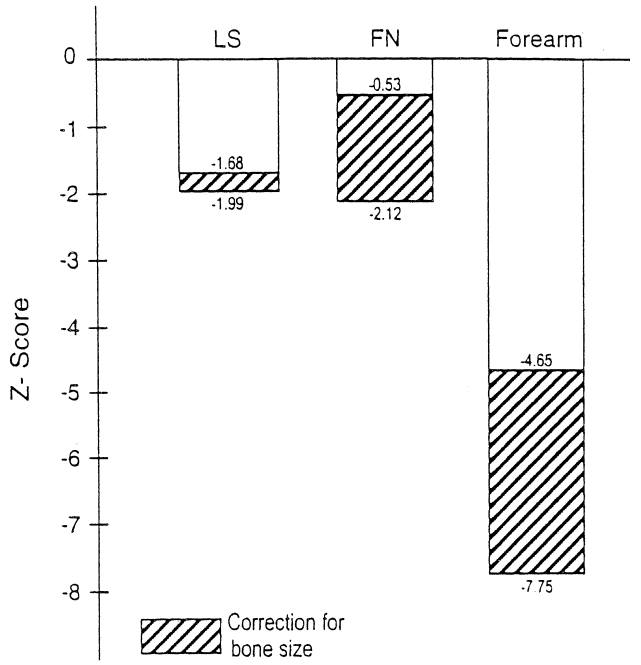

Figure 3 Bone mineral density at baseline in a man with aromatase deficiency. For each site, the bone mass is shown as the two-dimensional value (g/cm^2) and as bone mineral apparent density using correction factors for body size (Morishima *et al.,* 1995). For each site, estimated true bone density is substantially lower than the direct measurement, as shown in the shaded area of each site.

patient described by Morishima *et al.* is shown in Fig. 3. When these values were corrected because of his large bone size, bone mineral density was even more impressively reduced with *T* scores of −1.99 (spine), −2.12 (femoral neck), and −7.75 (forearm). Thus, the apparent bone density was even more evidently osteoporotic than the direct two-dimensional area density measurement.

Both men with aromatase deficiency responded to the administration of estrogen (Carani *et al.,* 1997; Deladoey *et al.,* 1999; Bilezikian *et al.,* 1998). Data are now available with a 5-year follow-up in the patient described by Morishima, Bilezikian, and their colleagues. Starting on low-dose conjugated estrogen, 0.3 mg, he was quickly raised to 0.75 mg daily, the dosage that he has consistently taken for most of the period of observation. Estradiol levels rose from undetectable into the normal range for men. Androgen levels fell from markedly elevated levels to normal. The rising estrogen levels and falling androgen levels occurred over the same period of time. The concomitant return of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), to normal illustrates well the role of estrogens in controlling gonadotropin production in the male (Fig. 4). He immediately stopped growing with closure of all open epiphyses within 6 months of starting estrogen therapy (Fig. 2). Bone markers that were elevated at baseline returned slowly to normal (Fig. 5). Most impressively, this man has experienced a dramatic increase in bone density with estrogen therapy. During the first 3 years, increases in the lumbar spine, femoral neck, and

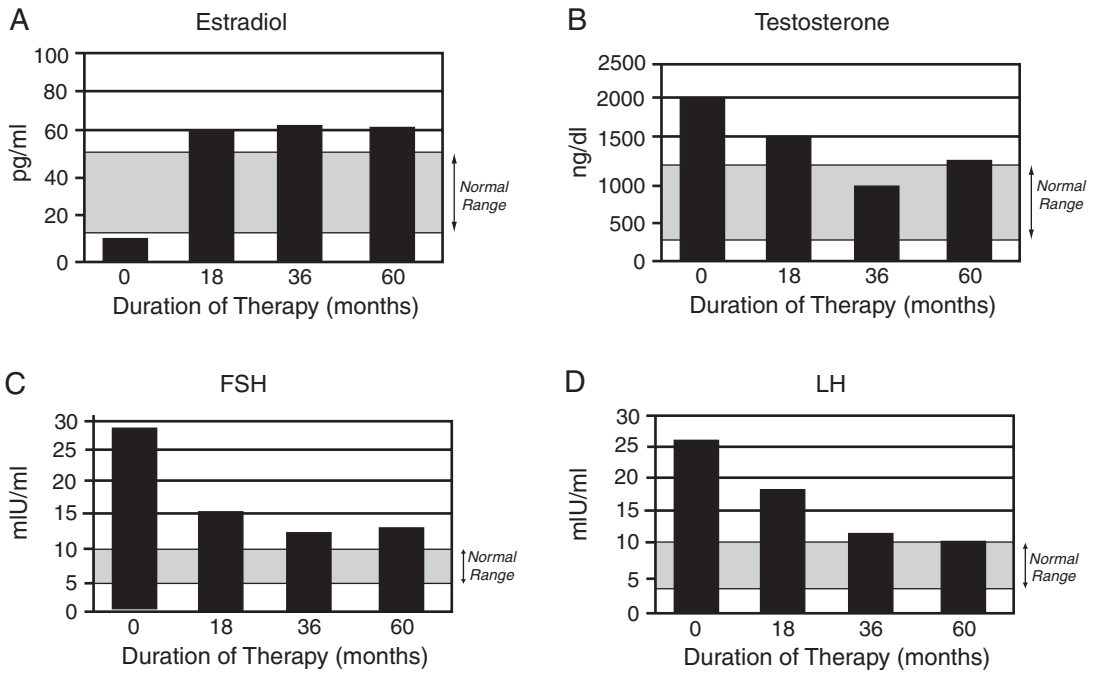

Figure 4 Changes in sex steroids and in gonadotropins with estrogen therapy in a man with aromatase deficiency. The data are shown for estradiol (A), testosterone (B), FSH (C), and LH (D). Over 5 years, estradiol levels initially rose and then were maintained at levels in the normal range for males when administered at 0.75 mg per day. Testosterone levels fell into the normal range, as did FSH and LH levels.

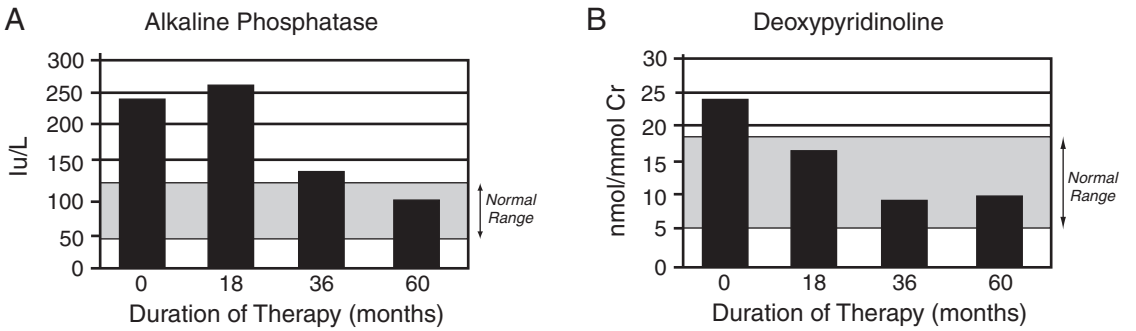

Figure 5 Changes in bone markers with estrogen therapy in a man with aromatase deficiency. The data are shown for alkaline phosphatase (A) and for deoxypyridinoline (B). The resorption markers, deoxypyridinoline, appear to fall faster over time than did the formation marker, alkaline phosphatase.

forearm were 20.7, 15.7, and 12.9%, respectively. Over the next 2 years of estrogen therapy, the gains in the lumbar spine and femoral neck were maintained with further marked increases in the forearm bone density, now totaling 26% (Fig. 6). The improvement in bone mass is seen also by the change in *T* score with normal values at the lumbar spine and femoral neck and from markedly osteoporotic levels in the forearm to mildy osteopenic levels ($T = -4.65$ to -1.84). The effect of estrogen to improve bone mass is best described as anabolic because of the magnitude of the change and also because further bone growth did not occur. Without further bone growth, the change in bone mineral density is more likely to reflect improved mineralization per unit area. It should be noted that this action of estrogen to stimulate the acquisition of bone mass through an anabolic effect is quite different from its effects in the postmenopausal woman, in whom the estrogen effect is more accurately described as antiresorptive. A summary of the skeletal aspects of aromatase deficiency is shown in Table III. A comparison between the syndromes of estrogen resistance and estrogen deficiency in the male is shown in Tables IV and V.

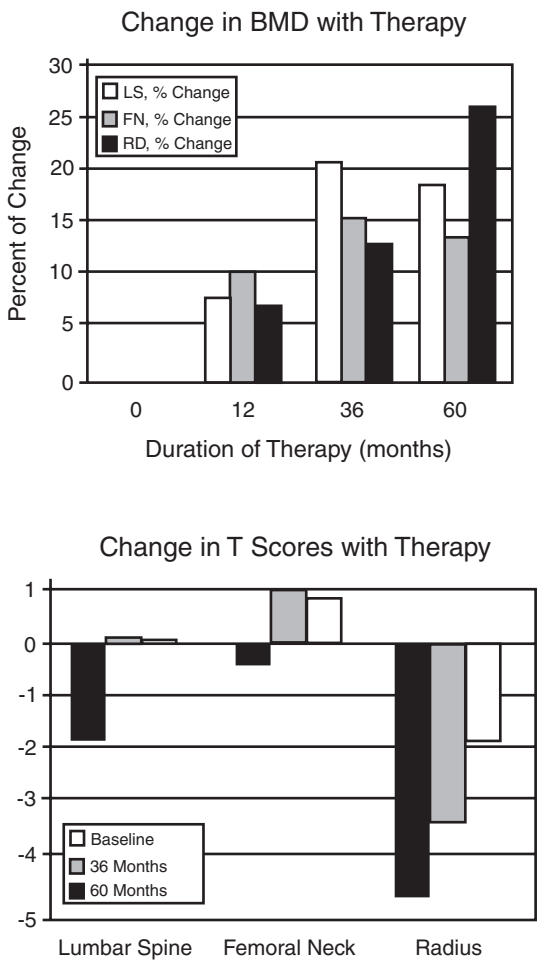

Figure 6 Changes in bone mineral density with estrogen therapy in man with aromatase deficiency. Percentage (A) and *T* score (B) changes are shown for lumbar spine, femoral neck, and distal radius. The data are shown not corrected for bone size.

Of special note is the relationship between the pubertal growth spurt and estrogen. In the syndromes of estrogen deficiency or resistance, the tallness and continued growth well into adulthood was not associated with a pubertal growth spurt. If estrogens are important in this regard, syndromes of estrogen excess would be expected to be associated with premature or early growth spurt. In the example of aromatase excess, due to an activating mutation of the aromatase gene, with elevated estrogen levels, puberty occurs early and is accompanied by skeletal maturation. This occurs despite normal testosterone levels (Hemsell *et al.,* 1977; Bulun *et al.,* 1999; Stratakis *et al.,* 1998; Shozu *et al.,* 1998). Additional evidence for an important role of estrogens in the pubertal growth spurt is the testicular feminization syndrome in which

Table III Importance of Estrogens in Male Skeletal Development

Linear bone growth
Pubertal growth spurt
Epiphyseal maturation
Optimal peak bone mass

Table IV Skeletal Aspects of Aromatase Deficiency in the Male

Constant linear growth
Lack of pubertal growth spurt
Delayed skeletal maturation
Markedly reduced bone mass

XY males do not respond to androgens because of a mutation in the androgen receptor. Responsiveness to estrogens is normal. In this syndrome, the pubertal growth spurt is seen (Grumbach and Auchus, 1999; Zachmann *et al.,* 1986). Finally, in estrogen-secreting tumors, similar observations of premature skeletal maturation have been made (Coen *et al.,* 1991; Simpson *et al.,* 1994; Grumbach and Styne 1998). These observations, therefore, argue rather persuasively that in the male, as in the female, the pubertal growth spurt is a function of estrogens, not androgens.

It is interesting to speculate that the difference in height among women and men may reflect the difference in the time of onset of puberty. Boys gain a few more years of linear growth because their puberty occurs later. The notion, namely, that the additional stature in the male is due to estrogen, not androgens, is particularly intriguing.

From these human experiments of nature, it seems reasonable to conclude that in the male, estrogens are important in the control of linear growth, in the pubertal growth spurt, in epiphyseal maturation, and in the achievement of optimal peak bone mass (Table VI). Such conclusions, obviously, must be tempered by an appreciation that androgens are also important in the acquisition of peak bone mass. In the testicular feminization syndrome, for example, the peak bone mass achieved is consistent with expectations for an estrogenic influence only since these XY boys have bone density most consistent with expectations for an XX girl (Kelch *et al.,* 1972). In either event, the failure to achieve peak bone mass may be a risk factor for the development of osteoporosis later in life. While one does not expect to "find" many examples of complete estrogen deficiency or resistant syndromes, there are possible more subtle abnormalities in those genes that would render men at risk for osteoporosis on this basis.

Table V Comparison of α-Estrogen Receptor and Aromatase Deficiency Syndromes

	α-Estrogen receptor	Aromatase deficiency
Testosterone	Normal	High
Estrogen	High	Low
Gonadotropins	High	High
Calciotropics	Normal	Normal
Bone turnover	High	High

Table VI Comparison of α-Estrogen Receptor and Aromatase Deficiency Syndromes

	α-Estrogen receptor	Aromatase deficiency
Tallness	+	+
Eunuchoid	+	+
Masculinized	+	+
Testicular size	Normal	Large
Bone age	Reduced	Reduced
Bone mass	Reduced	Reduced
Response to E	No	Yes

Animal "Knockouts" of the Estrogen Receptor and Aromatase Genes

Animal models that reflect the human gene defects in the alpha-estrogen receptor and in the aromatase enzyme are now available. The alpha-estrogen receptor knockout mouse displays a 20–25% reduction in bone density (Lubahn *et al.,* 1993; Korach *et al.,* 1996; Couse and Korach, 1999), consistent with the human patient described by Smith *et al.* (1994). In contrast, the beta-estrogen receptor knockout male mouse shows no skeletal abnormalities (Windahl *et al.,* 1999). It is of interest that the same beta-estrogen receptor defect in the adult female mouse, however, does show changes with an increase in bone mineral content due to an increase in cross-sectional cortical area.

Oz *et al.* (2000) have recently characterized the skeletal features of the aromatase knock out mouse (first reported by Fisher *et al.,* 1998). The male mice show radiographic evidence for reduced bone mineral density. Histologically, the mice show significant reductions in trabecular bone volume and in trabecular thickness. By histomorphometry, male knockout (but not female counterparts) show reductions in both osteoblastic and osteoclastic surfaces. Female mice showed a picture more consistent with high bone turnover. This sexual dimorphism with respect to these histomorphometric features has no ready explanation at this time. Nevertheless, these animal knockout experiments provide general confirmation of the human gene knockout disorders, documenting further the important role of estrogens in male skeletal development.

Studies in Humans—Observational Data

The data noted above from the human "experiments of nature" clearly indicate that estrogen plays a significant role in the male skeleton. In particular, they demonstrate that estrogen is required for epiphyseal closure, that it has significant effects on skeletal modeling during growth, and that it is necessary for optimal acquisition of bone mass during puberty in

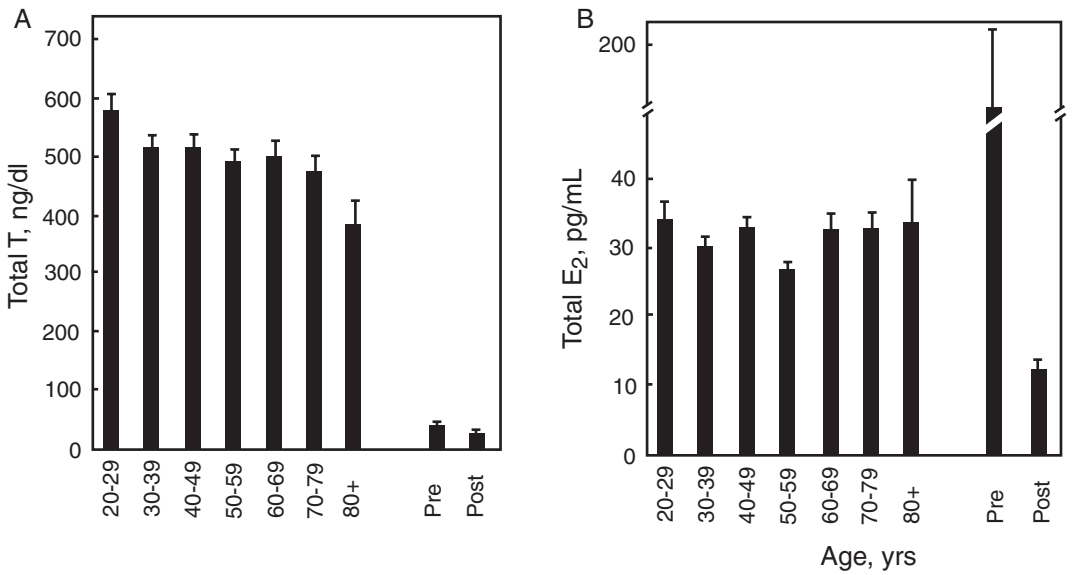

Figure 7 Serum total testosterone (A) and estradiol (B) levels as a function of age in a population-based sample of men. Data are adapted from Khosla *et al.* (1998), with permission.

boys. These observations, however, leave several major questions unresolved. In particular, they leave open the issues of what, if any, are the roles of estrogen in regulating bone remodeling in adult men with mature skeletons, whether estrogen is necessary for the maintenance of bone mass in adult men, and what, if any, is the role of estrogen (or estrogen deficiency) in mediating age-related bone loss in men.

Before addressing these issues, it is useful to review the available data on age-related changes in serum sex steroid levels in normal men. As shown in Fig. 7A, serum total testosterone levels do not change significantly in otherwise healthy men until the ninth decade. Similarly, there is little or no change in serum total estradiol levels in men over life (Fig. 7B). By contrast, serum bioavailable (or non-sex hormone binding globulin (SHBG) bound) testosterone levels decrease by approximately 70% (Fig. 8A) and serum bioavailable estradiol levels decrease by approximately 50% (Fig. 8B) over life. To the extent that the non-SHBG bound sex steroid fraction is the one that has access to target tissues (Manni *et al.,* 1985; Giorgi and Moses, 1975), these data indicate that while men do not have the equivalent of the menopause, they clearly have significant (but gradual) age-related decreases in bioavailable testosterone and estradiol levels

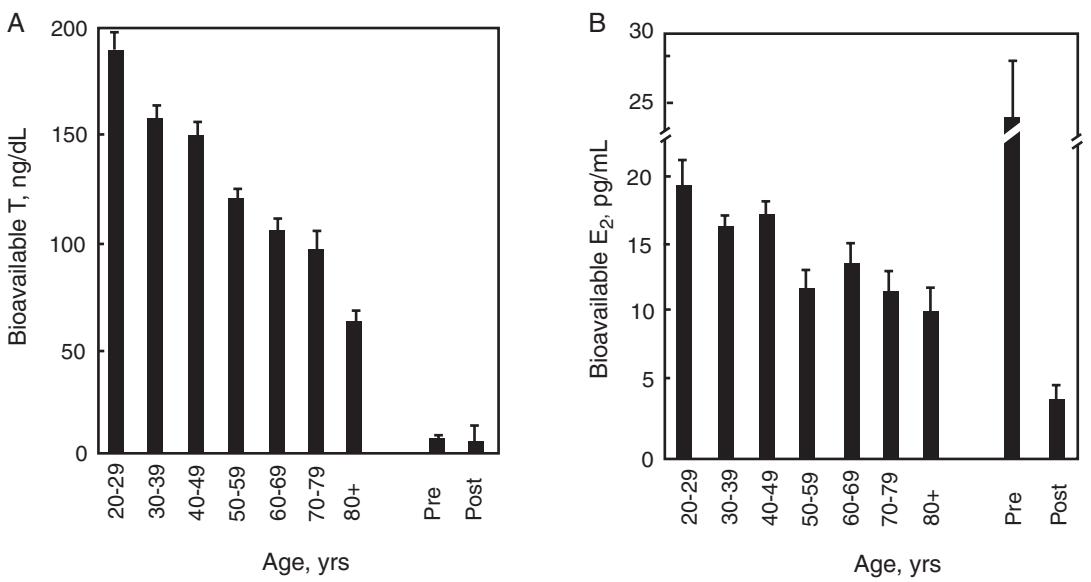

Figure 8 Serum bioavailable testosterone (A) and estradiol (B) levels as a function of age in a population-based sample of men. Data are adapted from Khosla *et al.* (1998), with permission.

over life. These changes are due primarily to a large increase (of approximately 125%) in serum SHBG levels over life in men (Khosla *et al.,* 1998). While the reason(s) for this increase in SHBG levels are unclear, it is apparent that the age-related increase in SHBG serves to limit the availability of testosterone and estradiol to target tissues in elderly men.

One approach to assessing the relative roles of estrogen versus testosterone in the male skeleton has been to measure sex steroid levels in adult men and to relate them in cross-sectional studies to bone mineral density (BMD) at various sites. Using this approach, Slemenda *et al.* (1997) found that in men over the age of 65 years, BMD was significantly positively associated with serum estradiol levels at all skeletal sites (correlation coefficients of 0.21 to 0.35). Serum testosterone levels were actually negatively associated with BMD in this study (correlation coefficients −0.20 to −0.28). Similar findings were noted by Greendale *et al.* (1997) in a study of 534 community-dwelling men ages 50–89 years. In this study, serum bioavailable estradiol and testosterone were directly measured, and serum bioavailable estradiol levels were most strongly associated with BMD at all sites, even after adjusting for age, body mass index, and other possible confounders. In a subsequent study, Khosla *et al.* (1998) also found that serum bioavailable estrogen levels were the most consistent independent predictors of BMD in both young (less than 50 years) and older (over 50 years) men. These and several other cross-sectional studies (Ongphiphadhanakul *et al.,* 1998; Center *et al.,* 1999) thus clearly indicate that, at least using this approach, BMD is more closely associated with serum estrogen levels than with serum testosterone levels in adult men.

While these findings add to the evidence from the ER-alpha and the aromatase-deficient males in building the case for an important role for estrogen in the male skeleton, there are clear limitations to cross-sectional observational data. Thus, since BMD in adult men is a function both of peak bone mass and bone loss with aging, these studies cannot dissociate the effects of estrogen on the acquisition of peak bone mass in early adulthood from its effects on continued bone loss later in life.

To address this limitation in the cross-sectional data, Khosla *et al.* (2000) recently assessed longitudinal rates of change in BMD in young (age less than 40 years) and elderly (greater than 60 years) men and related these to serum sex steroid levels. They found that the rate of increase in mid-radius and ulna BMD in the young men as well as the rate of decrease in BMD at these sites in the elderly men were more strongly associated with serum bioavailable estradiol levels than with serum testosterone levels. Together with the cross-sectional data, these longitudinal data thus provide additional support for a role for estrogen both in the acquisition of peak bone mass in young men and in bone loss in elderly men. However, these associations were found only at the forearm sites and not at the spine or hip, perhaps due to the greater precision of the forearm BMD measurements. Moreover, even the longitudinal findings are limited by the fact that correlation does not prove causality, and direct interventional studies are necessary to dissect out the relative contributions of estrogen versus testosterone toward bone metabolism in adult men.

Studies in Humans — Interventional Data

In order to definitively dissect out estrogen versus testosterone effects on the adult male skeleton, Falahati-Nini *et al.* (2000) studied 59 elderly men (mean age 68 years) in whom they eliminated endogenous testosterone and estrogen production using a GnRH agonist (leuprolide acetate) and an aromatase inhibitor (letrozole). Physiologic testosterone and estrogen levels were maintained by concurrently placing the men on testosterone and estradiol patches. Following baseline studies in which markers of bone resorption (urinary deoxypyridinoline, Dpd, and N-telopeptide of type I collagen, NTx) and formation (serum osteocalcin and amino-terminal propeptide of type I procollagen, PINP) were measured, the men were randomized into one of four groups: group A had

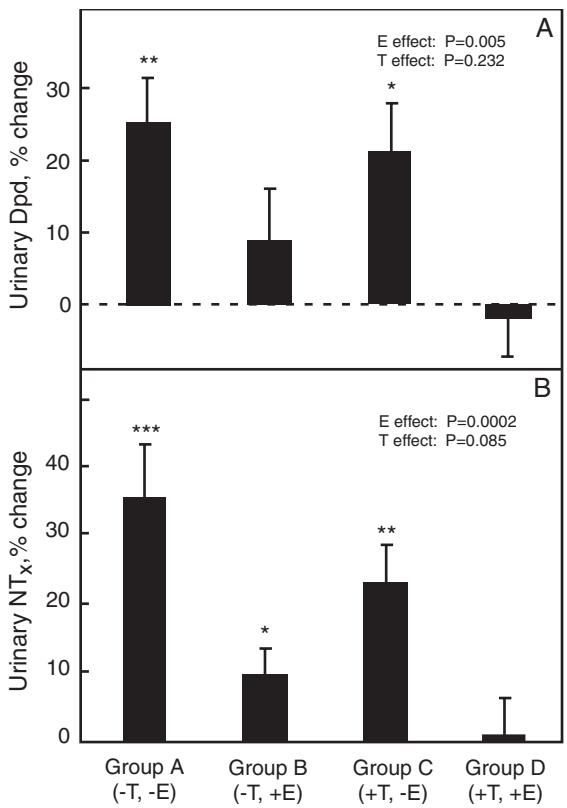

Figure 9 Changes in urinary Dpd (A) and NTx (B) excretion between the baseline and final visits in the four groups as described in the text. $^{***}P < 0.001$, $^{**}P < 0.005$, and $^{*}P < 0.05$ for change from baseline. The overall estrogen (E) and testosterone (T) effect on the bone markers was analyzed using a two-factor ANOVA model. Adapted from Falahati-Nini *et al.* (2000), with permission.

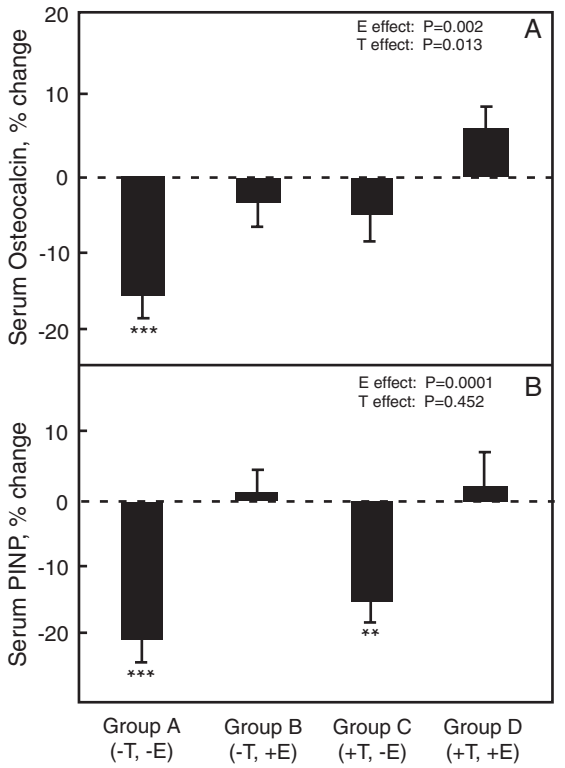

Figure 10 Changes in serum osteocalcin (A) and PINP (B) levels between the baseline and final visits in the four groups as described in the text. $^{***}P < 0.001$ and $^{**}P < 0.005$ for change from baseline. The overall estrogen (E) and testosterone (T) effect on the bone markers was analyzed using a two-factor ANOVA model. Adapted from Falahati-Nini *et al.* (2000), with permission.

both patches withdrawn, group B continued the estradiol patch but had the testosterone patch withdrawn, group C continued the testosterone patch but had the estradiol patch withdrawn, and group D continued both patches. After 3 weeks, the baseline studies were repeated. As shown in Fig. 3, estrogen had the dominant effect in preventing the increase in both urinary Dpd (Fig. 9A) and NTx (Fig. 9B) excretion that was observed in group A. These studies thus definitively established that estrogen was the major sex steroid regulating bone resorption in normal elderly men, although the data could not completely exclude a small effect of testosterone on bone resorption.

As shown in Fig. 10A, serum osteocalcin levels decreased following acute sex steroid withdrawal in these men, and either estrogen or testosterone was able to prevent the decrease in serum osteocalcin levels. By contrast, estrogen, but not testosterone, was effective in preventing the decrease in serum PINP levels (Fig. 10B). Since osteocalcin is produced primarily by mature osteoblastic cells and osteocytes (Lian *et al.,* 1999), these findings are consistent with an important role for both estrogen and testosterone in maintaining the functional integrity of these cells, perhaps by preventing apoptosis (Manolagas, 2000). Type I collagen, by contrast, is produced by cells of the entire osteoblastic lineage (Orwoll, 1995b), and these data would suggest that it is primarily estrogen that regulates this process.

Consistent with these findings, several other interventional studies have found significant effects of estrogen on the adult male skeleton. Thus, in a preliminary study, Taxel and Raisz *et al.* (1997) treated 9 elderly men with either 0.5 mg or 2.0 mg daily of micronized 17β-estradiol and found significant reductions in bone resorption markers. In addition, Anderson *et al.* (1997) treated 21 eugonadal men with osteoporosis with intramuscular testosterone and found a significant increase in lumbar spine BMD, which was correlated with changes in serum estradiol, but not testosterone levels.

Collectively, then, the observational and direct interventional data now provide conclusive evidence for an important and indeed, dominant role for estrogen in the male skeleton. Moreover, given the data noted earlier on age-related decreases in serum bioavailable estradiol levels over life in men, a plausible hypothesis is that this decline is primarily responsible for age-related bone loss in men. Clearly, further studies are needed to address this issue.

Acknowledgments

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CHAPTER 86

Mechanisms of Exercise Effects on Bone

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"The law of bone remodelling is the law according to which alterations of the internal architecture . . . as well as secondary alterations of the external form of the bones . . . occur as a consequence of primary changes in the shape . . . or in the stressing of the bones".

Julius Wolff, *The Law of Bone Remodelling (1892)*

Introduction

The skeleton is a multifunctional organ. It enables locomotion, neutralizes metabolic acid, surrounds and protects brain and viscera, and most importantly, permits organisms to withstand gravitational and other forms of mechanical stress. To accomplish the latter in a manner that optimizes bone strength while at the same time not unduly increasing its weight, bones accommodate the loads that are imposed on them by undergoing alterations in mass, in external geometry, and in internal microarchitecture. The first enunciation of this adaptive principle is generally credited to the German scientist Julius Wolff, whose paraphrased statement appears at the top of this page (Wolff, 1892). In recent years, considerable energy has been directed toward elucidating the biological and molecular mechanisms by which perturbations in mechanical loading are recognized and transformed into changes in skeletal architecture and mass. Skeletal adaptation has been reviewed by many authors (Currey, 1984; Frost, 1987; Fyhrie and Carter, 1986; Lanyon, 1984; Rubin, 1984; Turner, 1991), and a variety of models to explain it have been proposed. Frost (1987) offered the term *mechanostat* to indicate the mechanism(s) that sense and respond to mechanical signals in bone. Although this term has helped to focus the attention of clinicians and exercise physiologists on mechanical loading, it must be pointed out that the mechanostat model was preceded by other more rigorously quantitative and formal models of functional adaptation, such as those of Firoozbakhsh and Cowin (1981) and Fyhrie and Carter (1986).

A general, albeit imprecise, recognition of skeletal adaptation, combined with repeated observations of relatively high bone mass in athletes, has engendered the view that physical exercise is beneficial to bone, a view that has achieved axiomatic stature. However, as formal tests of this concept, controlled exercise trials to date have produced relatively disappointing improvements in bone mass. To understand why this is so, it is necessary to achieve better understanding of the fundamental mechanisms by which bone responds to mechanical stimulation. In this chapter, I discuss current views regarding the skeletal response to mechanical loading, summarize recent experience with exercise as a means of promoting bone mass, and suggest approaches for future work. Comprehensive analysis of quantitative adaptive models lies beyond the scope of this chapter, and discussion of them will be given in general and qualitative terms.

Relationship of Bone Mass to Habitual Loading

If, as stated in Wolff's law, bone accommodates the loads imposed on it, steady-state bone mass should reflect habitual loading history, a principle that is applicable when comparing bone mass among individuals, different bones within an individual, and even different regions within a single bone. A substantial body of published work has addressed this prediction. These studies are of two general types: comparisons of bone mass of athletes to that of sedentary controls and descriptions of associations between level of physical activity or fitness and bone mass within a general population. The first type of study generally considers only very active or sedentary subjects, hence extreme differences in activity are represented. In the latter case, a broader range of physical activity levels is examined.

Exercisers vs Sedentary Controls

Considerable evidence indicates that elite athletes and chronic exercisers have higher bone mineral density (BMD) than age-matched, nonexercising subjects, a finding, not surprisingly, that applies primarily to sites that undergo loading during the exercise. Compared to sedentary individuals, chronic long-distance runners have increased total body calcium (Aloia *et al.,* 1978), calcaneal BMD (Daléan and Olsson, 1974), and vertebral trabecular bone density (Lane *et al.,* 1986; Marcus *et al.,* 1985). Even competitive runners in the sixth decade and beyond have been reported to show ~40% higher vertebral trabecular BMD than nonexercising matched controls (Lane *et al.,* 1986). By contrast, more casual recreational running may have only a limited effect on bone mineral, especially in older women (Kirk *et al.,* 1989; Nelson *et al.,* 1988).

Activities associated with high load intensity at low number of cycles are associated with substantial increases in bone mass. World-class and recreational weight lifters have 10–35% greater lumbar spine BMD (Block *et al.,* 1986, 1989; Colletti *et al.,* 1989; Granhed *et al.,* 1987; Heinrich *et al.,* 1990) than sedentary age-matched controls. It is not certain whether weight lifting is also associated with increased hip BMD, as indicated by some (Colletti *et al.,* 1989; Heinrich *et al.,* 1990) but not all (Block *et al.,* 1989) reports.

Athletes whose activity involves loads at relatively high impact may gain particular skeletal benefit. The BMD values of collegiate gymnasts significantly exceeded those of equivalently trained runners or of age-based normative predictions, even though the former showed a high prevalence of oligo- and amenorrhea, conditions known to result in bone loss (Robinson *et al.,* 1995). The high impact associated with gymnastics training may have provided an adequate stimulus to overcome hormonal disruption. Other cross-sectional studies also support a skeletally beneficial effect of high-impact activities (Pettersson *et al.,* 2000).

Bassey and colleagues (1998) directly assessed the role of high-impact loading in a prospective study of a jumping program in healthy women. The exercise consisted of 50 8.5-cm vertical jumps each day, with loads equivalent to 3–4 body weights per jump as estimated by measurement of ground reaction forces. Five months into the trial, premenopausal women showed an increase in femoral neck and trochanteric BMD of 2–3%. By contrast, postmenopausal women, irrespective of HRT status, showed no change in BMD, even among those who completed a full year of training. Thus, jumping exercise improved BMD in young, but not older women and the failure of the latter to respond to impact loading seemed not to be determined by estrogen deficiency.

On the other hand, it appears that swimming, a trivial-impact activity, does not increase BMD (Heinrich *et al.,* 1990; Jacobson *et al.,* 1984; Taaffe *et al.,* 1995). In one study of elite university athletes (Taaffe *et al.,* 1995), swimmers actually had a lower bone mass than gymnasts or nonathletic controls, despite increased muscle bulk and regular weight training. Young athletes who spend >20 hr each week in a buoyant environment for many years may simply not experience sufficient gravitational stress to promote fully the expected degree of bone acquisition. Whether swimming activity provides effective muscular loading for skeletal maintenance in adults remains unclear, since Orwoll *et al.* (1989) showed that middle-aged long-term male, but not female, swimmers have higher vertebral trabecular bone density than sedentary, age-matched subjects.

A special case of cross-sectional study is represented by comparing dominant to non-dominant limbs in athletes whose sport involves unilateral loading. Jones *et al.* (1977) reported ~30% greater thickness of the humeral cortex in the playing arm than in the nonplaying arm of tennis players, a finding that has been repeatedly confirmed (Daleán *et al.,* 1985; Heinonen *et al.,* 1995; Huddleston *et al.,* 1980; Pirnay *et al.,* 1987).

The durability of training-related increases in BMD remains to be established. Taaffe *et al.* (1997) observed female collegiate gymnasts to show substantial BMD plasticity even during the course of a single year. When training diminished during summer months bone was lost, but with reinitiation of training BMD was regained. In a study of retired elite gymnasts (~25 years of age), the BMD at multiple sites was 0.5–1.5 SD higher than the predicted means for controls and no attenuation of this advantage was observed across the years since retirement despite the lower frequency and intensity of exercise (Bass *et al.,* 1998). Similar retention of BMD advantage into retirement and old age has also been reported for different types of athletes, although results have not been uniformly positive (Karlsson *et al.,* 1999).

This discussion has focused on the relationship of exercise to bone mineral density, but recent studies have brought attention to the possibility that early childhood exercise might lead to permanent biomechanically advantageous changes in bone geometry. Haapsalo *et al.* (1996)

compared the mineral density and geometry of the humerus in Finnish adult long-term tennis players. As expected, the athletes showed exaggerations of normal dominant–nondominant differences in bone mineral content and density. However, tennis players who had commenced training in their sport as children (age 9–10 ± 2 years) also showed significantly greater humeral width. As overall humeral dimensions would not be expected to change with age, and as the bending strength of a bone is related to its cross-sectional area, this geometrical advantage could provide a permanent degree of protection against fracture in later years.

Habitual Physical Activity and Bone Mass of Nonathletes

Evidence from several laboratories points to a significant skeletal effect of physical activity in children and on acquisition of peak bone mass in the third decade (Kroger *et al.,* 1992; Recker *et al.,* 1992; Ruiz *et al.,* 1995; Slemenda *et al.,* 1991). Slemenda *et al.* (1991) described stepwise increments in BMD at multiple sites in children stratified according to activity tertile, and Ruiz *et al.* (1995) reported that time devoted to recreational exercise became a significant predictor of bone mass when children reached the Tanner stage II phase of pubertal maturation. Bailey *et al.* (1998) conducted a longitudinal study of adolescent boys and girls. Participants were stratified into quartiles depending on a standardized gender-specific physical activity score. The authors estimated that an average of 26% of adult total body bone mineral was accrued during the 2-year period at which bone accrual velocity was highest. At that time, when maturational and size differences between groups were controlled for, they observed a 9 and 17% greater total body BMC for active boys and girls, respectively, over their inactive peers. Recker *et al.* (1992) showed that physical activity independently predicted the terminal phase of bone acquisition in healthy women over the age span of 18 to 28 years. In that regard, a recent longitudinal study of Caucasian teenaged girls showed a significant relationship of physical activity history, but not calcium intake, to proximal femur BMD at age 18 (Lloyd *et al.,* 2000).

The situation is less clear for moderately active adults, however, in whom no consistent relationship between *current* activity level and bone mass has been established. Many laboratories exploring this question have reported positive relationships for some age groups at some skeletal regions, but not at others (Aloia *et al.,* 1988; Buchanan *et al.,* 1988; Fraúndin *et al.,* 1991; Greendale *et al.,* 1995; Mazess and Barden, 1991; McCulloch *et al.,* 1990; Metz *et al.,* 1993; Sentipal *et al.,* 1991; Snow- Harter *et al.,* 1992; Sowers *et al.,* 1991; Stillman *et al.,* 1986; Suominen and Rahkila, 1991; White *et al.,* 1992). Mazess and Barden (1991) carefully studied a large group of healthy young women in whom daily activity was quantified by motion sensors. No difference was observed in BMD or in change in BMD over time at any site according to activity quartile. In young women, Kanders *et al.* (1988) described a positive correlation between lumbar spine BMD and total daily energy expenditure. However, the authors did not normalize energy expenditure for body weight, an established independent predictor of bone mass. In our own work (Bouxsein, Snow-Harter, Marcus, unpublished data), strong relationships between estimates of daily energy expenditure and BMD at the spine and hip were completely negated by normalizing the data for body weight or lean mass.

Several reports document positive relationships between *lifelong* physical activity patterns and bone status (Astroúm *et al.,* 1987; Greendale *et al.,* 1995; Kriska *et al.,* 1988; McCulloch *et al.,* 1990; Tylavsky *et al.,* 1992). Astroúm *et al.* (1987) reported that postmenopausal women with hip fracture had undertaken a lower level of occupational and leisure time physical activity between ages 15–45 years, and Greendale *et al.* (1995) observed highly significant associations between BMD at the hip and early-life exercise in a large group of older men and women.

Thus, cross-sectional studies generally support the notion that elite athletes and chronic exercisers have increased bone density relative to age-matched, sedentary individuals. The magnitude of this difference likely depends on the type and intensity of exercise; the age, sex, and hormonal status of the individual; and the number of years spent in training. Bone sites that undergo mechanical loading during exercise show the greatest advantage in bone density. However, data concerning the skeletal effects of *moderate* physical activity remain uncertain, particularly with regard to the role of *current* activity levels.

Cross-sectional comparisons of athletes and control subjects must be interpreted with caution. Since no measurements of bone mass are made prior to initiation of the exercise program, a *causal* relationship between exercise and bone cannot be established. It may be that individuals with higher bone density are more apt to succeed in athletic endeavors; therefore, they become the athletes and chronic exercisers. Conversely, elite swimmers may have excelled in buoyant activity because of a lighter skeleton. In many studies, important characteristics of the matched controls have been neglected. Factors such as menstrual status, nutrient intake, and use of tobacco or alcohol may confound the results. Finally, skeletal status is most frequently expressed as the areal bone mineral density (BMD, g/cm^2), a term that overestimates BMD in persons with large bones and underestimates it in smaller people (Carter *et al.,* 1992). Thus, if exercisers and controls are not well-matched for bone size or factors affecting it (gender, race, age, height, and weight), conclusions based on BMD measurements may be spurious.

Estimating Habitual Skeletal Loads

Studies relating activity level to bone mass pose interpretive difficulties. The instruments used to assess physical activity vary widely and few data establish their validity or reliability. Many instruments currently in use, designed

originally to assess aerobic work or energy expenditure, may simply fail to reflect the loads actually experienced by the skeleton. Even devices that quantify the number of steps taken in a 24-hr period do not distinguish the intensity of impact, and therefore do not fully describe skeletal loading.

Estimating habitual skeletal loads from activity records is not a trivial undertaking. Early attempts to provide such estimates assumed typical conditions to which a bone is regularly exposed, for example, the single limb stance phase during walking. These studies proved useful in understanding relationships between form and function in trabecular bone. However, a more complete understanding required consideration of the fact that bone is loaded under many different conditions and with great variation in the number of cycles during daily activity. Carter *et al.* (1987) introduced a general method of defining loading history as a series of discrete loading events. As a first approximation, they assumed that each loading condition can be best expressed by its load magnitude [s] and number of cycles [n] and that the daily loading experience can be decomposed into histograms of load histories that consist of i discreet loading conditions ($i = 1,2,3 \ldots$), each associated with n_i applications of load. For example, $i = 1$ may represent the activity of single-limb stance during walking with $n_1 = 5000$ cycles. Getting out of a chair 37 times may be expressed by $i = 2$, $n_2 = 37$ cycles. Thus, the steady-state loading history may be expressed as a constant daily stimulus [STIM], representing a summation of all loading events over a 24-hr period, each defined by its load intensity and by its number of cycles (Eqs. [1] and [2]):

$$[\text{STIM}] \propto (s_1n_1 + s_2n_2 + s_3n_3 + \ldots s_nn_n)$$

or

$$[\text{STIM}] \propto \Sigma(s_in_i).$$

Assuming that an individual is at equilibrium, that is, the skeleton is neither gaining nor losing bone, the loading history for each bone, or indeed for each region of a bone, will indicate the stress stimulus necessary for bone maintenance, and quantitative differences in bone density between bones and regions of a bone would be predicted from differences in stress stimulus.

Skeletal Adaptation

Various models have been proposed to explain the responsiveness of bone to loading. Most of these are predicated on the view that adaptive responses lead to optimization of some objective function. Such a function could be the ratio of peak stresses to a bone's maximum tolerable stress (ultimate breaking stress) or, as proposed by Fyhrie and Carter (1986), strain energy density, defined as the concentration of mechanical energy stored in a material. Optimization of strain (the deformation induced by a load or stress) seems particularly attractive (see also Chapter 3), in view of the remarkable uniformity characterizing the magnitude of bone strains experienced by diverse animals undergoing their usual activities. Whether it is measured in galloping horses, flying geese, swimming fish, or chewing macaques, peak strains consistently approximate 2000–3500 microstrain (μE), where 1 E represents a 1% deformation (Rubin and Lanyon, 1984) (Table I). Such *dynamic strain similarity* suggests that skeletal morphology and loading patterns together elicit a specific and beneficial habitual level of strain. By a strain-dependent model, bone would regulate its trabecular orientation and mass as a means for preventing habitual strains at any one site from exceeding an optimal range, above which fracture is more likely.

Table I Peak Functional Strains (PE_q in Microstrain) Measured from Bone-Bonded Strain Gauges in Animals Undergoing Customary Activity

Bone	Activity	PE_q
Horse		
Radius	Trotting	−2800
Tibia	Galloping	−3200
Metacarpal	Accelerating	−3000
Dog		
Radius	Trotting	−2600
Tibia	Galloping	−2100
Goose		
Humerus	Flying	−2800
Cockerel		
Ulna	Flapping	−2100
Sheep		
Femur	Trotting	−2200
Humerus	Trotting	−2200
Radius	Galloping	−2300
Tibia	Trotting	−2100
Pig		
Radius	Trotting	−2400
Fish		
Hypural	Swimming	−3200
Macaca		
Mandible	Biting	−2200
Turkey		
Tibia	Running	−2350

Note. While the primary role of all these bones is load-bearing rather than protection or display, it appears that, regardless of location or origin of loading, maximum peak strains induced during functional activity are similar. Adapted from Rubin and Lanyon, (1984).

Frost (1988) called attention to the very large difference between the loads in cortical bone that induce fracture (~25,000 μE) and those that are typically encountered, and proposed the existence of a minimum effective strain (MES), above which the system would be overloaded and adaptive mechanisms would be called into play. He suggested a range for MES, based on published strain measurements, of ~1500–3000 μE. When loads are within that range, no adaptive response is evoked, even if applied repeatedly and in a sustained manner. By contrast, using an elegant model of skeletal immobilization, Rubin and Lanyon, (1985) defined a strong inverse relationship between strain magnitude and number of cycles needed to induce adaptive remodeling, and showed that prolonged stimulation clearly initiates adaptive

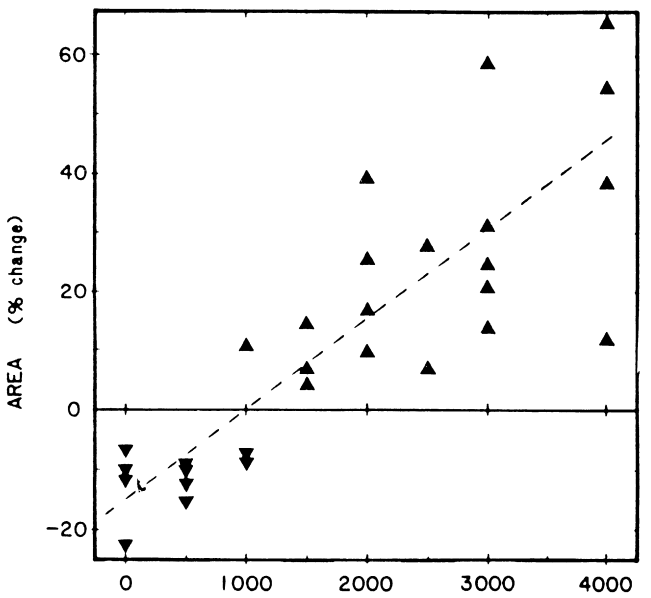

Figure 1 Changes in cross-sectional area of experimental turkey ulnae, loaded for 100 load reversals/day, to peak strains up to 4000 μE. These areal changes reflect comparisons of the left (experimental) to right (intact control) ulnae following an 8-week experimental period. Reproduced with permission from Rubin and Lanyon (1985).

remodeling, even at very low strain magnitudes. By this model, turkey ulnae are surgically isolated from their metaphyseal ends while retaining normal neurovascular connections. The immobilized segments are then placed into a loading device that delivers bending loads under highly defined conditions. Following 8 weeks of immobilization, ulnar cross-sectional area (CSA) decreased by ~20%. Application of 100 load cycles each day led to maintenance of CSA when peak magnitudes were 1000–2500 μE, and actually increased CSA by ~40% when peak strains of 4000 μE were employed (Fig. 1).

Characteristics of Effective Mechanical Loading

Mechanical loads may be characterized by several independent parameters, including load magnitude, number of cycles, and the rate at which strain is induced. To be an effective initiator of remodeling, mechanical stimulation must be dynamic. Using the immobilized turkey ulna model described above, Lanyon and Rubin (1984) showed that simple application of a static load produced no adaptive remodeling and did not protect the bone from atrophy, whereas application of 100 cycles per day of the same load induced bone deposition and increased ulnar cross-sectional area.

Load Intensity versus Cycle Number

Whalen and Carter (1988) introduced a model for comparing the relative effects of load intensity and cycle number on bone mass. Progressing from Eq. [2] (above), they derived an expression to approximate bone apparent density (ρ) from the load history (Eq. [3]):

$$\rho \propto [\Sigma n_i s_{mi}]^{1/2m}$$

By this formulation, the superscript [m] represents a weighting factor that reflects the relative importance of load magnitude to number of cycles. Assume that a 70-kg man walks 1 hr each day. If $m = 1$, magnitude and number of loads have an equal effect on stress stimulus, so walking 2 hr each day would be equivalent to walking 1 hr with a 70-kg backpack. If $m < 1$, cycle number would be more effective than increasing the load, but if $m > 1$, load magnitude would provide a more effective stimulus. Based on a careful analysis of two published human studies in which running exercise was imposed, they predicted values for m that ranged from 2 to 6. Thus, Whalen and Carter concluded that load intensity would be far more important than cycle number.

This conclusion is certainly in line with indications from clinical literature, in which highest bone density values have been observed in athletes whose activities include lifting of heavy loads and application of high-impact forces (Block *et al.,* 1989; Heinonen *et al.,* 1995; Robinson *et al.,* 1995; Snow-Harter *et al.,* 1992). It is also consonant with animal data indicating that the number of loading cycles necessary to maintain bone mass is relatively small (Lanyon and Rubin, 1984), that although modest running activity is associated with higher bone mass in rats, running 3 or 18 km/day has the same effect on bone mineral content (Newhall *et al.,* 1991), and that increasing the magnitude of loads with weighted backpacks is a more effective stimulus for increasing bone mass than increasing the duration of treadmill running (van der Wiel *et al.,* 1995).

Rate of Strain

Peak load magnitude does not itself describe the intensity of loading nor does it determine skeletal response. *Rate of strain* is a term used to describe the time over which strain develops following load application, and is roughly comparable to what is meant by "impact." In several experimental models, rate of strain has been shown to be of critical importance to skeletal response, a principle that applies even at large peak strains (O'Connor *et al.,* 1982; Turner *et al.,* 1993). Turner *et al.* (1995) applied loads of 54 N[1] at 2 cycles per second for 18 sec each day to rat tibias and measured the effect of varying the rate of strain on bone formation and mineral apposition. Results showed a marked linear rise in both variables as rate of strain increased.

The Curvilinear Nature of Skeletal Response

Complete immobilization, as seen with high level spinal cord injury, leads rapidly to devastating bone loss, with deficits that approach 30–40% over several months. By contrast, imposition of even substantial training regimens on normally ambulatory people or animals increases bone mass by only a few percent over a similar period. This is illustrated in Fig. 2, where the effect of walking on bone mass is schematized. As an individual goes from immobility to full ambulation, duration of time spent walking becomes a progressively less efficient stimulus for increasing bone

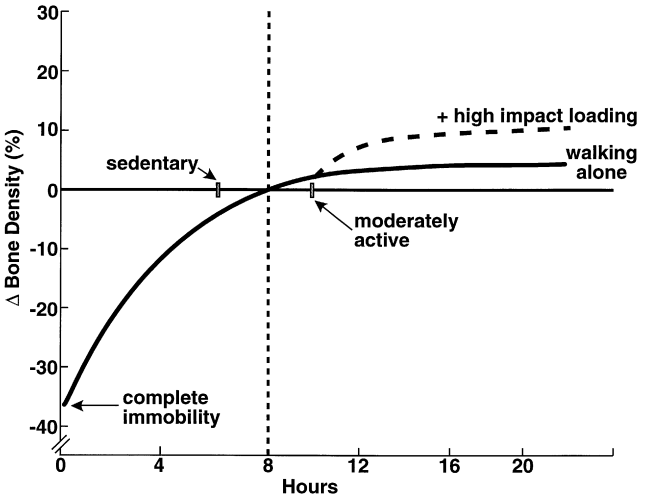

Figure 2 The curvilinear nature of skeletal response. The effect on bone mass of immobility is far greater than that of adding additional walking to an already ambulatory subject. It is more effective to provide a higher intensity stimulus than simply to extend the duration of ordinary loading activity. Ordinary activity, as shown by the region between "sedentary" and "moderately active," defines most individuals, and is characterized by a relatively modest dose–response slope.

mass. A person who habitually walks 6 hr each day might require another 4–6 hr just to add a few more percent BMD. On the other hand, adding a more rigorous stimulus, such as high-impact loading, for even a few cycles would increase the response slope.

The Cellular Basis of Skeletal Adaptation

Since the demonstration in 1975 by Rodan *et al.* (1975) that compressive forces of physiological magnitude lowered the tissue concentration of cyclic 3′5′-adenosine and -guanosine monophosphates (cAMP and cGMP) in incubated avian bone, considerable work has been done to elucidate the biochemical basis for this mechanical transduction. These studies have involved incubation of osteoblast-like cells and bone explants, as well as analysis of bones removed from mechanically loaded animals.

STUDIES IN INCUBATED CELLS

Mechanical loads can be delivered to incubated cells by several techniques, including application of hydrostatic and shear pressures, osmotic swelling, and stretch. Cells can be cultured on specially designed dishes fitted with a flexible bottom. When these are subjected to vacuum or other mechanical stimulation, the entire cell layer is stretched. Stretch can be applied cyclically under highly controlled conditions of magnitude, duration, and frequency. One potential difficulty with this approach is that strain magnitudes are not uniform across the bottom of each well. In one study, for example, strains varied from 0 μE at the edge to 120,000 μE at the center of the well (Harter *et al.,* 1995). In this circumstance, it is necessary to assume the validity of averaging strains over the entire well.

Results of cellular loading experiments have not been consistent. Some perturbations have increased cellular proliferation, whereas others have suppressed proliferative activity while promoting differentiated functions. After examining the available data, Burger and Veldhuijzen (1993) concluded that proliferative activity results from high applied strains, while lower strains promote differentiated activities, such as production of bone matrix proteins. Further consideration of these results led to the suggestion that selective activation of proliferative versus differentiated responses could determine a bone's mechanical characteristics. If an osteoblast is subjected to strains within a favorable range, no requirement for increased mass or change in trabecular orientation would be present, and maintenance of differentiated function would occur. For example, Harter *et al.* (1995) incubated human osteoblastic cells under sustained low-frequency strain, and showed a coordinated upregulation in production of type I collagen, osteopontin, and osteocalcin. On the other hand, if strain magnitude or orientation change substantially, a proliferative response leads to a change in trabecular orientation and density until the magnitude and distribution of strain per osteon returns to the former state.

STUDIES WITH INTACT BONE

A brief period of load application initiates a complex series of biochemical events in bone, resulting several days later in a substantial rise in bone-forming activity (Pead *et al.,* 1988). In cortical bone, mechanical loading stimulates increased bone formation on the periosteal surface, apparently without stimulating an initial resorption phase. Several reports document changes in cellular total mRNA content from 6 to 24 hr following mechanical loading of bones in organ culture (Dallas *et al.,* 1993; Haj *et al.,* 1990). Raab-Cullen *et al.* (1994) examined RNA blots for specific sequences shortly after applying external loads to rat tibias. A rapid but transient increase in mRNA for the oncogene c-*fos* was evident by 2 hr, with increases in message for TGF-β and IGF-I by 4 hr. At the same time, there was a decline in mRNAs for specific differentiated bone matrix proteins, alkaline phosphatase, osteopontin, and osteocalcin. The authors concluded that mechanical loading had induced periosteal cell proliferation.

Turner *et al.* (1995) outlined a cascade of four steps through which mechanical loads couple to skeletal response: mechanical sensing, biochemical coupling, cellular communication, and effector response. There is broad agreement that perception of strain must be a cellular phenomenon and that the cells in bone most likely involved are osteocytes, osteoblasts, and bone surface-lining cells. However, the identity of the mechanosensor itself has not been elucidated. The cells responsible for transducing mechanical signals into chemical mediators may not themselves experience the applied strains, but may receive the signal through strain-induced alterations in canalicular fluid flow (Reich

et al., 1990; Weinbaum *et al.,* 1994) or by generation of electrical potentials (Bassett and Becker, 1962).

The sequence of molecular events in biochemical coupling is not defined, but appears to involve the generation of classical messenger molecules, such as cyclic nucleotides or prostaglandins, by load sensing cells. Lanyon (1992) summarized evidence that stimulation of glucose 6-phosphate dehydrogenase (G6PD), a marker of overall cellular metabolism, occurs in osteocytes and lining cells within a few minutes of load application. This increase appears to be mediated by prostaglandins, since PGE_2 is rapidly and transiently released by mechanically loaded bone (Rawlinson *et al.,* 1991), and the activation of G6PD is blocked by coadministration of indomethacin. In addition, Binderman *et al.* (1984) showed that direct application of physical strain stimulated rapid synthesis of PGE_2 in cultured osteoblast-like cells and also found that electric stimulation of these cells may circumvent the requirement for PGE_2 and directly trigger the adenylate cyclase system.

Cellular communication is required for signal-sensing cells to direct osteoprogenitor and other effector cells to proliferate or to alter their production of bone matrix constituents. Such communication may occur through release of growth factors or other mediators into the canalicular fluid system, as well as by direct cellular contacts. Vander Molen *et al.* (2000) addressed the latter issue in studies of a gap-junction (connexin 43) deficient osteoblastic cell line. Cells were exposed to a 30-Hz, 1.8-mT magnetic flux sufficient to induce a 2 mV/m electric field. Treatment of control cells inhibited cell growth and induced alkaline phosphatase activity. The connexin-deficient cells showed significant treatment-dependent growth inhibition, but alkaline phosphatase induction was impaired. The authors concluded that induction of alkaline phosphatase by electrical fields requires the presence of functional gap junctions.

Effector responses involve activation of a broad range of molecules, including genes for cellular proliferation, genes for such differentiated functions as type I collagen, osteocalcin, or osteopontin, or upregulation of mechano-sensitive ion channels that have recently been characterized in osteoblast-like cells (Davidson *et al.,* 1990; Duncan and Hruska, 1994).

Finally, it should be mentioned that although the focus of most work has been on loading-induced bone *formation,* Klein-Nulend *et al.* (1990) presented evidence that application of mechanical loads to incubated murine long bones also resulted in decreased bone *resorbing* activity. In an elegant *in vivo* demonstration of this phenomenon, Hillam and Skerry (1995) applied 2400 loading cycles/day for 4 days to young rat ulnae. The 7-N peak force was estimated to provide cyclic strains of about 400 μE. In control bones, the medial periosteal surfaces showed resorptive activity, with no fluorochrome incorporation (a measure of bone formation) and with high expression of the resorption marker, tartrate-resistant acid phosphatase (TRAP). In the loaded bones, substantial fluorochrome labeling was observed, representing an 18-fold increase in formation surfaces, and was accompanied by virtual disappearance of TRAP. Rubin *et al.* (1999) assessed the production of osteoclasts in marrow cultures plated on flexible membranes. Application of 5% strain for 10 cycles/min, for 24 hr per day resulted in 40% suppression of osteoclast formation compared to control cultures. By contrast, conditioned media from strained cultures did not inhibit osteoclast formation in unstrained cultures; suggesting that the inhibitory effect of strain requires direct application of strain to the cell rather than the intermediacy of humoral factors.

The Role of Electric Fields in Mechanical Signal Transduction

Induction of both piezoelectric and kinetic currents in bone by mechanical strain gave rise more than 30 years ago to the concept that skeletal adaptation is mediated by cellular responses to electric fields (Bassett and Becker, 1962). Induction of electrical current in bone was shown to prevent bone loss due to denervation (Brighton *et al.,* 1985) and to plaster cast immobilization (Martin and Gutman, 1978), and Rubin *et al.* (1989) demonstrated that pulsed electromagnetic fields not only prevent the bone loss of functional disuse, but also induce new bone formation. Moreover, this work suggested the existence of a distinct frequency range, ~10–30 Hz, at which skeletal response is optimal (McLeod and Rubin, 1992). Indeed, even at peak strains well below those typical of habitual physiological loading, application of 30-Hz signals were shown to be osteogenic, supporting the concept that inducing even very low strains, i.e., <500 μE, may generate an effective osteogenic stimulus, provided that they are induced at optimal frequencies, 15–40 Hz. Surprisingly, a more detailed analysis of these studies indicates that the induced electric field intensities at sites where bone tissue is responding to the stimulus must be very low, indeed, perhaps 1–2 μV/cm. Thus, bone appears to respond with great selectivity and sensitivity to the frequency and amplitude of electrical stimulation. McLeod and Rubin have proposed that strains arising from the ground reaction forces and muscle fiber resonance associated with normal functional activity could themselves provide the signal characteristics to which bone cells respond optimally (McLeod and Rubin, 1990).

Host Factors in Skeletal Adaptation Age

Using the isolated turkey ulna model, Rubin *et al.* (1992) showed impressive deficits with age in the skeletal response to mechanical loading, a finding that was confirmed in a rat model by Turner *et al.* (1995). In view of the complex nature of signal transduction, these deficits could reflect impairments at multiple loci. Adequacy of skeletal response reflects cell numbers and vigor as well as hormonal and cytokine milieu. Since cell populations, circulating growth factors, and production of bone matrix proteins all decline with age (Benedict *et al.,* 1994; Termine, 1990), age-related deficits in skeletal response should ultimately prove to be multifactorial.

The ability of strains at very low magnitude to contribute to skeletal maintenance so long as their cycle number is sufficiently great was alluded to above (McLeod and Rubin, 1990). Huang and colleagues (1999) considered the possibility that an age-related decrease in skeletal exposure to strains induced by normal muscle tone might contribute to bone loss. Histologic studies clearly document age-related decreases in number and cross-sectional areas of both type I and type II muscle fibers. These changes are accompanied by declines in muscle strength and functional capacity. To test whether changes in postural muscle dynamics in the frequency range of 0.1–50 Hz were also associated with age, the authors recorded vibro-myographic output from the soleus muscles of healthy adults, age 20–82 years. They found that muscle vibration frequencies below 15 Hz did not change with age, but frequency components from 30 to 50 Hz decreased substantially with advancing age in both men and women. As 30- to 50-Hz stimuli appear to be those that provide the greatest degree of skeletal response, the authors hypothesize that age-related loss of bone may to some degree reflect the loss of stimulation by postural muscles.

The above considerations notwithstanding, it remains unclear in humans whether or to what degree the BMD response to exercise is constrained by age. Exercise trials in women ranging from the third to the fifth decades have shown increases in bone mass of only a few percent (Friedlander *et al.,* 1995; Gleeson *et al.,* 1990; Lohman *et al.,* 1995; Snow-Harter *et al.,* 1992) or even a loss of BMD (Rockwell *et al.,* 1990), whereas some (Dalsky *et al.,* 1988; Notelovitz *et al.,* 1991) but not all (Prince *et al.,* 1995; Pruitt *et al.,* 1992) studies involving women in the sixth through eighth decades have been associated with increases in spine BMD of 5–8%. One must remember that the overall physical activity of older men and women is lower than that of younger people, so the exercise protocols may have imposed substantially greater incremental loading in the studies of older groups.

The adaptive response of growing bones includes expansion of bone size as well as increases in mass. Once linear growth ceases, plasticity of bone geometry is severely limited. Some ability does persist, even at later decades, for long bones to counteract the weakening effect of endosteal resorption by undergoing compensatory expansion at the periosteal surface, thereby increasing the cross-sectional moments of inertia (Bouxsein *et al.,* 1994; Martin and Atkinson, 1977). The basis for this increase in bone cross-sectional geometry remains obscure, although the hypothesis seems plausible that habitual loads per remaining osteon are increased when bone is lost from the endosteal surface, and that this increase in osteonal loading leads to compensatory periosteal bone apposition. Nonetheless, it seems likely that the greatest opportunity to increase bone dimensions as well as mass through increased physical activity would be during times of growth. It is therefore of interest that not only bone mass, but also the diameters of tennis players' dominant arms are increased if training commenced at an early age (Vuori *et al.,* 1994).

Reproductive Hormonal Milieu

The extent to which endogenous hormone adequacy limits the skeletal response to mechanical loading is not clear, although models of skeletal adaptation suggest that responsiveness may be modulated by hormonal milieu. By Frost's mechanostat model (Frost, 1987), interactions among various elements, including hormonal adequacy, alter a bone's perception of loading, thereby affecting its sensitivity and magnitude of adaptive response. Experimental approaches to this question have largely focused on the effects of estrogen. Estrogen-deficient animals retain the ability to respond to exercise (Barengolts *et al.,* 1994; Hagino *et al.,* 1993; Lin *et al.,* 1994; Yeh *et al.,* 1994). In a well-defined model of external tibial loading, Hagino *et al.,* (1993) subjected rats to a schedule of 36 cycles/day of physiologic strain (~1300 μE) for 3 weeks. Periosteal bone formation increased equally, whether rats had been oophorectomized or sham-operated. Although these results appear to conflict with the notion that loading is perceived differently when hormonal milieu is disrupted, the authors cautioned that since the rat tibial cortex under ordinary circumstances neither responds to changes in reproductive hormone status nor undergoes significant Haversian remodeling, the tibia may not provide an ideal model for testing this issue.

Human studies confirm the ability of estrogen-depleted women to respond to exercise. Although young endurance athletes who lose spontaneous menstrual function show decreased BMD at multiple sites (Drinkwater *et al.,* 1984; Marcus *et al.,* 1985; Myburgh *et al.,* 1993), trabecular bone mass is still higher than that of amenorrheic sedentary women (Marcus *et al.,* 1985). One question of great interest is whether exercise alone will suffice to maintain bone for women who are recently menopausal but who are not taking estrogen. In a controlled trial of resistance exercise, Pruitt *et al.* (1992) found that such women maintained bone mass at the lumbar spine, but did not achieve benefit at the forearm or proximal femur. For older postmenopausal women, estrogen replacement appeared to have no impact on the skeletal response observed by Dalsky *et al.* (1988). However, Khort *et al.* (1995) demonstrated additive effects of estrogen replacement and vigorous weight-bearing exercise in older women, and in another study (Notelovitz *et al.,* 1991), resistance training of postmenopausal estrogen-replete women led to substantial and unprecedented rises in vertebral BMD that have not been otherwise observed in women of similar age.

Constraints on Skeletal Response to Exercise in Humans

As suggested at the beginning of this chapter, improvements in bone mass achieved in well-designed exercise trials have been, with few exceptions, relatively meager. The multiple reasons for this can be considered in light of the principles described above. For an imposed load to effect

a change in bone mass, it must materially alter the daily stress stimulus from its baseline equilibrium state. The most effective way of doing that is to start with very sedentary subjects. When active young people initiate a new activity, it is important to establish that the imposed loads are truly incremental, and are not simply countered by a reduction in other types of activity. Although one cannot be certain in the absence of data, a possible explanation for the 4% *loss* of lumbar spine BMD observed in the resistance-trained subjects of one study (Rockwell *et al.,* 1990) may be that the women, most of whom ran recreationally prior to starting the study, cut back sufficiently on their previous activity to decrease their total loading history, even when the training regimen was taken into account.

A second factor concerns rate of strain. For good reason, resistance training studies have relied on machines that provide safe, highly quantifiable loading to isolated muscle groups. However, this strategy may be counterproductive with regard to generating adequate rate of strain, or impact, to the loaded bones. Several findings are of interest in this regard. The BMD of collegiate gymnasts was significantly higher than that of equivalently trained runners or than age-based normative predictions, even though these women showed a high prevalence of oligo- and amenorrhea, conditions known to result in bone loss (Robinson *et al.,* 1995). It may be that the high impact associated with gymnastics training provided an adequate stimulus to overcome hormonal disruption. In addition, Bassey and Ramsdale (1994) demonstrated a 3.4% increase in trochanteric hip BMD in young women following several months of jumping activity that produced high-impact calcaneal loading. Unfortunately, the safety of such a regimen for older people or for people with skeletal fragility is uncertain. Nonetheless, it seems reasonable to design future trials to include some activities with higher impact than have been applied heretofore. Such activities might include jumping rope or stepping briskly from modest elevation.

A third issue concerns duration of training studies. For logistical regions, and because of high attrition, most published exercise trials have been conducted for 1 year or less. The criticism has been raised that, as opposed to muscle strength acquisition, where gains are maximal within the first few months, skeletal adaptation may evolve more slowly, so longer duration studies would be required to see more impressive skeletal gains. Two studies provide little support for this criticism (Friedlander *et al.,* 1995; Lohman *et al.,* 1995). In one report, lumbar spine, femoral neck, and trochanteric BMD increased by only 1.3, 0.5, and 2.6% after 2 years of supervised training (Friedlander *et al.,* 1995). In the other, BMD gains seen by 5 months either remained stable or actually decreased over the next year, despite continued and fairly dramatic increases in muscle strength through 18 months of training (Lohman *et al.,* 1995).

A final issue concerns the fate of BMD gains when exercise training is stopped or decreased. The paper by Dalsky *et al.* (1988), which is frequently cited to show gains in BMD when exercise is undertaken, also shows clearly that this effect degrades within a few months after the training program terminates, by which time BMD has returned to its baseline values.

Conclusions

In this chapter, I have reviewed the conceptual basis for understanding the relationship between mechanical loading and skeletal adaptation. Complete understanding of this relationship in humans must await development and validation of accurate, quantitative estimates of mechanical loading history. Although this has not yet been accomplished, sufficient information is available to permit general conclusions, as well as speculation about the kinds of exercises that are likely to prove most osteogenic. The adage remains true that the greatest damage occurs when the skeleton is put to rest. The curvilinear nature of skeletal response shows that addition of even modest activity to an immobilized subject will increase BMD to a much greater extent than will a substantial increase in training for a highly active person. Therefore, one might best consider physical activity for most people to be a hedge against bone *loss,* rather than a means of achieving major increases in bone mass. However, should one wish to impose an exercise regimen for the purpose of increasing bone mass and strength, it would be most effectively initiated during years of growth, and include activities that apply loads in an unusual manner or direction, with higher than customary impact. Naturally, one's embrace of this strategy is limited by the subject's ability to undertake such activity with safety.

There is a range of activity in healthy, ambulatory populations in which considerable variation in habitual loading leads to little, if any, change in bone mass. This extremely useful feature assures that adaptive remodeling occurs when changes in *habitual* loading occur, rather than responding to day-to-day alterations in activity. It is this "lazy zone," as designated by Carter, that makes it difficult to show unambiguous relationships between activity level and bone mass in healthy, ambulatory, albeit nonathletic populations.

In medieval times, itinerant priests sold indulgences to counteract future sins. Unfortunately, the skeleton does not allow present good works to be applied against future sloth, but adapts to the demands that are currently made upon it. The experience with astronauts, spinal cord injury, and exercise detraining shows that highly active individuals rapidly lose bone when the loading environment changes.

Modern postindustrial society is becoming increasingly sedentary. In the United States, the President's Council on Fitness estimates that only one of five adults engages in a single episode of recreational exercise each week. More alarming, fitness scores of children are declining. The challenge to the scientific community for developing practical and acceptable exercise programs for the great majority of the population remains formidable. It seems highly unlikely that most people will ever be motivated to participate in multiple exercise

programs, one for aerobic fitness, others for muscle strength and flexibility, and yet another for bone health. Thus, a comprehensive approach is required to develop a single program that offers some, if not optimal, benefit for all these needs.

Finally, the fact that mechanical loads are transduced into electrical and chemical signals in bone suggests that specific pharmacologic or bioelectrical interventions might obviate the need for the initial mechanical stimulus. Such interventions might produce changes in bone mass that exceed what can be accomplished through exercise itself, and might also offer effective strategies for frail individuals in whom rigorous exercise constitutes an unacceptable risk.

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PART IV

Methods in Bone Research

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CHAPTER 87

Application of Transgenic Mice to Problems of Skeletal Biology

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Introduction

Transgenic mice provide an excellent setting for studying complex regulatory systems that cannot be modeled or fully appreciated in isolated experimental systems *in vitro* or *in cellulo.* This potential is particularly important in the analysis of the cells that synthesize the extracellular matrix because their response to regulatory signals from the external environment is dependent on their state of cellular differentiation. Because these variables cannot be reliably modeled in isolated systems, the challenge is to develop assay systems within an intact animal that can still ask a focused molecular question. This chapter is designed to illustrate examples where transgenic animals have answered subtle questions about protein structure and gene regulation affecting bone structure or bone cell differentiation that could not have been performed in any other experimental system. In addition, newly developed transgenic approaches will be discussed that have not yet been applied to bone but have great potential in this research arena.

Analysis of Structural Macromolecules in Bone

Type I collagen is the major structural protein of bone, while type II is the primary collagen of cartilage. Mutations affecting the structural integrity of these two molecules result in osteogenesis imperfecta (OI) and chondrodysplasias, respectively. Both of these classes of diseases are inherited as a dominant negative trait, indicating that it is the presence of a mutant gene product that causes the disease. While molecular genetic studies of humans affected with these disorders have illustrated that the spectrum of disease severity can be correlated to specific mutations (Prockop *et al.,* 1993, 1994) a clearer understanding of how these mutations affect protein function can be obtained from transgenic mice.

The concept of the dominant-negative effect of a mutant collagen gene was dramatically demonstrated in transgenic mice carrying an OI producing (glycine substitution) collagen transgene (Stacey *et al.,* 1988). These mice displayed perinatal lethal OI. Molecular analyses showed that lethality was associated with the expression of the transgene to levels as little as 10% of the endogenous normal collagen production. This illustrated that the formation of an extracellular matrix with even a small proportion of mutant molecules has a dramatic destabilizing effect on the entire skeleton. In contrast, another series of transgenic mice were evaluated in which a large internal deletion of the collagen molecule was expressed (Khillan *et al.,* 1991; Pereira *et al.,* 1993). In this case the severity was more variable, predominantly less severe, and correlated with the degree of expression. In the affected yet viable mice, transgene expression was approximately equivalent to the endogenous gene (Pereira *et al.,* 1994). These observations suggest that when the mutant collagen molecule is severely deformed, as in the case of the internal deletion, few of the mutant molecules accumulate in the matrix. Mutations which have a moderate effect on molecular assembly lead to greater accumulation of altered collagen molecules in the extracellular matrix and thus present a more severe phenotype. Comprehension of disease severity based on mutation analysis requires an understanding of how the molecule is assembled, secreted, and incorporated into the extracellular matrix. While these steps may be defined in *in vitro* systems (Bateman and Golub, 1994; Lamande *et al.,* 1995), the functional significance requires a transgenic approach.

Similar observations have been made for type II collagen. Glycine point mutations within this gene produce the phenotype of chondrodysplasia in mice (Garofalo *et al.,* 1991; Maddox *et al.,* 1997) and abnormal craniofacial morphology (Rintala *et al.,* 1993). Transgenic mice with a small deletion in the Col2a1 gene display retarded skeletal development (Savontaus *et al.,* 1996), abnormal craniofacial (Rintala *et al.,* 1997) growth, as well as disruptions in spinal development (Savontaus *et al.,* 1997). However, a large internal deletion has a more variable phenotype which may serve as a model for osteoarthritic changes in older animals (Vandenberg, 1991; Helminen *et al.,* 1993). Mice homozgyous for a knockout of the type II collagen gene die shortly after birth. These mice develop a skeleton with membranous and periosteal bone but no endochondral bone (Li *et al.,* 1995a). Also these null mice do not develop intervertebral discs due to an inability to dismantle the notochord (Aszodi *et al.,* 1998). Furthermore, excessive expression of a normal type II collagen gene can have an equally disorganizing effect on cartilage formation (Garofalo *et al.,* 1993). This important observation underscores a requirement in the design of any gene therapy approach for bone disease. A correcting collagen gene will require a tissue-specific and normally regulated promoter since constitutive high expression or tissue missexpression of a normal extracellular matrix gene can be as deleterious as a low level of expression of a mutant collagen gene.

In addition to illustrating the importance of mutations which disrupt the collagen helix and lead to defective extracellular matrix formation, mutations at other sites within the molecule can demonstrate biological properties of type I collagen (Kivirikko, 1993). The first step in the enzymatic turnover of collagen from the extracellular matrix is cleavage at a specific site within the helix by mammalian collagenase. Collagen molecules containing a mutation at the site of cleavage are more resistant to degradation (Wu *et al.,* 1990b). Embryonic lethality was observed in mice bearing collagen transgenes with this mutation although not as a result of accumulating uncleaved heterotrimers but possibly due to the presence of α1(I) homotrimers. When this mutation was placed within the endogenous gene by homologous recombination, a fibrotic phenotype similar to human scleroderma and increased bone density was observed (Liu *et al.,* 1995a). This model was also used to examine the role of collagenase in the parathyroid hormone (PTH)-stimulated resportion of bone. Interestingly, in mice homozygous for the targeted collagenase resistant allele, the PTH-induced bone resportion responses were blunted. This observation suggests that collagenase degradation of type I collagen is necessary for PTH induction of osteoclastic bone resportion (Zhao *et al.,* 1999). Most recently it has been demonstrated that the altered skeletal remodeling seen in these mutant mice is associated with elevated ostocyte and osteoblast apoptosis, suggesting that matrix degradation may be involved in modulating osteocyte and osteoblast survival (Zhao *et al.,* 2000b).

Chance inactivation of an endogenous gene by insertional mutagenesis has been another important tool for studying the importance of a macromolecule in organ and tissue development. The Mov-13 mouse was discovered as an embryonic lethal in homozygous mice derived from a heterozygous breeding pair containing an experimentally introduced retroviral insertion (Lohler *et al.,* 1984). Further studies revealed that the retroviral insertion had disrupted the Col1a1 locus. Homozygous inactivation of type I collagen resulted in embryonic lethality due to arterial rupture well before bone formation. These mice have had many useful applications in studying collagen regulation and bone biology (Kratochwil *et al.,* 1989). The potential of gene therapy for a collagen mutation was demonstrated by the introduction of a normal collagen transgene which facilitated full embryonic development of the homozygous Mov-13 embryo (Wu *et al.,* 1990a). Initially, the heterozygous Mov-13 mouse was not appreciated as being abnormal. However, sensitive imaging and mechanical testing did demonstrate that mild weakening of the bone was present which spontaneously improved as the bone remodeled in early adulthood. These mice appear to be an excellent murine model of human type I OI (Bonadio *et al.,* 1990).

Cells derived from homozygous Mov-13 mice are valuable tools to study *in vitro* interactions of transfected collagen genes with endogenous macromolecules. For example, the binding of collagen to fibronectin was directly demonstrated by the ability of normal transfected collagen genes to form fibronectin collagen aggregates, in contrast to the failure of these aggregates to form when transfected collagen genes were mutated at sites important to fibronectin binding (Dzamba *et al.,* 1993). From human studies it was apparent that there is specificity of the N-terminal propeptide cleavage site because mutations which inactivated the cleavage region produced altered collagen molecules resulting in Ehlers–Danlos type VII (Weil *et al.,* 1990). However, no human examples of mutations affecting the C-terminal propeptide have been observed. *In vitro* studies in Mov-13-transfected cells suggest that mutations affecting C-terminal cleavage may not have a deleterious effect because secondary sites of cleavage are utilized (Bateman *et al.,* 1990). However, until these studies are performed by mutagenesis of the endogenous gene in a mouse model, the true importance of this region will not be appreciated.

In addition to the major contribution of types I and II collagen in numerous tissues, minor collagens and noncollagenous proteins interact with the major collagens to modify their structural role in tissues of similar composition. This modifying function is best appreciated in transgenic mice or naturally arising mutations in a minor collagen gene. An internal deletion within the type X collagen gene, which normally is expressed in hypertrophic chondrocytes, results in a spondylometaphyseal dysplasia (Jacenko *et al.,* 1993) as opposed to mutations in type II collagen which cause an epiphyseal dysplasia. Further examination of these transgenic mice bearing this dominant interference mutation in type X collagen showed moderate craniofacial skeletal abnormalities (Chung *et al.,* 1997). A somewhat surprising outcome was that a type X null mutation introduced by homologous recombination did not have a detectable phenotype in mice

either heterozygous or homozygous for the null mutation (Rosati *et al.,* 1994). Although a milder phenotype from a heterozygous null mutation would be predicted from studies of type I and type II collagen, lack of any phenotype was unexpected. Recently another group generated type X collagen-deficient mice (Kwan *et al.,* 1997). In these mice abnormalities were observed including a reduction in growth plate thickness in the resting zone and articular cartilage as well as abnormal trabecular bone. Most recently a study reexamined the original knockout mice and has documented a variable skeleto-hematopoietic phenotype that included growth plate compressions primarily within the proliferative zone (Gress and Jacenko, 2000). Type IX collagen is known to bind to type II collagen and promote its interaction with noncollagenous proteins of cartilage. Expression of a helix disrupting mutation within the type IX collagen cDNA in transgenic mice results in a mild form of chondrodysplasia that predisposes to degenerative osteoarthritis (Nakata *et al.,* 1993). A null mutation of the same gene results in an even milder degenerative joint disease (Fassler *et al.,* 1994). The analogous human disease appears to be a form of multiple epiphyseal dysplasia (Briggs *et al.,* 1995). The importance of type XI collagen to cartilage structure and subsequent bone formation has been demonstrated in the cho/cho mouse which harbors a null mutation in the α1(XI) gene and results in a perinatal lethal phenotype with severe bone deformity and shortening (Li *et al.,* 1995b).

Type V collagen is associated with type I in skin and tendon and may regulate type I fiber formation in the same way that type IX affects type II collagen fiber formation. The expression of an α2(V) chain with an N-terminal globular deletion in transgenic mice appears to result in an Ehlers–Danlos type I like phenotype (Andrikopoulos *et al.,* 1995). Bone formation appears to be normal although severe kyphoscoliosis results from ligamentous laxity. As other minor collagens are discovered, their role in the structure of the extracellular matrix will be revealed by mutagenesis in a manner similar to the examples given above.

The noncollagenous proteins of connective tissue are also likely to have a modifying role on type I and type II collagen function. In some cases, the gene knockout approach has not revealed dramatic phenotypes even though the genes are highly expressed in bone. For example, tensacin is strongly expressed in bone (Mackie and Tucker, 1992), and yet the tenacin knockout mouse does not have a connective tissue phenotype (Saga *et al.,* 1992; Forsberg *et al.,* 1996). Similarly, matrilin 1, or cartilage matrix protein, does not have a phenotypic affect when deleted. Homozgygous matrilin 1 knockout mice develop normally with no apparent anatomical or histological abnormalities. This observation suggests a redundancy of function of other matrilin family members (Aszodi *et al.,* 1999). Osteocalcin knockouts have also been generated and do not have a dramatic phenotype, although recent studies suggest there may be a problem in bone remodeling (Ducy *et al.,* 1996) as well as alterations in mineralization in older animals (Boskey *et al.,* 1998). The initial evaluation of osteonectin-deficient mice did not reveal any obvious skeletal changes (Gilmour *et al.,* 1998), however, a more recent study revealed an osteopenia associated with a decrease in bone formation (Delany *et al.,* 2000). Based on the experience of the major and minor collagens that a expressed mutation is more severe than a null mutation, the importance of the noncollagenous proteins may be more dramatically demonstrated by expressing mutations in regions thought to participate in interactions between the noncollagenous proteins and other matrix components. Further, it is clear that often minor but important changes in phenotype are sometimes not detected on first evaluation. It is only when more sophisticated examinations are made or older age groups are studied that differences between wild-type and mutant mice are detected.

Cis Regulation of Structural Macromolecules in Bone and Cartilage

Production of major structural macromolecules constitutes the major synthetic activity of a bone or cartilage cell and can be used to define a particular stage of differentiation. While transient and stable transfection studies can provide insight into the biochemical and molecular interaction, it is only within the intact tissue that the true biological importance of the promoter and transcriptional environment can be appreciated. This is the rationale for the extensive and expensive effort required to carry out a promoter analysis in intact mice. However, when a promoter fragment is identified that controls expression of a transgene in defined subpopulation of cells, it has great value for studies in which tissue directed expression is required as discussed in the following sections.

The Col1a1 and Col1a2 promoters have received the greatest attention. Sequences extending a minimum of 2.3–3.6 kb upstream of the minimal promoter are required for high expression in most type I collagen-producing cells (Krebsbach *et al.,* 1993; Rossert *et al.,* 1995), while sequence as distant as 17 kb are required for strong Col1a2 activity (Antoniv *et al.,* 2001). The role of the first intron is subtle but does direct expression to a subset of type I collagen producing cells. Specifically, knockout of most of the intron produces a subtle reduction on type I collagen synthesis in skin and lung which was insufficient to have a physical phenotype (Hormuzdi *et al.,* 1998). However, when these transgenic mice are challenged with a stimulus for pulmonary fibrosis, an exaggerated transciptional response from the mutant allele was observed (Hormuzdi *et al.,* 1999). While the interpretation of this result is not fully understood, this experimental approach reveals regulatory subtleties that would not be appreciated in cell culture experiments.

A similar type of analysis has demonstrated that there are distinctly separate DNA elements that are important for high expression in different type I collagen-producing cells. The complex nature of the promoter appears to allow a single gene to be differentially regulated in different type I collagen–producing cells such as bone, tendon, and skin (Bogdanovic *et al.,* 1994). The best defined element is the one required to

regulate type I expression in bone. This "bone" element has been delineated to a 6-bp-segment homeobox binding domain located 1.7 kb from the transcription start site (Dodig *et al.,* 1996; Rossert *et al.,* 1996). Mutations within this 6-bp segment inactivates the transgene in bone while transgene activity persists in tendon. The Col1a1 promoter contains another domain upstream of the bone element that is essential for high expression in skin and tendon. An element that is essential for high expression in vascular smooth muscle cells is not located within the 5′ region of the gene (Bedalov *et al.,* 1994). Mapping these elements and defining the transcription factors that interact with them will be an essential step in understanding developmental pathways and signals used by cells that express the same collagen gene to varying levels in different tissues.

The osteocalcin (OC) promoter has received major attention in transient transfection studies to map transcription factor binding domains. A more complex regulatory structure becomes apparent in trangenic studies. A broader spectrum of tissue expression is seen in versions of the transgene than occurs in the endogenous gene and it has been difficult to ensure that all osteocalcin producing cells express the OC promoter transgene (Kesterson *et al.,* 1993; Clemens *et al.,* 1997; Frenkel *et al.,* 1997). A similar complexity is seen for the bone sialoprotein promoter (Chen *et al.,* 1996, 1999; Benson *et al.,* 2000).

The analysis of the type II collagen gene first drew attention to large fragments necessary for activity followed by identifying specific transcription factor binding domains. The importance of the first intron became fully appreciated in transgenic mice (Metsaranta *et al.,* 1995; Seghatoleslami *et al.,* 1995; Zhou *et al.,* 1995). Specific transciptional domans that bind Sox 9 and the SRY-related high-mobility group (HMG) have now been identified (Zhou *et al.,* 1998). Analysis of the type XI promoter (Li *et al.,* 1998; Tsumaki *et al.,* 1998; Liu *et al.,* 2000) in chondrocytes and the type X (Beier *et al.,* 1996) in hypertrophic chondrocytes has also been performed.

Use of Tissue Directed Transgenes to Assess Function of Genes Affecting Skeletal Development and Maintenance of Bone Mass

While targeted inactivation of genes by homologous recombination has contributed greatly to understanding the role of genes to embryonic development, the importance of these genes to bone and cartilage development is often obscured by the fact that other developmental systems are more severely affected and thus preclude analysis of the gene in bone. Examples include knockouts of the wnt and notch pathways, the bHLH family of transcription factors including twist and id and extracellular modifying factors such as noggin all have skeletal components to their embryonic phenotype, indicating an essential role in skeletal biology. This section will review transgenic approaches that had been used to overcome problems associated with global knockouts. In addition the section will review approaches for directing expression of growth factors to the bone environment.

The fundamental tool that is used in this experimental approach is a promoter that has tissue-restricted activity. This strategy creates a biological situation that rarely occurs in nature and can be considered a targeted gain of function mutation. The outcome of transgenic expression can be dramatic, but the interpretation of the biological meaning of the experiment can be complex. Uncertainties include the spectrum of cells that express the transgene. For example, the OC and bone sialoprotein (BSP) expression is specific to bones and thrombocytes, but many versions of the OC and BSP promoter have ectopic expression that includes brain. Within the lineage of bone and cartilage cells, the type I and type II collagen promoter can be designed to have preferential expression at specific stages of differentiation. However, low level expression at other stages of differentiation or for that matter in other cell types may exist and even the low level of activity may be sufficient to disrupt cells in an unintended manner. The other uncertainty is the manner in which the expressed transgene acts, i.e, either within the cell of synthesis (cell autonomous) or on neighboring cells (cell nonautonomous). For example OC-driven production of transforming growth factor (TGF)-β will influence the entire bone cell and osteoclasts lineage (Serra *et al.,* 1997) while OC-driven expression on a dominant negative TGF-β receptor construct will limit its biological affect to the cells that express osteocalcin (Filvaroff *et al.,* 1999). Experimental strategies that achieve inappropriate expression of a growth or transcription factor may produce levels of the product that are never found in a normal cell and affect pathways that normally would never be utilized. This concern always arises when constructs that act in the dominant negative manner on an endogenous transcription complex or signaling pathway in which multimeric interaction exert subtle changes in gene regulation. It cannot be assumed, particularly when the dominant negative protein is highly expressed within its cell, that only the targeted partner will be affected by the interaction.

Despite these reservations, valuable insights into the role of growth and transcription factors and signaling molecules have been achieved using targeted expression techniques. For example, the global knockouts a cbfa1 dramatically demonstrated its central role for bone cell differentiation because mice deficient in this transcription factor fail to develop a mineralized skeleton. Unclear from that work was the effect of cbfa1 on the development of the hypertrophic zone of the growth plate and the role of this structure on endochondral bone formation. When cbfa1 is expressed under control of type II collagen promoter in the background of the cbfa1 knockout mouse, the hypertrophic zone does develop accompanied by vascular invasion and osteoclasts. However, osteoblasts still do not differentiate (Takeda *et al.,* 2001). This experiment is strong evidence against transdifferentiation of the hypertrophic chondrocyte to osteoblasts. In contrast, when a dominant negative cbfa1 is targeted to the chondrocyte with a different Col2a1 promoter, the cartilage remains in a

proliferative stage without development of hypertrophic chondrocytes and osteoblasts fail to differentiate in a pattern very similar to that of the global cbfa1 knockout (Ueta *et al.,* 2001). These observations suggest that the formation of the hypertrophic zone is essential for endochondral bone, but that cbfa1 expression within the osteoblast lineage is required for differentiation in the presence of an intact growth plate.

To assess the role that cbfa1 has in maintaining the osteoblastic phenotype, a dominant negative cbfa1 was expressed under control of the OC promoter. This ingenious design allows the mice to complete embryogenesis normally because OC is not expressed during prenatal life. Osteoblasts do differentiate and the dominant negative is only expressed in late osteoblast differentiation. Mice have an osteopenic phenotype with normal osteoblastic cell number, and primary osteoblastic cultures derived from these mice fail to form mature bone nodules. While this work supports the role of cbfa1 for maintenance of osteoblast lineage even in adult life, it is likely that the dominant negative can act on other members of the family of cbfa1 transcription factors all of which can influence osteoblast differentiation (Harada *et al.,* 1999).

The role that other transcription cofactors have within the osteoblast lineage have been studied by expressing them with promoters that are more broadly expressed but which include the osteoblast lineage. The most surprising result is the role that the fos subfamily (fra-1 and fos B) of the AP-1 helix–loop–helix transcription family have in the osteoblast lineage. Even though these factors are widely expressed and their knockout either has no bone phenotype (c-*fos* knockouts have osteopetrosis, while c-*fos*-overexpressing mice get osteosarcomas and chondrosarcomas), their overexpression produce osteosclerotic bones by stimulating bone formation without enhancing bone resorption (Jochum *et al.,* 2000). A supporting observation is the reciprocal increase in bone mass and reduction of fat tissue in mice transgenic for FosB overexpression (Sabatakos *et al.,* 2000). While the observations are important because this is a biological effect that has great appeal as a therapeutic agent for diminished bone mass, their interpretation of the molecular mechanism is extremely difficult. Because the factors act by forming either homodimers or heteodimers with other members of the AP-1 family and are very dosage sensitive, it is difficult to know if they work directly by partnering with another factor to stimulate an osteogenic molecular pathway or titrate out another factor that is inhibitory to an osteogenic pathway (Aubin, 2001).

Overexpression studies point to the complexities that can influence the differentiation pathway of the osteoblast. While the role of PTHrP has received the greatest attention in the growth plate (see below), it clearly has an equally important effect on the osteoblast lineage. Targeted expression of the constitutively active form of the PTHrP receptor to the osteoblast with a Col1a1 promoter results in a profound increase in trabecular and endochondral bone formation but loss in cortical bone (Calvi *et al.,* 2001). In addition osteoclastic activity was increased in the marrow compartment. Because of the cell autonomous effect of the transgene within the osteoblast lineage, the primary effect of PTHrP in this experimental model is at the level of the osteoblast and the osteoclast response is a consequence of the activated osteoblast lineage. Although the physiological implication of the model will require further evaluation, this well understood pathway in cartilage will have important implication for manipulation of the bone lineage.

Targeting expression of a factor secreted by osteoblast cells affect the regulation of the entire osteoblast and osteoclast lineage making interpretation of the biological role of factor difficult. The first example was the expression of TGF-β under control of OC promoter producing the paradoxical outcome of high bone turnover osteoporosis with an additional component of impaired matrix mineralization. When the effect of TGF-β was limited to the osteoblasts lineage either by addition of bisphosphonates or expression of the transgene in a c-*fos* $-/-$ background, enhanced differentiation of cells into mature osteoblasts was observed. When the deficiency of TGF-β was limited to mature osteoblasts by expression of a dominant negative form of the TGFß receptor driven by OC promoter, osteoclastic activity was diminished and resulted in an increase in trabecular bone mass even though the number of mature osteoblasts was reduced (Erlebacher *et al.,* 1998; Filvaroff *et al.,* 1999). While this experimental approach further underlines the complex interrelationship between bone formation and resorption, it does not adequately discriminate the opposing cellular regulators of bone mass. Although expression of a dominant negative or constituitively active receptor can limit the growth factor effect to a particular lineage, the subpopulation of cells within the lineage, the manner (intermittent vs continuous) in which the stimulus is applied and age/growth rate of the mouse when the analysis is performed can all impact on the phenotype observed. The same type of experiments have now been performed with IGF1 driven either with the OC (Zhao *et al.,* 2000a) or the Col1a1 promoter. Both enhance bone formation and increase bone turnover leading to a similar problem of interpretation as TGF-β forced expression. Targeted expression of noggin (producing bone loss) and the calcitonin gene-related peptide (increase bone mass) (Ballica *et al.,* 1999) are other examples of targeted secretion of a factor affecting the coupling of bone formation and resorption. Even over expression of the tartate-resistant acid phosphatase (TRAP) enzyme results in a phenotype of high bone turnover osteoporosis (Angel *et al.,* 2000). The model utilized the SV40 enhancer and TRAP transgene and resulted in high TRAP activity in osteoclasts of bone and macrophages in nonosseous sites. While defining the cellular control mechanisms for bone remodeling can be fully appreciated only in the integrated system of intact mouse bone, the experimental design has to be improved so as to limit the effect of the probing transgene to a defined subset of cells before an unambiguous interpretation of the experimental data can be made.

Because structural organization of the growth plate provides definition of the stage of differentiation of cells within the cartilage lineage, overexpression studies are more easily understood. The global knockout of PTHrP and

its receptor leads to premature differentiation of hypertrophic chondrocytes while expression of a constitutive active from of the PTHrP receptor causes delayed maturation of hypertrophic chondrocytes. This fundamental observation has lead to a series of experiments in which targeted expression of the constitutive active PTHrP receptor in an ihh −/− or PTHrP receptor −/− background can separate to contribution of ihh and PTHrP to chondrocyte proliferation and hypertrophic differentiation (Schipani *et al.,* 1997; Karp *et al.,* 2000). A similar approach has been used to define the role of cbfa1 in the chondrocyte lineage beyond its role in supporting endochondral bone formation. Overexpression of cbfa1 via the Col2a1 promoter in an otherwise normal background is disruptive to normal joint formation and induces hypertrophic chondrocyte differentiation and vascular invasion at a site that normally never supports bone formation. Of particular interest is a disruption of the pattern of tenacin expression characteristic of the chondrocytes lining an articular cartilage with the reaction of the surrounding tissues suggestive of a degenerative joint. In contrast, targeting the dominant negative form of cbfa1 to chrondrocytes leads to failure of hyperchondrocyte differentiation and expression of tenacin throughout the articular cartilage (Ueta *et al.,* 2001). This work suggests that more subtle levels of chondrocyte differentiation can be controlled by cbfa1 and the possibility that it might play role in the development of degenerative joint disease (Liu *et al.,* 1995b; Ma *et al.,* 1996). TGF-β may also play a role in maintaining the articular cartilage in its appropriate state of differentiation. When same dominant negative TGF-β receptor transgene that was used to remove TGF-β signaling to the osteoblast is targeted to the chondrocyte a state of degenerative joint disease is observed with hypertrophic chondrocytes and synovial thickening (Serra *et al.,* 1997).

Thus ability to assess the state of differentiation by their position or morphology adds to the interpretation of the transgenic experiments. In regards to the osteoblastic lineage, the level of differentiation is probably best appreciated in the sutures of the calvaria. Murine phenocopies of human forms of craniodystosis caused by either overexpression of the normal gene or an activated form of the transcrition factor Msx2 lead to early fusion. These studies suggest that Msx2 acts to keep the cells of the osteogenic front in a proliferative and nondifferentiated state (Dodig *et al.,* 1999; Liu *et al.,* 1999). This conclusion is supported by studies of Msx2 −/− mice that fail to close their suture and demonstrate a diminished number of osteoprogenitor of the osteogenic front (Satokata *et al.,* 2000). The generalized reduction in bone mass seen in the calvaria and axial skeleton is likely to result from a similar cellular mechanism but would not have been appreciated without the analysis of the lineage within the calvarial suture. It would not be surprising that misexpression of other molecular pathways affecting calvarial suture development such as FGF2, twist, and id are likely to act by regulating proliferation and differentiation of the osteogenic front (Rice *et al.,* 2000).

Use of the GFP Transgene Family to Assess Osteoblast Lineage

The previous section stressed the value of recognizing the level of cellular differentiation with the osteoblast and chondrocyte lineage for interpreting the effect of transgenic animal models that alter the biology of bone or cartilage by affecting the regulation of lineage progression. When position or cell morphology is not available to assist in assessing the state of differentiation with a cell population, *in situ* hybridization, immunohistochemistry, or lac Z staining for activity of stage-specific promoter-β-gal transgene can be employed (Rossert *et al.,* 1995; Antoniv *et al.,* 2001). *In situ* hybridization can localize a signal to a specific population of cells and immunohistology is best suited for extracellular molecules, but both procedures are laborious and technically demanding. β-galactosidase has been the traditional enzymatic marker for assessing transgene expression and has been most useful in development studies. However, its use for marking cells an adult tissue is limited because of background endogenous activity and variation of staining intensity due to uncertainties in the diffusion of the substrate into mature tissue. The same techniques can be used to assess lineage in primary culture, but the culture has to be terminated so that progression of a specific cell to later stages of differentiation cannot be recorded.

The green fluorescent protein (GFP) family autofluorescent proteins may provide an approach to overcoming limitations of β-galactosidase in both intact bone and primary cultures derived from transgenic mice. Despite some reports that GFP can have toxic effects in transgenic mice, there are sufficient numbers of examples where viable lines have been produced and have been useful for studying cell lineage and transplantation experiments. Currently there are fluorescent colors that can be distinguished using appropriate filter cubes. Three are isomers of GFP (topaz or yellow, sapphire, cyan) and the fourth is from another species (DS red). The most widely used form of GFP is enhanced GFP (eGFP) because its autofluorescent properties are very similar to fluoroscein-conjugated probes commonly used in most immunohistological studies. However, its emission spectrum spills into GFP sapphire and GFP topaz limiting its use in multicolor experiments.

Sample preparation appears to be important for preserving GFP fluorescence in a histological section. eGFP does not appear to tolerate standard paraffin embedding and instead requires frozen sections to be prepared from histological studies. However, GFP topaz, sapphire, and emerald do maintain a strong fluorescent signal in paraformaldehyde-fixed, EDTA-decalcified, and paraffin-embedded sections of bone. This allows regions of fluorescence within the histological section to be examined under optimal tissue preserving conditions. Once the fluorescent image is recorded, standard histological staining is performed for optimal interpretation of the section. Bone has the additional problem of a high autofluorescent background in the bone marrow and to a lesser

degree in the bone matrix. Use of a dual filter cube optimized for GFP and in the Texas red or rhodamine spectrum allows the green GFP signal to be separated from an orange to yellow autofluorescent color. The section can be examined by either laser confocal microscopy or standard mercury bulb illumination.

The power of the GFP transgenes in primary culture is the detection of the fluorescent signal in the same plate as it progresses from initiation of the culture to full osteoblast differentiation. In this setting the time that the cells activate the transgene can be accurately determined and the transcription profile of that subpopulation of cells can be determined by FAC sorting for the GFP positive cells. For example, a pOBCol3.6 promoter fragment activates GFP concomitant with the expression of alkaline phosphatase and type I collagen synthesis, which is at the stage of preosteoblast differentiation. Once bone nodules form and begin to mineralize the pOBCol2.3, promoter fragments become active along with osteocalcin expression, marking the stage of mature osteoblast/osteocyte differentiation. The two promoter GFP constructs have a different pattern of cellular fluoresence in sections of bone. The pOBCol3.6GFP transgenic mice show expression in the periosteal fibroblasts and lining osteoblast but with little expression in osteocytes. The pOBCol2.3GFP is not expressed in the periosteum but is found on lining osteoblasts and throughout the bone matrix. We envision building a series of promoter-GFP transgenic mice in which different stages of cellular differentiation are marked by a different color of GFP. These mice will provide the ability to recognize stages of differentiation within a complex tissue much like the cell surface markers that are used to identify precursor sells within a hematopoetic lineage. If these reagents become widely disseminated, they can provide a way of standardizing the analysis of factors affecting bone biology at the level of lineage control whether in intact animals or in primary culture derived from the mice.

The availability of a GFP transgene marking stages of cell differentiation will have a wide variety of uses. In primary cell culture, the activation of GFP transgenes can be used to assess the tempo of differentiation and the proportion of clusters of preosteoblasts that form nodules with full bone cell differentiation. Transgenes marking differentiation of adipocytes, smooth muscle cells, chondrocytes (Grant *et al.,* 2000), and vascular and endothelial cells (Kishimoto *et al.,* 2000) are available and need to be modified to a GFP that will function in paraffin-embedded tissues. These markers will allow the investigator to appreciate the complexity of primary marrow stromal cultures and factors that can modulate the culture to various lineages. Possibly its most useful application will be in microarray analysis of the osteoblast lineage. Interpretation of a complex array pattern is extremely difficult in a heterogeneous cell population. Is the change in an expression pattern a consequence of the change that occurred in a subpopulation that only represents 25% of the entire cell population? Are important patterns lost because of an increase in one subpopulation and a fall in another? The GFP also allows the isolation of a specific cell population from a heterogeneous population. The cells can be analyzed by FACS and even collected by cell sorting for biochemical or cellular analysis. This isolation may be an essential requirement for interpreting a microarray experiment.

In intact bone, direct visualizing of the cells that express the transgene, which in turn reflect a level of attained differentiation during development, in a knockout model or in response to a drug intervention will assist in the interpretation of experimental data. Building mosaic mice in which the identity of donor cells are distinguished by transgene expression can reveal cell–cell interactions that could not be appreciated in any other way. For example mosaic mice created with β-Gal marked chondrocytes obtained from the PTHrP receptor $-/-$ mouse produce islands of β-gal-positive hypertrophic chondrocytes within an elongated zone of proliferating and β-gal-negative chondrocytes. This outcome affirmed the model molecular pathway that coordinates lineage maturation within the growth plate. Loss of the PTHrP pathway induced early differentiation and these hypertrophic cells secrete ihh, which stimulates the surrounding PTHrP intact cartilage and color osteoblastic cells into a proliferative and nondifferentiating state (Chung *et al.,* 2001). A third application is in transplantation studies of marrow stromal cells for somatic gene therapy for diseases of bone. Most of these studies to date have used nonspecific marker genes, which did not reflect differentiation into the bone lineage. Recently, engraftment of cells expressing the OC CAT transgene has been demonstrated by *in situ* hybridization, providing the most convincing data that osteoblastic precursor cells can engraft bone and participate in endogenous bone formation (Hou *et al.,* 1999). In the future, GFP will be a more robust and versatile marker for quantitative studies of cellular engraftment and demonstration of transplanted stem cells by direct marrow stromal cells culture of the transplanted mice.

Transgenes as Tools to Ablate a Specific Population of Cells Affecting Bone Biology

The interaction of cells from different lineages is central to organogenesis and bone is no exception. The advent of transgenic technology has led to the development of experimental systems designed to eliminate or destroy specific cell types. The basic concept to direct the synthesis of a gene product that is lethal (the A chains of diphtheria toxin) (Breitman *et al.,* 1990; Breitman and Bernstein, 1992) or conditionally lethal (herpes thymidine kinase, htk transgenic mice) (Borrelli *et al.,* 1989; Heyman *et al.,* 1989) have been the two most widely used strategies. In the case of the viral tk, the viral enzyme can be expressed in mammalian cells without deleterious affects and conditional lethality is achieved by the addition of nucleoside analogs that are phosphorylated only by the viral enzyme. Cell killing occurs when these analogs are incorporated into DNA, thus limiting the lethality to dividing cells. The problem of male sterility in mice transgenic with

the htk gene can be overcome by using a truncated version of the gene in which intrinsic promoter elements within the coding region of the gene are removed without affecting enzymatic activity (Salomon *et al.,* 1995).

The first application of this approach to bone utilized the OC promoter driving an the unmodified form of htk (Corral *et al.,* 1998). After a 4-week treatment protocol with ganciclovir, an osteoporotic phenotype developed in which osteoblasts were destroyed and osteoclasts persisted. Upon removal of the drug, bone mass recovered. The authors interpreted their finding to indicate that the coupled process of bone formation and bone resorption can be dissociated in their model system. Subsequently another model using the truncated form of tk was developed in which a Col1a1 promoter fragment with restricted activity to early osteoblasts was used (Visnjic *et al.,* 2001). In this case a 2-week exposure to ganciclovir resulted in complete loss of osteoblasts and preservation of osteocytes. Osteoclasts were absent and a major disruption in the hemopoietic cell compartment developed. Upon removal of the drug, an osteosclerotic reaction developed in the endochondral and trabecular bone. The remarkable difference in the two models probably relates to level of differentiation within the osteoblast lineage at which the htk was expressed. Late lineage osteoblasts/osteocytes do not contribute the synthesis of new osteoid and probably do not influence the activity of osteoclasts. Osteoblasts actively making osteoid and probably undergoing a limited degree of cell division are more susceptible to ganciclovir toxicity. In addition their cellular activity is either directly or indirectly linked to the activity of osteoclasts and the maintainence of the bone marrow elements. Interpretation of these two models will be helped when GFP lineage markers are incorporated to assess which cells within the lineage survive and continue to influence bone microenvironment.

Probing Skeletal Biology with Strategies that Disrupt the Endogenous Genes

The complete sequencing of the human genome is near completion and will be followed shortly by the sequencing of the entire mouse genome. These achievements have changed the landscape of mammalian genetics. Attention is now focused away from genome structure to the challenge of completely understanding genome function. An obvious area that will be exploited is gene targeting. Systems have been developed and are being further refined as tools to permit the temporal and spatial modulation of a given genetic unit. The Cre/loxP recombinase system developed from the P1 bacteriophage is one such tool. The Cre (causes recombination) recombinase specifically directs recombination at loxP (locus of X-ing-over) recombination recognition sites not only in bacteria but also in eukaryotic cells. A segment of DNA flanked by two directly oriented loxP sites can be excised by the Cre recombinase. When applied to transgenic models, one transgenic line bears the Cre recombinase gene driven by an inducible or tissue-specific promoter. A second transgenic line carries a target transgene. One type of target transgene is an endogenous gene that has been engineered by homologous recombination to contain loxP sites in a desired location so as to produce a predetermined modification of the endogenous allele in the presence of the Cre recombinase. Often the target transgene is "silent" in the absence of the Cre recombinase. By breeding the Cre recombinase-bearing line to the target line, F1 progeny are produced that now contain a modified target transgene. Depending on the nature of the design of the target allele, Cre recombination can either activate or inactivate the targeted locus. This system has been recently employed to develop the first knock-in mouse model for osteogenesis imperfecta (Forlino *et al.,* 1999). Other systems are being developed to control spatial expression of targeted loci by directing of Cre expression to defined cellular components of the skeleton. A Col2a1–Cre fusion transgene has been developed with expression restricted to the chondrocytes (Sakai *et al.,* 2001). Important in the development of these systems is the evaluation of the Cre recombinase expression when driven by a specific promoter. Specialized lines of mice have been developed to serve as indicators of Cre recombinase expression. Information relative to these lines as well as others under development can be obtained from a web site maintained by Andras Nagy (*http:// www.mshri.on.ca/nagy/cre.htm*). While spatial control of Cre expression is a powerful paradigm for studying gene function, systems that allow both spatial and temporal modulation of Cre expression permit more elegant analyses. Several experimental paradigms have been developed to permit the temporal regulation of Cre recombinase expression, including two systems that involve the linking of Cre recombinase to a ligand-binding domain. One model utilizes human estrogen receptor fused to Cre (Cre–ERT). In this system Cre expression is activated by tamoxifen but not by natural hormone, estradiol (Brocard *et al.,* 1997). Recently a modified version of the Cre–ERT has been developed (Cre–ERT2) that is approximately 10-fold more sensitive to tamoxifen induction than Cre–ERT (Indra *et al.,* 1999). The Cre-ERT2 may be particular useful in studies where the impact of the inducing agent is a possible concern since much lower concentrations of tamoxifen are required for recombinase expression. A similar system fuses Cre recombinase to a truncated ligand-binding domain of the progestrone receptor. Like the Cre–ERT system, the Cre recombinase is activated by a synthetic steriod RU486 but not the endogeneous hormone progesterone (Kellendonk *et al.,* 1996). While these systems both permit the temporal and spatial regulation of Cre recombinase expression their value to bone biology may be of potentially limited because of unwanted affects of the tamoxifen or RU486 on bone cell metabolism. Careful experimental design is required to be certain that the synthetic steriods are not impacting on the biological process being evaluated.

While gene targeting is a compelling tool for studying gene function, this gene-driven method requires that the genetic units to be studied have been cloned. Recently, a

second avenue, the phenotype-driven approach, has received a great deal of attention. This research paradigm involves large-scale mutagenesis programs using the chemical mutagen *N*-ethly-*N*-nitrosourea (ENU) and the development of gene trap strategies. While very expensive and labor intense, ENU mutagenesis is important for evaluating a broad spectrum of gene function. Functions associated with a locus may be discovered in an ENU mutagenesis screen that would not be revealed in a gene-targeting experiment. Several major ENU screening programs are underway worldwide and a list of the web site addresses has been published (Justice *et al.,* 1999). Of particular interest to the bone biologist is the program being conducted at the GSF-National Research Center for Environment and Health in Neuherberg, Germany. A component of their screen focuses on dysmorphology which will include alterations in skeletal structure (Hrabe de Angelis *et al.,* 2000). Finally, gene trap methodologies utilizing embryonic stem cells and various targeting vectors are being developed. Trapping vectors are designed to detect *cis*-regulatory elements, splice sites, or poly(A) addition signals. The objective of these programs is to create large libraries of genetically altered ES cells for the generation and analysis of mouse mutations. As with the ENU mutagenesis program, large-scale programs are being initiated worldwide (Cecconi and Meyer, 2000).

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CHAPTER 88

Use of Cultured Osteoblastic Cells to Identify and Characterize Transcriptional Regulatory Complexes

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Introduction

The molecular details of transcription and its regulation in osteoblasts have become the subject of intense interest for researchers in developmental biology, endocrinology, cellular physiology, clinical pharmacology, and biomechanics. Since the original version of this chapter, great strides have been made in our understanding of osteoblast transcriptional regulation. The gene encoding the runt domain factor, Runx2—a.k.a. type II Cbfa1/ Osf2, til-1, PEPB2αA, or AML3—has now been unambiguously identified as necessary for robust expression of the osteoblast phenotype (Ducy *et al.,* 1997, 2000; Komori and Kishimoto, 1998; Komori *et al.,* 1997). In the absence of this crucial transcriptional regulator, ossification programs cannot be elaborated that propagate orthotopic mineral deposition during development. Intriguingly, other transcriptional regulatory circuits—e.g., those modulated by Msx2 (Newberry *et al.,* 1998; Satokata *et al.,* 2000), Dlx5 (Acampora *et al.,* 1999; Benson *et al.,* 2000; Erceg *et al.,* 2000; Ryoo *et al.,* 1997), and Fra1 (Jochum *et al.,* 2000; Newberry *et al.,* 1997c)—that determine the level and temporospatial timing and global extent of osteoblastogenesis, osteoblast gene expression, and matrix synthesis have also emerged. Additionally, members of the Msx gene family may participate in the initiation and propagation of certain heterotopic mineralization programs associated with macrovascular arterial calcification (Towler *et al.,* 1998). Nevertheless, even though the rudimentary components of the osteoblast transcriptional machinery have now been revealed, the cellular physiology of transcriptional regulation in the osteoblast remains poorly understood. As highlighted previously, morphogenetic, metabolic, and mechanical (matricrine) signaling cues all contribute to the control of bone formation, and the molecular pathways modulating gene expression in response to these physiological stimuli remain to be detailed. Moreover, as is increasingly apparent, transcription factors such as Runx2, Msx2, and the AP1 family function not as single entities but as modular components of large nuclear macromolecular transcriptional complexes. While several coregulatory molecules have been identified for these factors, the native "holoprotein" complexes that assemble in osteoblasts and regulate Col1A1, Col1A2, alkaline phosphatase, osteopontin, bone sialoprotein (BSP), osteocalcin (OC), etc., remain undefined.

This chapter will build from the foundation established in the first edition that emphasizes studies immediately relevant to osteoblast gene expression. However, two new methodological approaches will be discussed that are only now receiving attention in the field of bone biology transcriptional regulation. The recent technological advances in protein mass spectrometry and data base analyses tools (Neubauer *et al.,* 1998) will be highlighted, since these approaches will

permit characterization of the nuclear holoprotein complexes and posttranslational modifications that regulate osteoblast gene regulation. Additionally, the application of chromatin immunoprecipitation (ChIP) assays (Kuo and Allis, 1999) to detail holoprotein complexes assembled by specific promoters in native chromatin contexts will be discussed. Dissection of the molecular details of gene expression in the osteoblast holds tremendous promise for development of novel therapeutic strategies for management of the prevalent musculoskeletal diseases, heterotopic ossification syndromes, skeletal neoplasms, and craniofacial dysmorphias that afflict the human population throughout life.

Osteoblast Cell Culture Models

Model systems developed using osteoblastic cell type-specific promoters have provided important general insights into transcription control by steroid hormones, retinoids, cytokines, growth factors, oncogenic proteins, and homeodomain transcription factors (Table I). For example, our understanding of how calcitriol regulates gene expression has been gleaned largely from studies of the osteocalcin and osteopontin promoters, two genes characteristically expressed in osteoblasts. Similarly, important insights into the influence of chromatin structure on gene expression during cell cycle progression have been elucidated using cultured osteoblastic cells (Drissi *et al.,* 2000; Stein and Lian, 1993; Stein *et al.,* 1994). However, much work remains to be done before an accurate and detailed working model of osteoblast-specific transcription control can be drawn.

A variety of cultured cell systems have been successfully used to study the role and regulation of osteoblast-specific elements in transfection assays (Table II). Three general classes of culture systems have been employed: osteoblastic osteosarcoma cell lines, spontaneously transformed and Papova virus antigen transformed cell lines, and primary cultures of osteoblastic cells derived from fetal rat calvaria or subperiosteal bovine fetal long bones. Each system has its advantages and disadvantages; osteoblastic osteosarcomas, MC3T3E1 cells, and primary calvarial cell cultures will be discussed below. The reader is referred to cited references and the earlier edition of this book for full discussion of other, less utilized culture models.

Osteoblastic Osteosarcomas

The most widely used system for studying transcription control in osteoblastic cells has been the ROS 17/2.8 osteoblastic osteosarcoma (Majeska *et al.,* 1980). This mineralizing subclone obtained from a rat osteosarcoma constitutively expresses a number of mature osteoblastic phenotypic markers, including alkaline phosphatase, PTH receptor, vitamin D receptor, osteopontin, BSP, OC, and lower levels of type I collagen (Majeska *et al.,* 1980; Rodan and Noda, 1991). Importantly, these cells are phenotypically very stable, respond to major osteotropic hormones (Rodan and Noda, 1991), and are readily transfected (Table II). Studies of transforming growth factor (TGF)-β regulation of the COL1A2 gene, calcitriol regulation of the osteopontin and osteocalcin genes, and retinoid, tumor necrosis factor (TNF)-α, and glucocorticoid regulation of the osteocalcin promoter have all used ROS 17/2.8 cells (Table I). Moreover, a number of nonmineralizing, nonosseous sarcomatous subclones from the same tumor provide convenient cellular backgrounds for comparison for identifying cell type specific elements. For example, the ROS 25/1 clone, which does not mineralize and does not express osteocalcin (Majeska *et al.,* 1980), has been successfully used in concert with ROS 17/2.8 cells to define the osteoblast cell type-specific transcriptional regulation and DNA protein interactions controlling the rat (Towler *et al.,* 1994a) and mouse (Ducy and Karsenty, 1995) osteocalcin promoters. Of note, comparison of basal OC promoter regulation in these two cellular backgrounds guided the search that ultimately identified Cbfa1/Osf2/Runx2 (Ducy *et al.,* 1997, 2000) and Msx2 (Newberry *et al.,* 1998; Towler *et al.,* 1994b) gene family members as important osteoblast transcription factors.

Transfection of ROS 17/2.8 cells by calcium phosphate and DEAE dextran techniques is straightforward (section III). Electroporation has also been used successfully with ROS 17/2.8 cells for transient transfection analyses (Terpening *et al.,* 1991). However, when performing multiple independent transfections with a large number of systematically altered promoter constructs, DEAE–dextran is preferred because of ease of performance and reproducibility (Keown *et al.,* 1990; Lopata *et al.,* 1984) and low cost.

Similar to ROS 17/2.8 cells, the UMR-106 series of osteosarcomas expresses many aspects of the phenotypic characteristics of osteoblasts, including PTH responsiveness (Rajakumar and Quinn, 1996; Selvamurugan *et al.,* 2000), BSP expression (Lecanda *et al.,* 1998; Ogata *et al.,* 1995), and mineralization (Stanford *et al.,* 1995). However, the lack of significant osteocalcin expression in certain subclones suggests that UMR clones express a distinct, restricted spectrum of the osteoblast differentiation program continuum (Rodan and Noda, 1991). Transfection has been successfully achieved by electroporation (Davis *et al.,* 1994) and calcium phosphate transfection (Ernst *et al.,* 1991). A useful variant of UMR cells has been engineered by stably expressing a cyclic AMP (cAMP)-resistant form of the type I regulatory subunit of cAMP-dependent protein kinase (Bringhurst *et al.,* 1989), which specifically usurps this important signaling pathway in the osteosarcoma background. Such engineered cell culture systems may prove useful in the examination of the positive (Boudreaux and Towler, 1996) and negative (Tintut *et al.,* 1999) effects of cAMP signaling on osteoblast-specific transcription factor complexes.

Much less detailed information exists on transcriptional regulation in human osteosarcoma lines. Similar to the ROS 17/2.8 cell line, MG63 human osteosarcomas express alkaline

Table I Studies of Osteoblast Promoter Regulation and Associated Transcription Factor Biology

Promoter	Element	Reference
Human osteocalcin	Basal promoter	Kerner *et al.,* 1989
	Calcitriol stimulation	Morrison *et al.,* 1989; Ozono *et al.,* 1990
	Glucocorticoid response	Morrison and Eisman, 1993
	FGF response	Schedlich *et al.,* 1994
	Retinoic acid	Schule *et al.,* 1990
	TNF-α	Li and Stashenko, 1993
Rat osteocalcin	Basal promoter	Towler *et al.,* 1994a
	Calcitriol stimulation	Yoon *et al.,* 1988; Demay *et al.,* 1992
	NMP2/Cbfa1/AML/Runx2	Merriman *et al.,* 1995
	TNF-α	Nanes *et al.,* 1994
	FGF2/cAMP response	Boudreaux and Towler, 1996
	PTH response	Yu and Chandrasekhar, 1997
	Msx2	Hoffmann *et al.,* 1994; Towler *et al.,* 1994b
	Dlx5	Ryoo *et al.,* 1997; Newberry *et al.,* 1998
	Connexin 45/43 response	Lecanda *et al.,* 1998
	MINT	Newberry *et al.,* 1999
	Ku 70 / Ku 80/ FREB100	Willis *et al.,* 2000
Mouse Osteocalcin / OG2	Basal promoter	Ducy and Karsenty, 1995
	Osf2/Cbfa1/Runx2	Geoffroy *et al.,* 1995; Ducy *et al.,* 1997
	Ascorbate/ integrin response	Xiao *et al.,* 1997; 1998
	Calcitriol inhibition	Zhang *et al.,* 1997
	Osf1	Schinke and Karsenty, 1999
Bone sialoprotein	Basal promoter	Kim *et al.,* 1994
	Dlx5	Benson *et al.,* 2000
	PTH response	Yang and Gerstenfeld, 1996
	PTH response, Pit1	Ogata *et al.,* 2000
	Cbfa1/Runx2/AML	Javed *et al.,* 2000
	RORα response	Meyer *et al.,* 2000
	v-src response	Kim and Sodek, 1999
	TGF-β response	Ogata *et al.,* 1997
	Glucocorticoid response	Ogata *et al.,* 1995
Osteopontin	Basal promoter	Zhang *et al.,* 1992
	Calcitriol	Noda *et al.,* 1990
	v-src	Tezuka *et al.,* 1992
	Ets1, Cbfa1/Runx2	Sato *et al.,* 1998
	Hoxc8, Smad1, Smad 6	Yang *et al.,* 2000; Bai *et al.,* 2000
Col1A1	Basal	Dodig *et al.,* 1996; Rossert *et al.,* 1996
	Cbfa1/Runx2/AML	Kern *et al.,* 2000
	FGF response	Hurley *et al.,* 1993
	PGE2 response	Raisz *et al.,* 1993
	cAMP response	Fall *et al.,* 1994
	Calcitriol response	Pavlin *et al.,* 1994
	PTH response	Alvarez *et al.,* 1998
	Msx2	Dodig *et al.,* 1996
	RHox/Prx1/Mhox	Hu *et al.,* 1998
	IL1 response	Harrison *et al.,* 1998
IGF1	Basal; C/EBP-delta	Umayahara *et al.,* 1999
MMP13/ Collagenase 3	Basal / Cbfa1/Runx2	Winchester *et al.,* 2000; Winchester *et al.,* 2000
	PTH response, Cbfa1/Runx2	Selvamurugan *et al.,* 2000
	PTH response, Ets1, CREB, CBP	Quinn *et al.,* 2000; Rajakumar and Quinn, 1996
	FGF response	Varghese *et al.,* 2000
	PDGF response	Rydziel *et al.,* 2000
MMP1 / Collagenase 1	FGF2 response, Fra1	Hurley *et al.,* 1995; Newberry *et al.,* 1997c
	PE1/ Ets2 repressor factor-2/ERF2	Bidder *et al.,* 2000
Osteoprotegrin / OPG	Runx2/Cbfa1	Thirunavukkarasu *et al.,* 2000
PTH/PTHrP	Basal promoter	Minagawa *et al.,* 2000
Biglycan	Basal promoter	Heegaard *et al.,* 1997
Alkaline phosphatase	Retinoic acid response	Heath *et al.,* 1992
	Cbfa1/Runx2/AML	Harada *et al.,* 1999

Table II A Selected List of Useful Cell Culture Models for Transcription Control in Osteoblasts

Cell culture systems	Phenotypic makers expressed	Transfection modalities	References
ROS 17/2.8 (rat)	COL I, AP, OP ON, BSP, OC	Ca PO_4/Gro shock DEAE dextran/DMSO shock Electroporation Polybrene	Ernst *et al.,* 1991 Yoon *et al.,* 1988 Terpening *et al.,* 1991 Kerner *et al.,* 1989
UMR (rat)	COLI, AP, OP ON, BSP, MMP13	Ca PO_4/Gro shock Electroporation	Ernst *et al.,* 1991 Davis *et al.,* 1994
RCT1 (rat)	COL I, AP, OP (RA dependent)	DEAE–dextran/DMSO shock with chloroquine	Ernst *et al.,* 1991
RCT3 (rat)	COL I, AP, OP	Ca PO_4/Gro shock	Ernst *et al.,* 1991
MG63 (human)	COL I, AP, OP BSP, OC (OC calcitriol dependent)	Liposomes Retrovirus	Cervella *et al.,* 1993 Thacker *et al.,* 1994
MC3T3-E1 (mouse)	COL I, AP, OP ON, BSP, OC, MMP1	Ca PO_4, Gro shock DEAE–dextran/DMSO shock	Hurley *et al.,* 1993 Towler *et al.,* 19994b
Primary calvarial Osteoblasts (rat)	COL I, AP, OP ON, BSP, OC, MMP1, MMP13	Ca PO_4, Gro shock Liposomes	Pash *et al.,* 1995 McCarthy *et al.,* 1995 Newberry *et al.,* 1998
Primary cortical Osteoblasts (bovine)	COL I, AP, OP ON, BSP, OC	Ca PO_4, Gro shock	Ibaraki *et al.,* 1992, 1993

Note. COL I, type I collagen; AP, alkaline phosphatase; Op, osteopontin; ON, osteonectin; BSP, bone sialoprotein; OC, osteocalcin; Ca PO_4, calcium phosphate; Gro, glycerol, DEAE, diethylaminoethyl; DMSO, dimethylsulfoxide; RA, retinoic acid.

phosphatase, bone sialoprotein, MMP1, and osteocalcin (Bonewald *et al.,* 1992; Lajeunesse *et al.,* 1991; Riikonen *et al.,* 1995). However, these cells are much more difficult to transfect; DEAE–dextran and calcium phosphate techniques are generally not useful. Cationic liposome-mediated transfection (Cervella *et al.,* 1993; Riikonen *et al.,* 1995) and retroviral vectors (Thacker *et al.,* 1994) have been successfully used for transfection of MG63 cells. MG63 cells have been widely used in the study of integrin-mediated cell–matrix interactions and regulation by cytokines and hormones (Broberg and Heino, 1996; Riikonen *et al.,* 1995). Studies of β-1 integrin expression in MG63 cells introduced the notion that osteoblastic cell-type specificity exists for one of the two known β-1 integrin gene promoters (Cervella *et al.,* 1993). Moreover, the MG63 line has been used as a convenient source for purification and identification of known and novel osteoblast transcription factor complexes (Newberry *et al.,* 1997a). However, very little published information exists on osteoblast-specific promoter regulation and transcription factor biochemistry in this human osteosaroma line. The MG63 osteosarcoma has yet to reach its full potential as one model cell culture system for studying osteoblast-specific gene expression. Future studies in this cellular background promise to be rewarding.

Another human osteosarcoma, Saos-2 (lacks endogenous tumor suppressor p53), can be transfected by the calcium phosphate technique. The Saos-2 line has been widely used in the studies of p53-activated (Morris *et al.,* 1996; Tang *et al.,* 1998; Thornborrow and Manfredi, 1999) and repressed (Sun *et al.,* 2000) genes that control cell growth, apoptosis, and extracellular matrix turnover. Additionally, estrogen receptor signaling has been examined in this cellular background (Ernst *et al.,* 1991). However, the Saos-2 line expresses a more limited subset of osteoblast phenotypic markers when compared with MG63 cells.

MC3T3-E1 Calvarial Osteoblasts

For a variety of reasons, the MC3T3-E1 cell line provides one of the most convenient and physiologically relevant culture systems for studies of transcription control in calvarial osteoblasts. The MC3T3-E1 cell line is a spontaneously immortalized (not transformed) cell line selected by the 3T3 passaging protocol (Sudo *et al.,* 1983). MC3T3-E1 cells behave as immature, committed osteoblast cells, which go on to differentiate in response to intracellular and extracellular cues. Upon reaching confluence, this clonal cell line differentiates along the osteoblast lineage, sequentially expressing characteristic osteoblast phenotypic markers (including bone sialoprotein and osteocalcin) in a manner closely mimicking that of primary cultures of fractions 3–5 calvarial osteoblasts (Franceschi and Iyer, 1992; Franceschi *et al.,* 1994; Vary *et al.,* 2000). Moreover, postconfluent cultures of MC3T3-E1 cells will mineralize—accumulating hydroxyapatite—when cultured in the presence of ascorbic acid and β-glycerol-phosphate (Sudo *et al.,* 1983). MC3T3-E1 cells respond to osteotropic hormones, prostaglandins, cytokines, and numerous growth factors, including FGF, IGF, and BMP family members. The phenotype of this cell line is very stable as long as stocks are rigorously maintained (in fetal calf serum) by passaging before confluency. Finally, MC3T3-E1 cells

are readily transfected by DEAE–dextran (at higher cell densities) and calcium phosphate modalities (Newberry *et al.,* 1997b; Towler *et al.,* 1994a). With regard to detailed analyses of transcription control, MC3T3-E1 cells have been used most widely in the study of the osteocalcin (Boudreaux and Towler, 1996; Newberry *et al.,* 1999; Towler *et al.,* 1994a; Xiao *et al.,* 2000), type I collagen (Hurley *et al.,* 1993), interstitial collagenase (Bidder *et al.,* 2000; Hurley *et al.,* 1995; Newberry *et al.,* 1997c), bone sialoprotein (Benson *et al.,* 2000), and prostaglandin G/H synthase-2/Cox-2 (Kawaguchi *et al.,* 1995) promoters. This cell line holds tremendous promise as a physiologically relevant model for future studies outlining growth factor-activated signal transduction cascades and their interactions with the calvarial osteoblast transcriptional machinery.

Cultures of Primary Osteoblasts

The most widely used source of primary osteoblasts are proteolytically released cells derived from fetal and neonatal rat calvaria. Cells released at later times of digestion have great osteogenic potential, sequentially expressing osteoblast phenotypic markers and forming mineralizing nodules with time in culture (Kawaguchi *et al.,* 1995). These cells in early passage are neither immortalized nor transformed. The population of cells released from calvarial tissue is inherently heterogenous; however, enrichment of cultures for committed osteoblasts can be obtained by passage and treatment with 0.1 μM dexamethasone (Aronow *et al.,* 1990). Cultures of primary calvarial osteoblasts are readily transfected by calcium phosphate (Newberry *et al.,* 1998; Pash *et al.,* 1995) or lipofection (McCarthy *et al.,* 1995) techniques. The regulation of the insulin-like growth factor (IGF)-I promoter by cyclic AMP in primary calvarial osteoblasts identified the unique role for C/EBP-δ in this signaling cascade, and proved the utility of this culture model for studies of promoter regulation and signal transduction in osteoblasts (Umayahara *et al.,* 1999). More recently, AP1 complex protein/DNA interactions mediating FGF2 regulation of the MMP13/collagenase-3 promoter have been studied in this culture model (Varghese *et al.,* 2000), confirming the important role of the Fos and Fra family members in FGF2-dependent control of osteoblast matrix metalloproteinases (Bidder *et al.,* 2000; Newberry *et al.,* 1997c).

In a similar fashion, cultured primary bovine osteoblasts have been used in studies of the human osteonectin promoter (Dominguez *et al.,* 1991). Subperiosteal bovine osteoblasts express phenotypic markers and mineralize in culture (Ibaraki *et al.,* 1992). Young and coworkers used this system for identifying and characterizing an element consisting of repetitive GGA motifs, which were recognized by a nuclear factor present in primary osteoblasts but not in kidney cell lines; the identity of this factor is unknown (Ibaraki *et al.,* 1993) and cell-type specificity conveyed by the element may be species-specific (Hafner *et al.,* 1995). Finally, chick calvarial osteoblast cultures have also been used for detailed studies of Msx2 actions on osteoblast differentiation programs (Dodig *et al.,* 1999); generally, retroviral transfection is required for efficient use of this system (*vide infra*).

Transcriptional Analysis

A wide variety of techniques exist to allow monitoring of steady-state mRNA levels in bone cells. These include Northern blot assays, S1 nuclease mapping, RNAse protection assays, reverse transcription–polymerase chain reaction (RT-PCR) assays quantified by traditional competitive template analysis or real-time fluorescence signals, differential display-PCR, or the powerful new method of gene arrays and DNA microarrays. A description of all these techniques is beyond the scope of this chapter; however, it should be mentioned that their use and the results that they generate represent the initial step in the study of the control of the expression of any given gene. They have been used extensively to obtain a profile of gene expression in bone cells during cellular differentiation, and in response to a wide variety of signals as diverse as extracellular matrix attachment, cytokine and hormone treatment, drug treatment, and even microgravity during space flight (Bidwell *et al.,* 1998; Carmeliet and Bouillon, 1999; Carulli *et al.,* 1998; Damsky, 1999). Once a condition has been identified that modulates the steady-state mRNA levels of the gene of interest, the real task of determining which mechanisms contribute to the regulation of the expression of the gene can begin.

Transcriptional Control vs mRNA Stability

Detecting increased levels of steady-state mRNA does not necessarily mean that gene transcription is increased. Indeed, bone cells can control the abundance of any given mRNA through a variety of means: transcription initiation, transcription elongation, mRNA stability, pre-mRNA splicing, mRNA transport, and polyadenylation. Translation and posttranslational modifications represent other means available to the cell to ultimately regulate the expression of a given gene product. Changes in splicing efficiency, transport rates, and polyadenylation are not easy to evaluate, and these mechanisms are usually invoked when direct measurements of mRNA stability and transcription initiation have yielded negative results. They still represent bona fide control mechanisms, however, as exemplified by the differential expression of the liver/bone/kidney isozyme of alkaline phosphatase in osteoblasts that is controlled via a posttranscriptional mechanism not involving increased cytoplasmic mRNA stability (Kiledjian and Kadesch, 1991; Manji *et al.,* 1995). Similarly, the major stimulation of bone sialoprotein expression following glucocorticoid treatment of bone cells involves a nuclear posttranscriptional mechanism (Ogata *et al.,* 1995).

Measuring the stability of a given mRNA represents a valid starting point for any study of the mechanisms regulating the expression of a gene in response to a particular

signal. The easiest method for measuring the half-life of an mRNA is to block transcription with an inhibitor such as actinomycin D, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), or α-amanitin. The abundance of the mRNA of interest is measured as a function of time after inhibitor treatment using Northern blotting, S1 nuclease, or RNAse protection assays. The amount of time required for 50% of the mRNA to degrade represents an approximate half-life for the mRNA. The technique can be used if the steady-state abundance of the mRNA is readily detectable and yields useful results when the half-life to be measured is relatively short ($<$2 hr). Longer half-lives are difficult to measure using this approach due to the toxicity of the inhibitors that induce cell death after a few hours of treatment.

This approach has been successfully used to establish that stabilization of the mRNA mediates the induction of Indian hedgehog (Ihh) following TGF-β treatment of primary calvarial osteoblastic cells (Murakami *et al.,* 1997) and cAMP regulation of OC mRNA accumulation (Noda *et al.,* 1988). Suppression of gene expression can also be mediated post-transcriptionally as exemplified by the destabilization of osteonectin mRNA in osteoblasts observed after treatment of the bone cells with basic fibroblast growth factor (Delany and Canalis, 1998).

Evaluation of Transcription Initiation by Nuclear Run-on Assays

If the modulation of steady-state mRNA abundance following treatment is not due to a change in mRNA stability, one likely regulatory step is the control of transcription initiation. This can be measured using the nuclear run-on transcription assay. The nuclear run-on assay was developed to determine if transcription initiation is involved in the regulated expression of mammalian genes (Derman *et al.,* 1981; Greenberg and Ziff, 1984). The method provides a measure of the frequency of transcription initiation and is largely independent of the effects of RNA stability. Isolated nuclei from samples of cells that contain different steady-state amounts of the mRNA of interest (following cytokine or hormone treatment, induction of differentiation, etc.) are incubated with radiolabeled UTP and cold NTPs to allow elongation and labeling of the transcripts that were being synthesized when the nuclei were harvested. It is generally assumed that new transcripts are not initiated during this incubation. The labeled RNA is purified and hybridized to a membrane on which DNA from the gene of interest has been spotted. The strength of the hybridization signal is proportional to the number of nascent transcripts.

Several variations of the nuclear run-on protocol have been developed (Celano *et al.,* 1989; Nepveu *et al.,* 1987; Schibler *et al.,* 1983). A critical parameter is the quality of the isolated nuclei. Intact nuclei must be obtained free of cell membrane and cytoplasmic material, as these contaminants result in poor incorporation of labeled UTP. Isolated nuclei can be snap-frozen as Tris-buffered/40% glycerol stock aliquots at -70°C for up to 1 year. Usually, 5 to 50 $\times$ 10^6 nuclei are used per assay and it is useful to aliquot the proper number of nuclei in the volume recommended by the chosen protocol (which can vary from 20 to 500 μl depending on the protocol selected). Upon thawing, the isolated nuclei are incubated with [α-^{32}P]UTP in reaction buffer (composition varies between protocols but basic constituents include about 1 mM each of cold ATP, GTP, and CTP). The nascent transcripts are labeled by allowing elongation for a short time; here again, conditions vary, but successful run-on reactions using nuclei isolated from osteoblasts have been reported using incubation at room temperature (Elango *et al.,* 1997; Tetradis *et al.,* 1997) or 30°C (Blanquaert *et al.,* 1999). Following elongation, the DNA template is digested with RNAse-free DNAse I, the proteins extracted with phenol/chloroform, and the labeled nascent RNAs purified either by trichloroacetic acid (TCA) precipitation (Nepveu *et al.,* 1987) or modifications of the acid phenol guanidinium isothiocyanate method (Celano *et al.,* 1989; Elango *et al.,* 1997). The radiolabeled RNA is hybridized to appropriate filter strips containing a slot or dot with the cDNA of interest. Genomic DNA fragments can also be used for slot blotting. The filter strips should include a suitable positive control ("housekeeping" gene such as glyceraldehyde-3-phosphate-dehydrogenase or 18S ribosomal RNA) and an appropriate negative control (empty plasmid vector). It is preferable to blot single-stranded DNA fragments (obtained by inserting the desired probe into M13 or phagemid vectors) complementary to the mRNAs to be detected rather than using double-stranded fragments. This is because anti-sense transcription, which is surprisingly common in mammalian cells (Nepveu *et al.,* 1987), can generate non-specific signals.

When suitable probes from various regions of the gene of interest are used, the nuclear run-on assay can assess the contribution of Pol II processivity (Uptain *et al.,* 1997) to postinitiation control of gene expression. This has been described for the vitamin D-induced expression of the c-*fos* proto-oncogene in osteoblasts (Candeliere *et al.,* 1991).

RT-PCR Analysis of Nascent Transcript Levels in hnRNA as an Alternative to Nuclear Run-on Assays

An alternative to the use of the nuclear run-on assay in determining the putative contribution of transcriptional regulation in the control of gene expression has been developed. In this method, RT-PCR is used to amplify the unspliced heterogeneous nuclear RNA (hnRNA) using at least one primer derived from intronic sequences. The method is not a direct measure of RNA transcription but increases in hnRNA are thought to reflect increases in nascent transcript synthesis, although stabilization of unspliced RNA species cannot be ruled out. Various modifications of this method have been used to study gene expression in osteoblasts (Dong and Canalis, 1995; Pash *et al.,* 1995), and the conclusions have been validated using nuclear run-on assays (Franchimont *et al.,* 2000).

Promoter Analysis

An important aspect of the analysis of a gene's transcriptional regulatory mechanisms is the identification of an assay for measuring the activity of putative *cis*-acting regulatory regions. The appropriate functional assay will mimic accurately the expression pattern of the endogenous gene. Mutations can then be introduced in the control region to identify important control elements and, eventually, relevant transcription factors.

Several types of functional assays have been developed to study transcriptional regulation. These include the transient transfection assay, the stable transfection assay, the transgenic assay, or the *in vitro* transcription assay. For the transient and stable transfection assays, plasmids containing the control region of interest are introduced into cultured cells using one of several transfection procedures (see below). When a promoter region is studied, it is usually subcloned upstream of a reporter gene whose mRNA or protein level can be readily measured. When an enhancer or other control region is being analyzed, a well-characterized minimal promoter (initiator region, TATA box, +/−CCAAT box) is placed upstream of the reporter gene, and the enhancer inserter upstream of the promoter or downstream of the reporter gene. A number of useful web-based tools are available to help identify potential regulatory regions by sequence analysis (Table III); however, functional analysis is absolutely required to unambiguously assign functional DNA–protein interactions to putative elements. To lighten up the text, the examples discussed here will be restricted to functional promoter analysis.

Assay Selection

TRANSIENT TRANSFECTION

The transient assay is rapid and simple to perform. Plasmid DNA containing the promoter of interest placed upstream of a convenient reporter gene (see below) is introduced into cells in culture. At a specific time point following transfection, the activity of the promoter is assessed by measuring the mRNA or protein synthesis from the reporter gene (Alam and Cook, 1990; Kricka, 2000; Schenborn and Groskreutz, 1999). The assay is considered to be transient because the plasmids do not integrate into the host genome and thus remain episomal.

The main limitation of the transient assay concerns the artificial configuration and copy number of the transfected plasmids that may lead to aberrant activity of the promoter region under study (Smith and Hager, 1997). This is a major disadvantage when studying regulatory regions that depend on a specific chromatin structure. Since the activity of the reporter gene must be measured within a short time period ranging from 2 to 5 days, the transient transfection assay is not useful for monitoring the activity of a promoter during an extended differentiation period.

STABLE TRANSFECTION

In this assay, the plasmid containing the reporter gene under the control of the promoter of interest becomes stably incorporated into the genome of the host cell (Smith and Hager, 1997). A selectable gene marker (e.g., antibiotic resistance—neomycin, hygromycin) is also required. This can be contained on the same plasmid as the reporter gene or within a separate plasmid. The plasmid or plasmids containing the reporter and selection genes are transfected into cultured cells. The cells that have stably integrated the plasmid into a chromosome are selected by adding a drug to the culture media to kill the cells that do not express the drug-resistance gene. The activity of the promoter region under study can then be assessed in pools of stably transfected cells or in several individual cell clones.

The primary advantage of this assay is that the promoter region being analyzed is usually in a more natural chromatin configuration and at a more natural copy number than when studied by transient transfection (Smith and Hager, 1997). The main disadvantage of the stable transfection assay is that it is more difficult and time-consuming than the transient assay. The second limitation is that the transcriptional activity of the promoter under study can be strongly

Table III Useful Website Resources for Transcription Factor Biologists

Analytical need	Web-based tool	URL
Nucleic acid or protein database sequence homology search	NCBI Basic Local Alignment Search Tool (BLAST)	*http://www.ncbi.nlm.nih.gov/BLAST/*
Identification of potential DNA binding cognates in promoter sequence	Transcriptional Element Search Engine (TESS)	*http://www.cbil.upenn.edu/tess/*
Translation of nucleic acid sequence to protein in sequence	Molecular Biology Shortcuts (MBS) Translator	*http://mbshortcuts.com/translator/*
Identification of potential sites of posttranslational modification	Expert Protein Analysis System (ExPASy)	*http://www.expasy.ch/*
Protein domain identification	ISREC ProfileScan or PFAM	*http://www.isrec.isb-sib.ch/* *http://pfam.wustl.edu*
Pairwise / multiple protein sequence alignment	Protein Information Resource (PIR)	*http://pir.georgetown.edu/pirwww/*
Mass spectroscopy peptide fingerprint analysis	Protein Prospector	*http://prospector/ucsf.edu/*

influenced by the integration site. It is thus necessary to characterize multiple clones derived from each stable transfection experiment and to interpret the results obtained with caution.

Transfection Methods

Calcium Phosphate

This method has been widely used for both transient and stable transfection experiments in osteoblasts (most examples in Table I). Plasmid DNA is mixed with calcium chloride in a phosphate buffer; this leads to the formation of a DNA–calcium phosphate coprecipitate, which is deposited on the cells, and subsequently taken up by endocytosis (Keown *et al.,* 1990). Briefly, the DNA is dissolved in a 0.25 M $CaCl_2$ solution, the added dropwise with bubbling agitation to a pH 7.05 Hepes (pH is critical) and phosphate-buffered solution to form a fine calcium phosphate precipitate. The precipitate is layered onto the cells, and cultures incubated for 4–20 hr. At the end of the incubation period, the media is aspirated and the cells are subjected to a 1- 2- to min osmotic shock in 15% glycerin/Hepes phosphate-buffered saline. Typically, cellular extracts are prepared for analysis of reporter gene activity 48-72 hr after the glycerol shock.

Diethylaminoethyl (DEAE)–Dextran

The negative charges of the plasmid DNA allows it to bind to the cationic DEAE–dextran. The soluble complex is endocytosed by the cells after it has been deposited on the culture. While this protocol is widely used for transient transfection, it is rarely utilized for stable transfection experiments due to toxicity that affects long-term culture of the transfected cells. The DEAE–dextran method has been succesfully used to introduce DNA into ROS 17/2.8 or MC3T3–E1 osteoblastic cells (Boudreaux and Towler, 1996; Towler *et al.,* 1994a). Typically, the cells are shocked with 10% DMSO in phosphate-buffered saline for 1.5 min, rinsed twice with media, and then refed with complete media. Reporter gene activity can be monitored 36–72 hr after DMSO shock.

Lipofection

In this method, the negatively charged DNA binds the positive charges of cationic lipid compounds and the liposomes, associating with the cell membrane, are readily taken up by the target cells (Felgner *et al.,* 1987; Mannino and Gould-Fogerite, 1988). Usually, the DNA is diluted in serum-free medium in one half of the transfection volume (dictated by the size of the tissue culture dish used). The lipofection reagent is diluted with the other half of the transfection volume, mixed with the DNA, incubated for 10–45 min, and then layered on the cells. Transfection is usually achieved within a short time span (3–5 hr), after which the cells are refed complete media. Expression assays can be performed within 24–72 hr following transfection. For some difficult to transfect osteoblast-like cell lines, such as the MG63 human osteosarcoma line, lipofection has been the only reliable technique for efficient transfection (Cervella *et al.,* 1993; Lin *et al.,* 1993). Proprietary lipid compounds are marketed by several different companies and new compounds keep arriving on the market, providing a wide range of reagents to test for transfection of cells that have typically been hard to transfect. The main drawback of the lipofection technique is its relatively high cost relative to inexpensive methods such as calcium phosphate precipitation and DEAE–dextran.

Electroporation

In this method, the plasmid DNA and the trypsinized cells are mixed in an appropriate buffer. This mixture is then subjected to a high-voltage electrical field that induces or stabilizes pores in the plasma membrane through which the DNA enters the cell (Chu *et al.,* 1987; Neumann *et al.,* 1982). The voltage, affecting the magnitude of the pulse, and the capacitance, affecting the duration of the pulse, need to be optimized for each cell line. The procedure can be conducted at room temperature (Chu *et al.,* 1987), although most protocols call for brief chilling of the cells before and after electroshocking. Electroporation has been used with osteosarcoma cell lines (Davis *et al.,* 1994) and cultured primary bone cells (Feng *et al.,* 1995). One drawback of this method is that successful transfection is often associated with significant cell death (Neumann *et al.,* 1982).

Viral Vectors

Amphotropic retrovirus, vaccinia virus, and adenovirus factors have found a wide range of applications for introduction of genes both *in vitro* and *in vivo* (Stone *et al.,* 2000). These vectors yield very high efficiency of transfection. So far, application of this technique to bone cells in culture has largely been limited to the generation of T-antigen-immortalized osteoblastic cell lines (Heath *et al.,* 1989; Keeting *et al.,* 1992; Harris *et al.,* 1995; Hicok *et al.,* 1998; Hofbauer *et al.,* 1997) and to infection of a few established lines of osteoblastic cells (O'Brien *et al.,* 1999; Thacker *et al.,* 1994). Retroviral vector preparation involves transfection of plasmid expression constructs (containing viral packaging signals) into packaging cell lines followed by preparation of infectious, replication defective virus particles from culture supernates. The maintenance of packaging lines and the necessity to generate infectious virus for each promoter construct limits its general use. Moreover, the virus only inefficiently infects nonavian cell types; however, this latter feature has been used to permit tissue-specific transfection *in vivo* by expression of the avian leukosis virus receptor (tv-a) from a muscle-specific promoter (Federspiel *et al.,* 1994). Of note, Lichtler and coworkers have use a novel transgenic tv-a transgenic line, bAKE, to express transvect Dlx5 in murine bone marrow stromal cells and primary cultures of murine calvarial osteoblasts (Erceg *et al.,* 2000).

Recently, a novel amphotrophic retroviruses have been described by Zheng and Hughes (1999), engineered by

replacing the avian env gene with the murine leukemia virus env gene. This novel virus, RCASBP-M2C, is now able to efficiently infect mammalian cells; however, it is replication competent only in avian cells—a feature that obviates the need for specialized packaging cell lines—and does not require the generation of a transgenic mouse expressing the ALV receptor.

MICROPROJECTILE PARTICLE BOMBARDMENT (BIOLISTIC) TRANSFECTION

Micrometer-sized gold or tungsten particles traveling at high velocity (ca. 500 m/sec) can penetrate cellular membranes (Heiser, 1994). When coated with DNA, microprojectiles can act as a transfection modality. This technology has been successfully used to transfect a number of cell types, both *in vitro* (Becker and MacDougald, 1999; Heiser, 1994), organ culture (Nakayama *et al.,* 2000), and *in vivo* (Sato *et al.,* 2000). Transient and stable transfectants can be obtained. Recently, gold microprojectile particle bombardment has proven useful for introducing DNA into cells that are less efficiently transfected by DEAE–dextran or lipofection techniques (Heiser, 1994). As with viral vectors, particle bombardment holds tremendous promise for introduction of DNA into primary cultures of stromal cells and osteoblasts populations that are difficult to transfect by other modalities. The cost of the microprojectile apparatus is significant (about $15,000).

Reporter Genes

Following introduction into the osteoblasts of the chimeric DNA placing the reporter gene under the control of the promoter region of interest, measurement of the reporter gene product (Alam and Cook, 1990; Schenborn and Groskreutz, 1999) provides an indirect estimate of the induction of gene transcription directed by the regulatory sequence. The reporter gene product assayed should be absent from the transfected cells. The assay itself should be simple and rapid, with a broad linear range to allow the analysis of large or small changes in transcriptional activity. These criteria are met by the reporter genes listed below, which have all been used to study gene expression in osteoblasts.

LUCIFERASE

A commonly used reporter gene is the luciferase gene from the firefly *Photinus pyralis* (de Wet *et al.,* 1987). The luciferase enzyme oxidizes D-luciferin in the presence of ATP, oxygen, and Mg^{2+}, yielding a fluorescent product that can be detected using a luminometer—or a liquid scintillation counter with coincidence circuitry inactivated to detect all photons. Convenient luciferase assay kits are commercially available but the required solutions can also be prepared inhouse.

The transfected cells are lysed either with a nonionic detergent (e.g., Triton X-100) containing lysis buffer or by repeated cycles of freeze–thawing in a phosphate buffer. The lysed cells are microcentrifuged and the supernatant containing the cell extract is collected. A volume of cell extract ranging from 20 to 100 μl is mixed with glycylglycine-buffered luciferase assay buffer containing dithiothreitol (DTT), luciferin, and ATP. The light output is measured at room temperature in a luminometer. Signal decay is rapid with ATP as substrate; adding coenzyme A to the reaction mixture produces a more durable light signal and increases the sensitivity of the assay.

An interesting improvement of the luciferase technology was provided by vectors that utilize the gene from the sea pansy *Renilla reniformis* (Liu *et al.,* 1997), forming the basis for Promega's dual-luciferase reporter assay system. The system utilizes the *Renilla* luciferase gene as an internal control for the efficiency of transfection. Transfected cells are harvested in Promega's passive lysis buffer (a buffer optimized to provide optimum performance and stability of both *Photinus* and *Renilla* luciferase reporter enzymes) and the activity of the reporter enzymes is measured sequentially with a luminometer. Normalizing the activity of the experimental firefly reporter to the activity of the internal control *Renilla* reporter minimizes experimental variability. Additionally, modifications have been made to the *Photinus* luciferase reporter vectors that preclude peroxisomal targeting of the luciferase, thus yielding better expression levels and thus sensitivity.

CHLORAMPHENICOL ACETYL TRANSFERASE (CAT)

A second commonly used reporter gene is the *Escherichia coli* CAT gene (Gorman *et al.,* 1982). The CAT enzyme catalyzes the transfer of acyl groups from acetyl- or *n*-butyryl-coenzyme A to chloramphenicol. There is minimal background activity in eukaryotic cells since CAT is a bacterial enzyme. The most common CAT assay uses cell lysates from transfected cells incubated with ^{14}C-labeled chloramphenicol. The acetylation is monitored by autoradiography after thin-layer chromatography to separate the acetylated from the unacetylated forms of the substrate (Gorman *et al.,* 1982). The assay can be quantified either by using a phosphorimager, by excising the radioactive spots and counting in a scintillation counter, or by analyzing the autoradiograph by densitometry. An alternative assay has been developed using [^{3}H]acetyl-coenzyme A and differential extraction of chloramphenicol from the acetylated derivatives, eliminating the need for chromatography (Seed and Sheen, 1988). Two nonisotopic detection methods for quantifying CAT activity have been developed. A CAT enzyme-linked immunosorbent assay (ELISA) is commercially available and a fluorescent derivative of chloramphenicol can be used (Alam and Cook, 1990; Schenborn and Groskreutz, 1999). Laboratories studying gene expression in osteoblasts have made extensive use of the CAT reporter gene. Thin-layer chromatography has been commonly used as well as alternative detection methods. The CAT reporter has also been used to assess promoter activity in bone from transgenic animals (Dodig *et al.,* 1996; Harrison *et al.,* 1998; Kesterson *et al.,* 1993).

β-GALACTOSIDASE/LACZ

The *lacZ* gene from *E. coli* is a versatile genetic reporter since both *in vivo* and *in vitro* assays are available to detect its gene product, the enzyme β-galactosidase. *In vivo* levels in fixed transfected cells or tissue sections from transgenic animals can be measured using the X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactoside) or Bluo-GAL (halogenated indolyl-β-galactoside) substrates. These are often used in transgenic assays to compare the pattern of expression of the reporter under the control of the reporter of interest to the endogenous expression pattern (Rossert *et al.,* 1996; Tsumaki *et al.,* 1996). The development of chemiluminescent 1,2-dioxetane substrates for β-galactosidase has greatly improved the utility of *lacZ* as a transcriptional reporter by increasing the sensitivity and extending the linear range of the assay (Bronstein *et al.,* 1996). Activity of the test *lacZ* reporter gene can be measured using the chemiluminescent Galacton substrate following the instructions provided with the Galacto-Light assay kit (Tropix). A light-emission accelerator (Emerald luminescence amplifier), supplied with the kit, is added and photon emission measured with a luminometer.

Recently, Tsien and coworkers described the development of a novel cell-permeant fluorescent lactamase substrate, CCF2, that undergoes intramolecular fluorescence resonance energy transfer (FRET) prior to cleavage by lactamase (Zlokarnik *et al.,* 1998). Following cleavage, intramolecular FRET is relieved, and the higher energy photons emitted following excitation are quantified as an index of promoter activity — assayable in real time in living cells. Since FRET-dependent lower energy photons released by excited uncleaved CCF2 substrate are in large excess, ratioing of the fluorescence signal arising from cleaved substrate to uncleaved substrate generates a normalized value that corrects for cellular uptake and cell number — valuable for high throughput screening analyses necessary for drug discovery efforts that seek to manipulate transcription factor signaling cascades. This technology has been applied to studies of *in vivo* gene expression in living teleost embryos (Raz *et al.,* 1998).

GROWTH HORMONE (GH)

Because the endogenous expression of growth hormone is restricted to the somatotropic cells of the anterior pituitary gland, the growth hormone protein was seen as an attractive choice as a genetic reporter for all other types of mammalian cells. Convenient vectors using human growth hormone (hGH) as reporter have been engineered (Selden *et al.,* 1986). Growth hormone is secreted into the culture medium from the transfected cells; this precludes the preparation of cell lysates. Additional advantages include the possibility of studying kinetics of gene expression by repeated sampling of medium from the same cultures, and low background levels. However, the hGH needs to be detected using a radioimmunoassay that has a relatively low sensitivity and a narrow linear range. The hGH reporter has been used to study the vitamin D-induced transcription of the osteocalcin gene in osteoblasts (Terpening *et al.,* 1991).

SECRETED ALKALINE PHOSPHATASE (SEAP)

A truncated form of the human placental alkaline phosphatase gene lacking the membrane-anchoring domain has served to engineer a series of vectors that can be used to express secreted alkaline phosphatase (SEAP) (Berger *et al.,* 1988). Levels of SEAP activity detected in culture medium parallel changes in SEAP reporter gene transcription. The SEAP reporter gene assays have the same advantages as hGH but sensitivity and linear range are significantly increased when chemiluminescent alkaline phosphatase substrates are used (Bronstein *et al.,* 1996). Because both SEAP and hGH are secreted and measured in culture medium, constructs using one of these reporters can be used as internal control to normalize transfection efficiency when the other gene is used as experimental reporter.

GREEN FLUORESCENT PROTEIN (GFP)

The 28-kDa green fluorescent protein from the jellyfish *Aequorea victoria* represents a recent addition to the selection of reporter genes (Chalfie *et al.,* 1994). GFP expressed in eukaryotic cells emits a bright green fluorescence when the cells are excited by UV light; conditions used for visualizing fluorescein are also suitable for GFP. The major utilization of GFP-based vectors has concerned the subcellular localization of fusion proteins; however, the ability to monitor GFP in real time has led to its use in the study of transcriptional changes (Chalfie *et al.,* 1994) and ligand-dependent protein–protein interactions directed by nuclear receptors (Day *et al.,* 1999; Schaufele *et al.,* 2000). Investigators in the bone field have used GFP to monitor gene expression in osteoclasts from transgenic mice (Luchin *et al.,* 2000).

Assays of DNA–Protein Interactions *in Vitro* and *in Vivo*

Transcription regulation is either directly or indirectly dependent upon sequence-specific DNA–protein interactions assembling on promoter elements. These DNA–protein contacts can be readily detected and easily assayed gel shift assay and DNAseI footprinting. However, although highly predictive when coupled with functional mapping studies using transfected promoter–reporter constructs, such biochemical studies to not unambiguously establish the nature of regulation in the physiological context of cellular chromatin. The application of ligation-mediated PCR to chemical or photo footprinting of DNA and ChIP assays directly assess protein–DNA contacts and complex identity at specific transcriptional regulatory elements *in situ.* This section is meant only to provide a didactic overview; the reader is referred elsewhere for a more all-inclusive treatise of DNA–protein interaction assays.

PREPARATION OF NUCLEAR EXTRACTS

To assess sequence specific DNA–protein interactions by gel shift or by footprinting analysis, extracts of intact nuclear protein must be prepared. The most widely used and reproducible protocol for preparation of nuclear extracts is that described by Dignam *et al.* (1983). Confluent monolayers of cells from 2 to 20 150-mm diameter dishes are harvested by scraping into isotonic buffered saline. Dounce homogenization of cells in hypotonic buffer and collection of nuclei by centrifugation is followed by 0.45 M KCl salt extraction of nuclear proteins off of chromatin. These extracts are then dialyzed against a Hepes-buffered 0.1 M KCl/25% glycerol solution for storage and gel shift analysis. This protocol precludes disruption of the nuclear membrane and subsequent release of nuclear DNA, which hinders detection of protein interactions with radiolabeled probes. Alternatively, whole cell extracts for gel shift can be made by lysis of cell pellets with nonionic detergents in high-ionic-strength extraction buffers. This permits reduction in the total number of cells required for gel shift analysis (e.g., from 35-mm-diameter dishes). Such modified gel shift protocols have been used to characterize the time course of FGF2-regulated DNA binding activities in MC3T3E1 cells (Newberry *et al.,* 1997c). However, for DNAse I footprinting studies or for detection of low-abundance DNA binding activities, nuclear extracts prepared by the standard protocol (Dignam *et al.,* 1983) followed by 55% saturation ammonium sulfate precipation/concentration and dialysis provide the best results (Towler and Rodan, 1995). Note, however, that modifications of standard nuclear extraction techniques are required to efficiently extract some proteins tightly associated with the nuclear matrix, which contains a number of sequence-specific DNA binding activities (Bidwell *et al.,* 1993; Newberry *et al.,* 1999; Thunyakitpisal *et al.,* 2001).

ELECTROPHORETIC MOBILITY GEL SHIFT ASSAYS (EMSA)

The gel shift assay is very sensitive technique for detecting sequence specific DNA–protein interactions (Ceglarek and Revzin, 1989; Fried, 1989; Revzin *et al.,* 1986). A ^{32}P-radiolabeled duplex oligonucleotide (typically 20,000 cpm/0.1 pmol per 20 μl reaction) containing the potential binding site for a transcription factor is mixed with nuclear extracts (5–50 μg of protein) prepared from the appropriate cellular background. Oligonucleotide bound by nuclear factors (migrates slowly) is separated from unbound/free oligonucleotide (migrates rapidly) by nondenaturing polyacrylamide gel electrophoresis and visualized by autoradiography. Several parameters can alter the binding of transcriptional regulators to protein (Fried, 1989). The two most common modifying factors are lowering ionic strength and decreasing temperature of electrophoretic separation to 4°C; these favor detection of DNA–protein interactions. This assay can be used to quantify the affinity of recombinant DNA binding proteins for altered DNA binding sites to developing consensus sequences for recognition. Moreover, the effects of coregulatory molecules on DNA–protein interaction are also readily assayed by this technique (Bidder *et al.,* 2000; Carlberg *et al.,* 1993; Newberry *et al.,* 1998).

Not infrequently, because of the information dense character of promoter elements, multiple DNA–protein interactions are observed. Specificity of the DNA–protein interactions is demonstrated by examining the capacity of unlabeled unrelated or mutated duplex oligonucleotides to compete for factor binding to the radiolabeled oligonucleotide. The inclusion of polymeric duplex poly deoxyinosine-deoxycytosine or sheared calf thymus DNA (0.2–1.5 μg per reaction) decreases nonspecific background DNA–protein interactions. Comparisons between transcriptionally active and nonactive cellular backgrounds can reveal functionally important, cell-type-specific DNA–protein interactions, as have been described for the rat and mouse osteocalcin promoters (Ducy and Karsenty, 1995; Towler *et al.,* 1994a). Because of the sensitivity of the gel shift assay, it is important to functionally assess the importance of any specific DNA–protein interaction by fine 5′-deletion analysis or point mutations, which selectively disrupt factor binding.

DNASEI FOOTPRINTING ASSAYS

DNAseI is a ca. 40-Å-diameter protein possessing nuclease activity that cleaves both strands of the 20-Å duplex B DNA with minimal sequence specificity. Stoichiometric occupancy of a DNA cognate by a transcription factor participating in sequence-specific DNA–protein interactions typically encompasses a 10- to 15-bp (ca. 34- to 51-Å) linear stretch of DNA, and thus sterically inhibits DNA cleavage by DNAseI. When an end-labeled duplex DNA template is factor-bound and digested with DNAseI, the radiolabled DNA fragments electophoretically resolved, and the subsequent autoradiogram compared to one arising from the naked DNA template similarly digested, regions of decreased DNAseI cleavage ("footprints") reflect sites of specific DNA–protein interactions. Segments as large as several hundred base pairs can be assessed for multiple simultaneous protein–DNA interactions that demarcate multiple promoter regulatory elements. Moreover, weak DNA–protein interactions poorly visualized and quantified by EMSA can be readily characterized by DNaseI footprinting. The technique is well established as previously detailed (Augereau and Chambon, 1986), and has been applied to analysis of either expressed recombinant proteins or crude biological admixtures. In brief, concentrated nuclear extracts are prepared as described above and then mixed (25–100 μg protein) in a typical binding reaction (see above) with ^{32}P end-labeled radioactive probe of ~0.3–0.5 kb length (20,000 cpm in $<$0.01 pmol of DNA fragment). Increasing amounts of DNAse I are then added to partially digest the probe and titrated to cleave ~50% of the intact probe, which gives rise to approximately one nick per molecule. Similarly, nuclear extract is added at increasing concentrations to titrate to saturation the binding sites on the probe. A control lane is run in which DNAse I is added to probe in the absence of nuclear extract. After termination of DNAse I digestion, cleaved DNA is subsequently analyzed by denaturing urea

polyacrylamide gel electrophoresis and autoradiography. Regions of the probe involved in sequence-specific DNA–protein interactions are sterically protected from DNAseI digestion indicated upon comparison with the control, unprotected probe. In general, the best footprints are obtained when the DNA–protein interaction is within 50–150 bp of the radiolabeled end of the footprinted fragment. Since some DNA–protein interactions preferentially occur along one strand of duplex DNA, separate footprinting reactions should be carried out using duplex DNA labeled on "sense" and "antisense" strands (Augereau and Chambon, 1986).

In Vivo Footprinting by Ligation Mediated PCR (LM-PCR)

Although EMSA and DNAseI footprinting identify, characterize, and quantify changes in DNA binding protein complexes that regulate transcription, these techniques do not directly assess occupancy of transcription factor binding sites in the native context of the cell nucleus. *In vivo* sequence-specific DNA–protein interactions can be identified in cultured cells by treatment with conditions that predispose DNA to "nicks" that are modified by bound proteins. Dimethylsulfate (DMS) methylates the N7 of guanine bases in the major groove of DNA when not sterically occupied by bound transcription factors; subsequent treatment of extracted DNA with piperidine (cleaves DNA at N7–methyl guanine nucleotides). Similarly, ultraviolet (UV) light induces pyrimidine dimer formation and DNA cleavage, altered by bound proteins that orient adjacent pyrimidine residues that either enhance or limit the quantum yield energy absorbed. Mueller and Wold initiated the widespread use of ligation-mediated PCR to identify with base-specific resolution the DNA–protein interactions sterically footprinted by either DMS or UV treatment (Mueller *et al.,* 1988; Mueller and Wold, 1989). The technique has been well-reviewed. Briefly (for DMS footprinting), a confluent cell culture (150-mm-diameter tissue culture dish) is typically treated for 2 min with 0.1% DMS freshly prepared in phosphate-buffered physiological saline solution. After rinsing thrice with phosphate-buffered saline (PBS), cells are lysed in a Tris-buffered saline/EDTA/sodium dodecyl sulfate (SDS)/proteinase K solution, genomic DNA is isolated by phenol extraction, spooling, and precipitation and finally cleaved as N7-methylated G residues by treatment with 1 M piperidine for 0.5 hr at 90°C. Following removal of the piperidine, the cleaved DNA is utilized as a template for primer extension with a gene-specific primer. The resulting termini—blunt end DNA fragments representing the sites of DMS + piperidine cleavage—are blunt-end ligated to an asymmetric (5′-overhang one end, blunt-end other end) synthetic adapter. Subsequent PCR using an adapter primer with a nested gene-specific primer amplifies the DNA fragments, and tertiary analysis with a radiolabeled, third-nested gene-specific primer results in generation of the DNA ladder that reveals the footprint following autoradiography of electrophoretically resolved fragments. Hererra and coworkers have used this methodology to demonstrate that at the c-*fos* serum response element, the serum-responsive promoter element is constitutively occupied by serum-inducible DNA binding activities of the Ets factor p62TCF and the MADS factor SRF (Herrera *et al.,* 1989). Similarly, while noting delayed (24-hr) increased occupancy of the bipartite Ets-AP1 element of the heparin-binding epidermal growth factor gene in response to raf-1 activation, McCarthy *et al.* (1997) observed kinetically more rapid (2-hr) increases in promoter accessibility at sites adjacent to the Ets and AP1 cognates subsequently occupied. These latter observations add to the accumulating data set that indicates that rapid activation and/or disruption of preformed protein–DNA complexes extant *in situ*—in addition to subsequent upregulation of novel DNA binding activities—contribute to many early transactivation events elicited by growth factor stimuli. Of note, similar footprinting technologies using $KMnO_4$ footprinting (10 mM in PBS for 2 min; modifies unpaired thymine bases, enhances subsequent cleavage by piperidine) have been successfully applied to identify unmelted regions of ssDNA stabilized by ssDNA binding proteins during transcriptional regulation of the vascular smooth muscle actin gene in fibroblasts (Becker *et al.,* 2000); the reader is referred to this excellent example of the application of this conceptually similar *in vivo* footprinting technique. To our knowledge, *in vivo* DMS, UV, or $KMnO_4$ LM-PCR footprinting techniques have not been applied to analysis of DNA–protein interactions in osteoblasts.

Chromatin Immunoprecipitation Assays (ChIP Assays)

A major challenge for the molecular biologist is to unambiguously identify and characterize the macromolecular complexes that regulate transcription in the native nuclear chromatin context. Recently, ChIP assays have become routinely applied to answer such questions (Fig. 1)—particularly in response to hormonal cues (Nissen and Yamamoto, 2000; Shang *et al.,* 2000). Briefly, cultured cells are rinsed, and then treated for 10 min at 37°C with 1% formaldehyde in phosphate-buffered saline to cross-link chromatin-bound macromolecular transcription factor complexes to DNA. Subsequently, the cross-linking reaction is quenched with ethanolamine and cells disrupted and DNA sheared to ca. 300–3000 nucleotide-length fragments by probe sonication. After adjustment of the sonicated cell lysate to the appropriate immunoprecipation conditions (typically 0.3 M NaCl in 10 mM Tris, pH 7.5, 1% Triton X-100, 0.2 mM EDTA, 1 mM DTT with protease inhibitors), specific endogenous DNA binding proteins identified as potential regulators in assays lacking the chromatin context (e.g., transient cotransfection, gel shift) are immunoprecipated. The immunoprecipitated DNA–protein chromatin complexes are washed, heated for 6 to 18 hr at 65°C to reverse the formaldehyde cross-links, extracted with phenol:chloroform to remove protein, and the remaining DNA ethanol precipitated. Using gene-specific primers directed toward a specific regulatory region of interest, the polymerase chain reaction is applied to provide a semiquantitative index of the amount of the

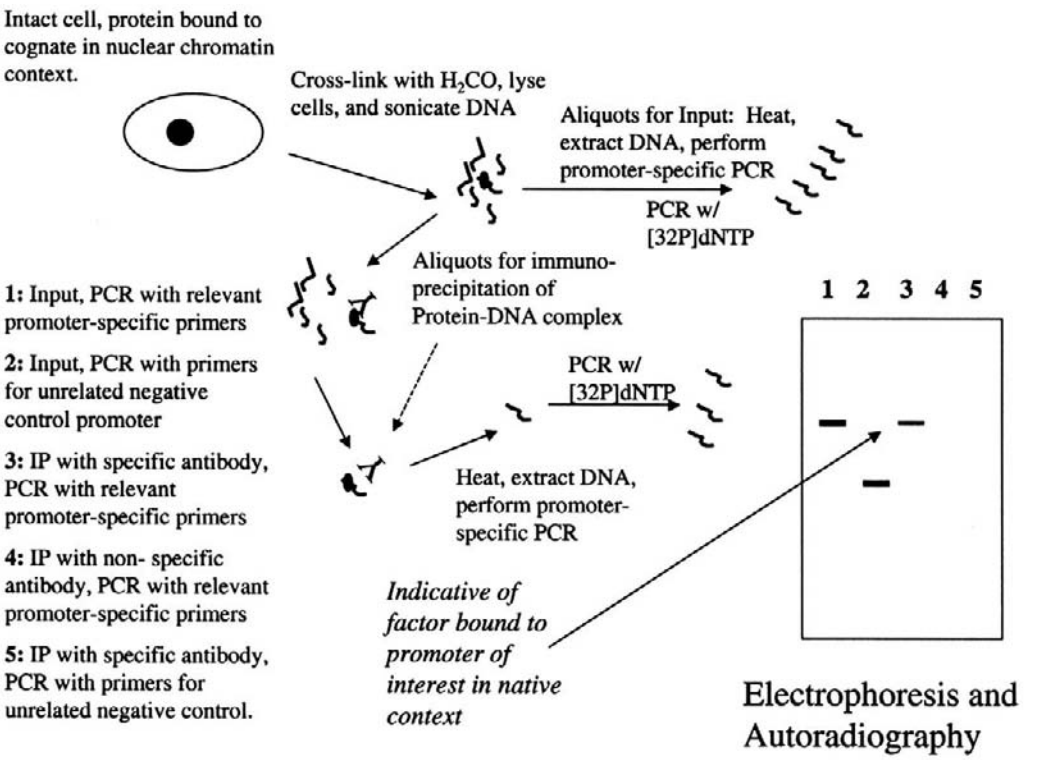

Figure 1 The chromatin immunoprecipitation (ChIP) Assay.

protein–DNA complex assembled. At a minimum, comparisons are made (1) between PCR results obtained using complexes immunoprecipitated from treated and untreated cell cultures; (2) with PCR results obtained from aliquots of extracts processed in parallel without precipitation to control for DNA input; and (3) with PCR results obtained after precipitation with a negative control, i.e., a transcription factor that does not recognize or regulate the promoter of interest (Fig. 1). In one application of this technique, multiple transcription factors are queried by immunoprecipitations performed in parallel to profile the complexes assembled after a stimulus (Shang *et al.,* 2000). For robustly activated promoters, chromatin immunoprecipitation can be performed sequentially with antibodies of differing specificity for establishing these profiles.

As an example of one of the best studies published to date, the protein complexes assembled by the pS2 promoter in MCF7 breast cancer cells were profiled in response to estrogen receptor ligands. In this detailed study by Lazar, Brown, and coworkers (Shang *et al.,* 2000), the estradiol-dependent recruitment of ER-α and a cadre of transcriptional coactivators and corepressors to the pS2 promoter was characterized. In the absence of ligand, the ER-α complex was not associated with the pS2 promoter. In the presence of the agonist estradiol, a sequential, time-dependent recruitment of coactivator complexes was elicited. Impressively, a two-phase transcriptional activation cycle was observed. The first phase waxed and waned within 1 hr, with both p300 and CBP platform protein HAT activities recruited subsequent to the appearance of the p160 coregulators. Of note, however, in the second phase, p300 was excluded from the complex while CBP was again recruited. Most importantly, in the presence of the SERM tamoxifen—an ER-α AF2 antagonist—corepressor complexes consisting of NCoR, SMRT, and HDACs (histone deacetylases) were recruited to the pS2 promoter. Thus, by application of the ChIP technology, this study unambiguously established for the first time that the anti-estrogen tamoxifen—rather that only passively inhibiting promoter activation by sterically preventing agonist binding to ER-α—additionally promotes the assembly of an active repression complex on estrogen-activated genes such as pS2.

Cloning of Transcriptional Regulatory Molecules

Several strategies have been designed to permit the cloning of genes encoding transcriptional regulatory factors. Each method has distinct advantages and drawbacks. This section presents an overview of each technique and reviews the pros and cons of each method.

Screening of an Expression Library with a Binding Site DNA Probe or Southwestern Blot

This strategy is based on the recognition of binding sequences by fusion proteins encoded by recombinant phages from a cDNA library constructed in expression vectors such

as λgt-11. Nitrocellulose lifts of the plated phages are blocked with protein, and then incubated with the binding site DNA probe in the presence of an excess of nonspecific competitor DNA. Washed filters are then exposed to film and further rounds of plaque purification enrich potentially positive clones. The method was simultaenously developed in 1988 in the laboratories of Phil Sharp and Steve McKnight (Singh *et al.,* 1988; Vinson *et al.,* 1988) and represented an important advance in gene cloning technology.

This approach has proved successful in the cloning of a number of diverse transcription factors. Its success relies on the ability of the transcription factor of interest to still be able to bind its recognition site as part of a fusion protein with a bacterial gene. Of course, the genes encoding transcription factors that bind DNA as heterodimers cannot be cloned by this strategy. Moreover, the factor must not require any post-translational modifications, such as tyrosine phosphorylation, in order to be able to bind DNA. Finally, the factor must be capable of binding its target site while immobilized on a nitrocellulose support. Biochemical assays, such as the so-called "Southwestern" assay (Miskimins *et al.,* 1985) can be used to test some of the specific requirements before attempting to use this method.

The expression library screening method presents an additional advantage. The fusion proteins can be crudely purified from lysogenized bacterial cells infected by the positive recombinant phages. This enables the easy characterization of the functional binding activity of the fusion protein and permits to ascertain that the recombinant clone really encodes the factor of interest. While it has been used successfully to clone several transcription factors, we are not aware of any bone transcriptional regulatory molecules that have been cloned using this "Southwestern" expression cloning strategy.

Farwestern Blot or Interaction Cloning Strategies

Recently, a similar expression cloning strategy has been used to clone MINT, novel nuclear matrix protein expressed in bone, brain, and testis that binds the osteoblast homeoproteins Msx2 and Dlx5 (Newberry *et al.,* 1999). The application of the Farwestern blot to transcription factor cloning was initially popularized by Blanar and Rutter (1992) to identify Fos interacting proteins. This strategy relies upon the identification of the interactions between a radiolabeled probe protein and phage-expressed fusion proteins — interactions stabilized by the summed energies of hydrophobic interactions, ion pairing, and hydrogen bond formation. The Towler laboratory utilized a ^{32}P-radiolabeled recombinant GST–Msx2 fusion protein to screen a λgt11 library to identify cDNAs encoding novel Msx2-interacting proteins (Newberry *et al.,* 1999). In this study, clonal phages plaque proteins were transferred to nitrocellulose membranes, denatured in buffered 6 M urea, and renatured in Hepes — buffered per the Blanar and Rutter protocol (Blanar and Rutter, 1992), and probed with [^{32}P]GST-Msx2[55–208] encoding the minimal Msx2 repressor domain. Well-characterized, random hexamer-primed λgt11 library from embryonic brain was utilized for the initial cloning stategy since no adequate calvarial osteoblast libraries existed. Following isolation of 20 clones at the level of secondary screens, a variation was incorporated to identify true positives from false positive clones; in proper orientation, subcloned inserts that initially had been expressed as β-galactosidase fusion protein from lamdba phage were now expressed from the pET23a T7 promoter as an N-terminally tagged T7 polymerase fusion protein. After expression and induction in BL21(DE3) *E. coli,* T7–fusion proteins from all unique subclones were immunoisolated with anti-T7 agarose resin, immunopurified fusion proteins eluted and resolved by SDS–PAGE, and electrotransferred to nitrocellulose for subsequent evaluation by Farwestern blot with [^{32}P]GST-Msx2[55–208]. The addition of this intermediate step permitted relatively rapid yet unambiguous identification of clones that were true positives — i.e., encoded protein domains that interacted with Msx2 — among the 20 or so clones isolated in primary and secondary screens. Standard library and PCR-based cloning techniques were then applied to obtain the full-length cDNA sequence (Newberry *et al.,* 1999). Given the role of Fra1 in augmentation of bone mass (Jochum *et al.,* 2000), and the original utilization of this Farwestern strategy to identify Fos interacting proteins (Blanar and Rutter, 1992), studies applying similar Farwestern strategies to identify Fra1 interacting proteins in the osteoblast promise to be very enlightening.

Purification and Sequencing of Muliprotein Transcription Factor Complexes

Tissue-specific gene expression is regulated by multiprotein complexes — a combination of sequence-specific DNA-binding transcriptional activators, general or basal transcription initiation factors, and associated cofactors. While the general initiation factors are sufficient for basal-level transcription, enhancement of transcription by activator proteins bound to DNA requires the presence of additional mediator proteins, known as transcriptional coactivators (Kingston and Narlikar, 1999; McKenna *et al.,* 1999). Although difficult, biochemical approaches permit the identification and characterization of both DNA binding constituents and the collaborative coregulators that functionally define the transcription factor complex. To identify complexes necessary for vitamin D receptor (VDR)-dependent transactivation, the Freedman laboratory applied an affinity column approach, immobilizing the ligand-binding domain (LBD) of the VDR (Rachez *et al.,* 1998, 1999, 2000). Since the LBD of nuclear receptors contains a transcriptional activation domain that interacts with auxiliary proteins that are required for activated transcription, this generated a VDR-specific cofactor affinity resin. When nuclear extracts from cells that support $1,25(OH)_2D_3$-enhanced transactivation were passed on the ligand-bound affinity resin, 13 proteins of molecular weights ranging from

33 to 250 kDa were selectively retained in the column (Rachez *et al.,* 1998, 1999, 2000). Sequences were obtained from proteolytic peptides using Edman degradation or mass spectrometry, and cDNAs identified either from the translated database or cloning using classical molecular biology techniques. This group went on to identify and clone all components of this DRIP (vitamin D receptor interacting proteins) complex using a combination of peptide sequencing/RT-PCR and antibody generation (Rachez *et al.,* 1998, 1999, 2000). Interestingly, the DRIP complex is almost indistinguishable from the ARC (activator-recruited cofactor) complex, which is recruited by other types of transcription activators to mediate transactivation on chromatin-assembled templates (Naar *et al.,* 1999). This remarkable finding indicates that ARC/DRIP is a multisubunit composite cofactor important for chromatin-dependent transcriptional stimulation by a variety of activators and suggests that selectivity of transcriptional control may reside in subtle changes in the composition of these large, multiprotein complexes.

Classical protein purification techniques have only recently been applied to the characterization of osteoblast transcription factor complexes; a novel heterotrimeric nuclear complex has been purified that participates in the regulation of the FGF response of the osteoblast-specific osteocalcin gene (Willis *et al.,* 2000). In immature osteoblasts, signals elaborated by FGF receptor-1 and -2 activation synergize with A-kinase-dependent pathways to upregulate OC gene expression via a bipartite motif, dependent upon DNA–protein interactions at −144 to −138 (GCAGTCA motif; osteocalcin FGF response element OCFRE) in the rat OC promoter (Boudreaux and Towler, 1996). The OCFRE binding complex is estimated to be ca. 150–200 kDa in size based upon size-exclusion chromatography. Using DNA binding to the GCAGTCA in EMSA to follow activity and classical protein purification techniques, 4 μg of the protein complex binding to the OCFRE was purified to homogeneity from MG63 human osteosarcoma cells (Newberry *et al.,* 1997a). Three protein constituents of 100, 80, and 70 kDa were identified and denoted OCFREB100, OCFREB80, and OCFREB70. The 80- and 70-kDa subunits were identified by LC-MS/MS sequencing of tryptic peptides as Ku70 and Ku80, DNA-binding proteins previously known to regulate DNA repair, transcription, and immunoglobulin gene rearrangement (Willis *et al.,* 2000). FGF regulates the phosphorylation of Ku70 in calvarial osteoblasts in a stage-specific manner. Importantly, skeletal growth and ossification defects are noted in both Ku80 (aka Ku86) (Vogel *et al.,* 1999) and Ku70 (Willis *et al.,* 2000) knockout mice; thus, biochemical and genetic analyses have converged upon Ku70 and K80 as nuclear factors mediating responses to growth factor-regulated signaling cascades that control skeletal growth, tissue-specific gene expression, and orthotopic mineralization. The final subunit, OCFREB100, was a completely novel protein (Willis *et al.,* 2000). Six peptide sequences were unambiguously obtained by LC-MS/MS sequencing (see Fig. 2 and Section 7.0 for overview of technique). After querying the translated EST database, two of the peptides were immediately identified and the EST sequences used in iterative BLAST screening of the database to assemble a novel contig of ca. 1 kb, encoding a contiguous open reading frame (ORF), with two peptides and a perfect nuclear localization signal motif. RT-PCR analysis of cDNA confirmed the contiguous protein ORF. By 5′- and 3′- RACE, the remainder of the coding region was obtained, sequenced, and a total of 23 tryptic peptide sequence tags identified, establishing the contiguity of the peptides sequenced in a single protein (Willis *et al.,* 2000). OCFREB100 is distantly related to a yeast *N*-acetyltransferase that is required for cell growth (Park and Szostak, 1992). Importantly, coexpression of OCFREB100 augments FGF-dependent transactivation of the OC promoter in osteoblasts. The identification of a NAT-related protein as constituent of this MG63 osteoblast transcriptional complex introduces the notion that, like HATs, NATs may play an important role in gene regulation. The powerful combination of classical protein biochemistry, mass spectrometry, and bioinformatics promises to dramatically accelerate the functional characterization of macromolecular transcriptional complexes in osteoblasts.

Cloning of Related Family Members

In the course of the characterization of the promoter region of the gene of interest, critical regulatory elements may be identified that should bind a previously identified transcription factor. However, expression patterns or immunological data reveal that this previously identified factor cannot be responsible for the expression of the target gene in osteoblasts. Such results would be consistent with the possibility that another member of the same protein family is the functionally relevant activator. Therefore, this related family member should now be cloned. Database mining may provide a useful starting point. A second approach is based on the observation that families of transcription factors contain conserved domains, such as the DNA binding domain (DBD). The DBD from a given factor can be used directly as a probe to screen a cDNA library from the tissue of interest using reduced stringency conditions. This approach was used to clone Osf2/Cbfa1, an osteoblast-specific transcription factor that is a member of the *runt*-domain family (Ducy *et al.,* 1997). Alternatively, degenerate PCR primers that account for the redundancy of the genetic code within the DBD can be used to identify and clone related family members. This strategy was successfully used to clone members of the helix–loop–helix transcription factor family (Tamura and Noda, 1999) from osteoblasts, *Krüppel* family members from chondrocytes (Dreyer *et al.,* 1999), and Ets family members from osteoblasts (Bidder *et al.,* 2000) and chondrocytes (Iwamoto *et al.,* 2000). The novel family members should be tested using the functional methods described

above, in addition to assessing their expression pattern, in order to determine their functional relevance in the control of the expression of the gene under study.

Characterization of Cloned or Purified Transcription Factors

Optimal Cognate Identification by Binding Site Selection

Determining the DNA-binding specificity of a transcription factor is a vital step in the characterization of its interaction with DNA and the identification of its regulatory targets. The advent of the polymerase chain reaction has led to the development of a number of techniques that can help in the identification of high affinity binding sites for DNA-binding molecules. Site selection techniques can be used to determine the consensus binding sites for proteins whose target sequences are unknown. It can also provide additional information about the protein–DNA interactions of previously characterized factors and help to identify all of the target sequences for a given DNA-binding domain. The methods can also be used for structure–function analysis, in which mutated DNA-binding domains are used to examine the contribution of individual amino acids or motifs to binding site recognition.

In general, all of the available methods include four similar steps: a DNA–protein incubation step, a protein–DNA complex separation step, a DNA elution step and a PCR DNA amplification step. The variation resides in the protein–DNA complex separation step, in which some methods use electrophoretic mobility shift assays (EMSA) (Blackwell and Weintraub, 1990), immunoprecipitation using a specific antibody (CASTing) (Wright *et al.,* 1991) or an antibody directed against an epitope tag (Pollock and Treisman, 1990), GST pull down (Chittenden *et al.,* 1991; Pierrou *et al.,* 1995), affinity columns (Ekker *et al.,* 1991), filter binding (Thiesen and Bach, 1990), or immobilization on nitrocellulose membranes (Norby *et al.,* 1992). Methods based on immunoprecipitation provide the additional advantage of using crude nuclear extracts instead of purified recombinant proteins in the site selection assay. Once selected, individual binding sites are cloned into plasmids and examined by sequencing.

A pool of random-sequence oligonucleotides (10 to 25 nucleotides in length), flanked by two specific PCR primers sequences, is used as the source of potential binding sites. The oligonucleotide pool is incubated in a binding reaction with the recombinant transcription factor of interest or with an extract containing the DNA-binding protein of interest. If optimal binding conditions for the factor of interest are already known, then these should be used; alternatively, general binding conditions of moderate ionic strength can be used as a starting point.

In the selection and amplification of DNA binding site (SAAB) method (Blackwell and Weintraub, 1990), the protein–DNA complexes are separated from the pool of unbound oligonucleotides using EMSA. The bound templates are isolated by excision of the gel slice directly from the dried gels, including the paper backing. Gel slices are then incubated at 37°C for 3 hr in 0.5 ml of 0.5 M ammonium acetate, 10 mM magnesium chloride, 1 mM EDTA, and 0.1% SDS. About half of the radioactivity present in the gel slice can be recovered by this procedure. After addition of tRNA or glycogen carrier, the eluate is extracted with phenol and chloroform and then precipitated with ethanol. The precipitated oligonucleotides are resuspended and subjected to 15–35 cycles of PCR. These selected and amplified binding sites can then be subjected to further rounds of EMSA. The method has been used to identify specific DNA binding sites for several transcriptional regulatory molecules controlling gene expression in bone cells: the CBF heterotrimer controlling collagen type I transcription (Bi *et al.,* 1997), the Smad3 and Smad4 proteins required for TGF-β signaling (Zawel *et al.,* 1998), and the α-NAC transcriptional coactivator (Yotov and St. Arnaud, 1996).

In the cyclic amplification and selection of targets (CASTing) method (Wright *et al.,* 1991), DNA–protein complexes are immunoprecipitated with a specific antibody. A protein A–Sepharose slurry makes it possible to separate the bound complexes from the excess of unselected binding sites. The bound DNA is eluted from the protein A–Sepharose beads pellet using recovery buffer (50 mM Tris–Cl, pH 8, 100 mM sodium acetate, 5 mM EDTA, 0.5% SDS) and incubation at 45°C. The DNA is extracted with phenol/chloroform, precipitated, and reamplified using PCR. If an antiserum specific for the DNA-binding protein of interest is not available, the factor can be tagged with an epitope and antibodies directed against the epitope tag can be used in the procedure (Pollock and Treisman, 1990). The antibody used should not disrupt DNA–protein complexes and therefore should not be directed toward the DNA-binding domain of the factor of interest. When available, antibodies that "supershift" in EMSA should be selected.

The immunoprecipitation-based method allows for a high degree of enrichment and provides the additional advantage to be able to isolate DNA–protein complexes from crude mixtures. The ability to use crude extracts offers several advantages: first, there is no requirement for purified or overexpressed target protein. Second, the method can be used with proteins that require posttranslational modifications in order to bind DNA. Third, binding sites can be selected for proteins that bind DNA only as components of heteromeric complexes. The method has been used successfully to identify the consensus binding sites for the muscle regulatory factor myogenin, and to identify potential interactions between myogenin and other members of the helix–loop–helix regulatory family (Funk, 1992). In applications more related to the bone field, the CASTing protocol made it possible to define DNA recognition elements for the vitamin D receptor (Perez-Fernandez *et al.,* 1993) and to identify novel consensus DNA binding sites for AP-2, a transcription factor

essential for cranial closure and craniofacial development (Gee *et al.,* 1998).

Characterization of Transcription Factor Complexes by Mass Spectrometry

The molecular biology revolution permitted application of low-stringency hybridization, expression cloning (Southwestern), and degenerative RT-PCR to the identification of novel transcription factors expressed in any tissue and is responsible for the massive explosion of transcriptional regulators discovered in the past 15 years. However, the DNA binding factors represent only one salient component of the macromolecular complexes that mediate tissue-specific gene expression, and important coregulatory transactivators that may or may not contact DNA are routinely missed, identified by subsequent two hybrid or "Farwestern" cloning approaches that reflect protein–protein interactions. Recently, concomitant improvements in mass spectrometry technology (sensitivity in the 10- to 100-fmol range) and the explosion of mammalian sequence information available (ESTs and completion of sequencing the human genome) have converged to restore biochemical characterization to the forefront of transcription factor research (Yates, 1998). This section will serve to briefly orient the reader to this emerging technology and its successful application to transcriptional regulation relevant to bone biology.

All mass spectrometry methodologies have three essential components: a molecular ionization method, a mass analyzer, and an ion detector. Ions are extracted and accelerated to identical kinetic energies, directed toward an ion detector typically placed at the end of a linear flight tube (time of flight or TOF mass analyzer) or an oscillating quadrupole field (functions like a "band-pass" filter for ions of specific kinetic energies). A detailed discussion of the instrumentation/technology available for these three components is beyond the scope of this chapter, but is nicely reviewed by Suizdak (Siuzdak, 1994) and Yates (Dongre *et al.,* 1997; Yates, 1998). However, TOF separation is readily conceptualized; since ions of identical kinetic energy will experience velocities that are inversely proportional to the square root of the molecular mass ($KE = 1/2mv^2$; $v = [2KE/m]^{0.5}$). Molecules of greater mass/charge (m/z) ratios but equivalent kinetic energy (KE) will take longer to arrive at the ion detector. The capacity to separate tryptic peptides by microbore reverse-phase HPLC and directly ionize and analyze the eluted peptides by tandem mass spectrometry (Fig. 2) has revolutionized protein microsequencing and transcription factor biology. Once tryptic peptides are resolved by HPLC and ionized by coulombic field forces applied to atomized eluate, peptides of specific m/z ratios are selected by the first of two tandemly linked spectrometers. Subsequently, the ionized peptide selected is bombarded with an inert noble gas in a collision cell. Since proteins are stereotypical polymers of peptide bonds, the alpha-chain backbone fragments in a predictable fashion—generally either from the N- or the C-termini. The fragmented ionized peptides are then resolved and analyzed by the second mass spectrometer placed in tandem. Since a single parental

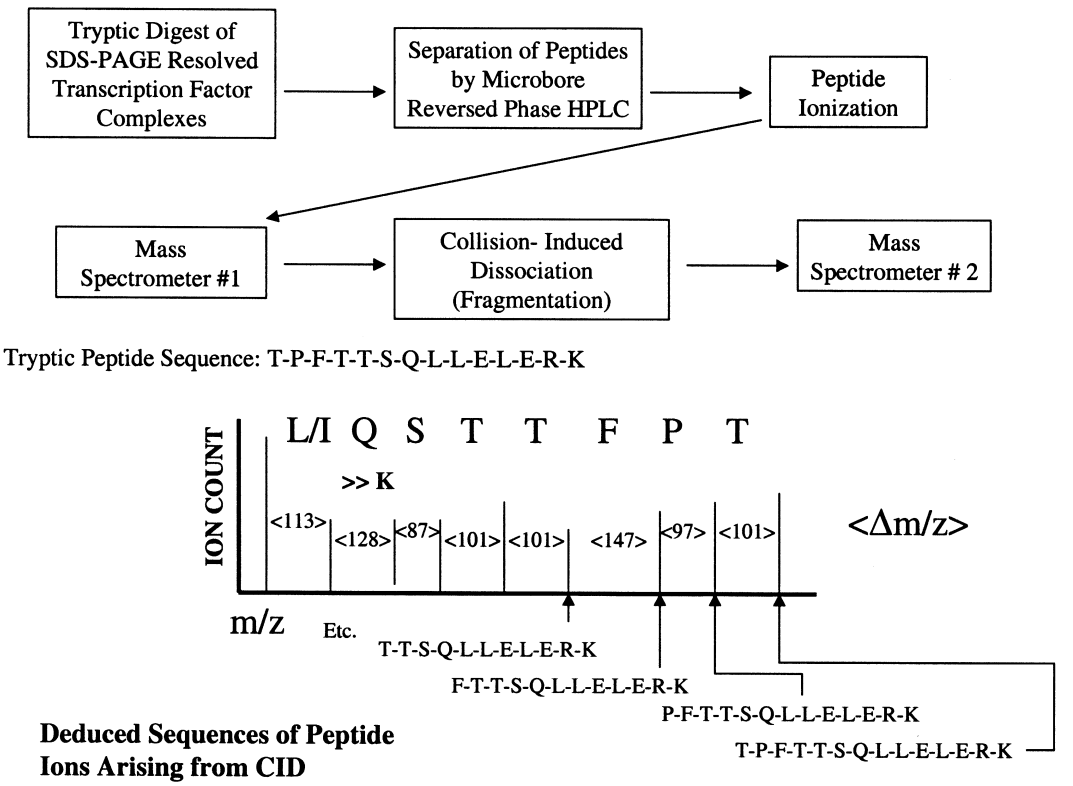

Figure 2 Tandem HPLC - MS / MS to characterize purified or partially purified transcription factor complexes.

Table IV Common Posttranslational Modifications of Transcription Factors and DNA Binding Proteins

Modification	Amino acid	Examples	References
Phosphorylation	Ser, Thr, Tyr	CREB, STAT, Pol II, ERF, Runx2, SREBP, VDR, ER-α/β, NFAT	Cheng *et al.,* 2000 Swanson *et al.,* 1999 Okamura *et al.,* 2000 Roth *et al.,* 2000
Acetylation	Lysine (epsilon N) N-Terminus	Histones, p53, SRC1, GATA, AR, HNF4	Abraham *et al.,* 2000 Soutoglou *et al.,* 2000 Fu *et al.,* 2000
Methylation	Arg	Histones	Koh *et al.,* 2001
ADP-ribosylation	Glu	Histones, TFIIF	Anderson *et al.,* 2000
Ubiquitination	Lys	NFKB, MyoD, ER	Nawaz *et al.,* 1999
Oxidation	Cys	NFKB, AR	Staal *et al.,* 1995
Nitrosylation	Cys	NFKB	Matthews *et al.,* 1996
Proteolysis	Various	SREBP, Notch	Schroeter *et al.,* 1998
Glycosylation	Ser, Thr	SRF, SP1, Myc, PolII	Reason *et al.,* 1992

polypeptide ion was selected, the fragmented species represent daughter products derived from that peptide, differing from each other by loss of amino acid residues. The differences in mass between adjacent daughter ion peaks permit assignment to the specific amino acid lost, and the iteratively determined differences between adjacent peaks (read from high to low mass when charge is placed on the amino terminus) provide a direct readout of the peptide sequence (Fig. 2; the reader is referred to Siuzdak for a more detailed description of the fragmentation process). While isobaric amino acid pairs (same molecular weight, i.e., leucine and isoleucine; lysine and glutamine; methionine sulfoxide and phenylalanine) cannot be discriminated, the remaining unique sequence is generally sufficient to identify the peptide from a translated EST data base (e.g., see Table III). When confirmed by comparison to the completed human and mouse genomes, this latter information, coupled with the MALDI MS fingerprint of the tryptic peptides, establishes sequence identity. Thus, an admixture of tryptic peptides obtained from a purified protein constituent can give rise to a unique peptide fingerprint and sequence. Such an approach was utilized to characterize the component of the osteocalcin FGF response element binding protein (OCFREB) purified from MG63 osteosarcoma cells (*vide supra*; Willis *et al.,* 2000).

A wide variety of posttranslational modifications regulate the magnitude and duration of transcriptional activation and repression phenomenon, generally by promoting or inhibiting specific protein–protein interactions, by regulating protein turnover, or by regulating subcellular localization (Table IV). Since modifications add predictable mass increments to amino acids when tandem MS/MS techniques are applied, mass spectrometry provides the rapid identification of modified residues and clues to the modifcation. Thus, in addition to providing a valuable tool for characterizing purified macromolecular complexes, protein mass spectrometry will catalyze the identification of important posttranslational modifications that function to modulate the enzymatic process that is transcription.

Conclusions

In recent years, a great deal of information has been added to our fund of knowledge concerning osteoblast-specific gene expression, osteoblast lineage allocation, and skeletal development. Cbfa1/Osf2/Runx2, Msx2, and Fra1 have emerged as important global regulators of skeletal gene expression, osteoblast matrix synthesis, and tissue mineralization. Other homeoproteins such as Alx4 , Cart1 (Qu *et al.,* 1999; Wu *et al.,* 2000), and Bapx1 (Lettice *et al.,* 1999; Tribioli and Lufkin, 1999) have been identified by genetic means to control craniofacial and axial mineralization programs, respectively, generating defects that are suggestive of interactions with the Msx2 and Runx2 transcriptional regulatory circuits. Similarly, high-molecular-weight platform proteins of the nuclear matrix have been strongly implicated in integrating morphogenetic, metabolic, and mechanical control of osteoblast gene expression. Thus, genetic and biochemical evidence point to the formation of large macromolecular transcriptional regulatory complexes that mediate elaboration of the osteoblast phenotype during skeletogenesis. The repertoire of known osteoblast transcription factors represents the "tip" of this macromolecular "iceberg"—an important set of rate-limiting regulators, but insufficient to explain or predict all osteoblast gene expression in detail. For example, while the expression of Runx2 can be well-documented at E12.5 during murine skeletogenesis, target genes such as osteopontin and osteocalcin are not expressed until the onset of mineralization at E14.5 (Bidder *et al.,* 1998; Jiang *et al.,* 1999; Komori *et al.,* 1997). The possibility that alternative N-terminal domains of Runx2 isoforms are required for robust osteoblast gene expression implicates

additional regulation at the level of coregulator adapter comolecules (Harada *et al.,* 1999). Moreover, as highlighted by Blau and Baltimore a decade ago (Blau and Baltimore, 1991) and exemplified by reciprocal Msx2–Dlx5 regulation of OC gene expression in the osteoblast (Ducy *et al.,* 2000; Newberry *et al.,* 1998), transcriptional derepression is a fundamental mechanism that controls the timing of terminal differentiation in virtually all cell types. Finally, the DNA binding activities responsible for polypeptide growth factor, biomechanical stressors, and integrin-mediated extracellular matrix regulation (matricrine) of osteoblast gene expression are only beginning to be explored in detail—and are particularly relevant to an integrated understanding of the relationship between osteoporosis and arterial macrovascular calcification. A more complete understanding of osteoblast gene regulation by anabolic endocrine and matricrine signal cascades should lead to truly novel osteoanaoblic therapies for the pharmacologic treatment of osteoporosis—without concomitant exacerbation of clinically relevant heterotopic ossification syndromes such as aortic calcification or osteoarthritis.

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CHAPTER 89

Current Methodologic Issues in Cell and Tissue Culture

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Introduction

The application of *in vitro* experimental approaches to the study of skeletal biology has proved to be a highly successful endeavor. Once practiced by only a few investigators, the techniques of bone organ and cell culture are now standard practice in many laboratories, graduate students are routinely trained in performing and interpreting *in vitro* experiments, and many of the results from such studies have now attained the status of dogma.

This chapter is aimed at providing a fairly short, selective overview of *in vitro* methods in skeletal biology. It will emphasize some of the strengths and limitations of current methods for addressing biological questions and suggest some areas where new methodologic approaches will likely be needed. It is not intended to be a technical manual or an exhaustive critique of specific methods—there are simply too many to cover adequately. Rather, this chapter will hopefully complement the more detailed reviews in this volume that target specific biological issues.

In Vitro Systems: "The more things change . . . "

Bone organ (tissue) cultures have been in use since the pioneering studies of Strangeways and Fell in the 1920s (Strangeways and Fell, 1926), while the isolation and culture of bone cells came into practice nearly half a century later (Peck *et al.,* 1964; Luben *et al.,* 1976). (see Table I). During their long histories, both *in vitro* approaches have been used extensively and adapted to suit a wide range of purposes. Yet despite the development of numerous variations on these initial methodologic themes, most of the *in vitro* culture systems used in skeletal biology today are remarkably similar in principle and procedure to their early ancestors. The day-to-day techniques of tissue isolation, feeding, subculturing, cloning, and preservation are much the same as they were decades ago, with the exception that most materials are now made of disposable plasticware rather than glass, and most media are purchased ready-to-use rather than prepared from scratch. Most of the culture media now in common use were formulated in the 1960s and 1970s, and for the most part medium supplementation remains almost purely traditional. Serum-free media are used mainly for organ culture; most practitioners of cell cultures rely on "undefined" media supplemented with fetal calf serum for most purposes, although defined supplements (e.g., insulin–transferrin–selenium mixtures) are available at reasonable cost. Serum lots are still screened to make sure they properly support cell growth.

The fact that so many fundamental aspects of cell and organ culture have changed so little makes a number of points in addition to illustrating the fairly strong tendency among *in vitro* biologists not to change successful methods (i.e., "if it ain't broke don't fix it"). It demonstrates that the basic principles of *in vitro* methodology are sound, it validates the careful work of those who painstakingly established the systems, and it generates confidence that *in vitro* data from different laboratories can be readily compared and understood based on a common set of accepted methodology. Finally, it demonstrates that cell and organ culture techniques as we know and practice them are far from becoming obsolete. There is a substantial supply of important biological information available to be mined with these methods,

Table I Examples of Bone Organ Culture Systems

Tissue	Source	Use(s)	References
Fetal/neonatal calvaria	Chick Rat Mouse	Regulation of development, bone formation and resorption	Jacenko and Tuan, 1995; Kream *et al.,* 1985; Meghji *et al.,* 2001
Fetal limb bones	Mouse radius/ulna	Bone resorption	Raisz, 1963
	Rat metatarsal	Formation/resorption	Chamoux *et al.,* 1997; Coxam *et al.,* 1996
	Mouse embryo	Development	Hall, 2000
Folded periosteum	Chick Rat	Bone formation	Birek *et al.,* 1991; Hui *et al.,* 1993; Tenenbaum *et al.,* 1992

without a need for dramatic new methodologic advances. Yet having made the case in favor of the now-familiar techniques of cell and organ culture, there are certain areas where these methods limit our ability for further investigation. In the following sections, we will review the types of experimental systems currently used to investigate the biology of the major cell types in bone and their principal functions. We will examine a few specific uses for these model systems, identify some of the limits imposed by the current methodologies, and describe areas for possible future development.

Current Techniques and Applications

Organ Culture

General Considerations

Organ cultures offer investigators the opportunities to study a tissue system with much of its architectural hierarchy intact, but without interference from the rest of the animal and its variable systemic influences (e.g. diurnal rhythms, episodic hormonal fluctuations). The investigator's major concerns with organ culture are first that the explanted tissue remains viable, and second that it maintains its *in vivo* behavior during culture. Because tissues are often removed by a surgical procedure, some damage inevitably occurs to the explant; hence, both viability and phenotype are likely to decline with time in culture. It then becomes necessary to establish these time courses. Assessment of viability if often carried out by staining with vital dyes, such as the colorimetric redox dye MTT (Mosmann, 1983). Alternatively, fluorescent dyes selectively taken up by viable cells (CMFDA) or by dead cells (ethidium homodimer) offer sensitive means for assessing tissue viability by two simultaneous, complementary means (Poole *et al.,* 1996).

Methods for measuring tissue function or phenotype vary, depending on the traits of interest. Organ cultures of fetal or embryonic tissues frequently continue to undergo morphogenesis in culture (Gronowicz *et al.,* 1989) and these processes are usually assayed by histologic methods. Metabolic endpoints such as collagen synthesis (Kream *et al.,* 1985) or bone resorption (Raisz, 1963) are evaluated by chemical methods. Interestingly, the need to maintain a phenotype as close to that *in vivo* as possible often precludes the use of serum supplements from organ cultures, as the growth factors tend to stimulate unwanted proliferation of cells, similar to a wounding response *in vivo.* The ability of tissue explants to continue growth or tissue function *in vitro* is undoubtedly due to production by the resident cells of growth factors and cytokines.

While organ cultures maintain tissue architecture, they are somewhat limited by inherent cellular heterogeneity; few skeletal explants (with the possible exception of some cartilage biopsies) contain a cell population considered to be uniform. As a result, biochemical analyses must be interpreted with a certain degree of caution unless at some point they are well correlated with morphologic investigations. For example, how much collagen synthesis in explanted calvariae is due to osteoblasts, and how much is due to periosteal fibroblasts is not readily apparent from purely biochemical assays. A second limitation of organ culture systems (discussed further in the next section) is imposed by their need for adequate nutrition. Since organ and cell cultures rely on diffusion to provide nutrition and remove waste from tissues, the explanted tissue needs to be small and is often limited to fetal tissues.

Current Uses and Limitations

Bone organ culture systems have been used primarily to study dynamic events and their regulation; in particular, these include bone formation and matrix synthesis (Gronowicz *et al.,* 1989; Kream *et al.,* 1985), bone resorption (Raisz, 1963), and tissue morphogenesis (Vaahtokari *et al.,* 1996). All of these processes are associated with tissue growth and development, and thus are amenable to study using embryonic, fetal, or newborn tissues that have high metabolic activities and can be maintained easily over short periods in organ culture.

While bone organ cultures derived from embryonic, fetal, or neonatal tissues has been successful, cultures of postneonatal bone tissue have not. This limitation appears to preclude organ culture systems from investigations of features associated with adult, rather than neonatal or fetal, bone. These include adaptive responses to mechanical stress, cortical bone remodeling, and changes in the tissue due to age itself. The paucity of adult bone organ culture systems likely reflects more than postnatal reductions in metabolic activity. Older and larger tissues are more difficult to maintain, since in culture nutrients are supplied and wastes removed by diffusion. This becomes a particular difficulty for studies of

postneonatal bone, since the mineralized extracellular matrix poses a substantial barrier to diffusion. As a result, access of osteocytes within the bone to nutrients is limited, and accumulation of waste metabolites may occur. Bone *in vivo* is perfused by tissue fluids flowing through its matrix and its lacunar–canalicular systems. This flow is not diffusion-dependent, but is driven by blood pressure and by mechanical loading (Bronk *et al.,* 1993; Knothe *et al.,* 1998). It may also be essential to maintaining the viability of the osteocytes inside the bone. Recent findings have implicated osteocyte apoptosis, brought about either by hormonal deprivation (Tomkinson *et al.,* 1997) or by tissue microdamage (Verborgt *et al.,* 2000) as part of the trigger mechanism that initiates bone resorption. Thus the health of osteocytes may have a significant influence on the behavior of cells at both periosteal and endosteal surfaces. This finding also seems to rule out one of the traditional means of overcoming diffusion-limited nutrition in organ—using very small explants of tissue. The consequences of tissue damage caused by making small pieces of mineralized bone for explant cultures are not known, but may be substantial, especially if the biological questions under study depend on extensive cellular interactions throughout the tissue—the type of problem normally well suited to investigation in organ culture. Possible approaches to the problem of studying adult bone in organ culture include the use of very small bones (e.g., mouse or rat metatarsals) that might be maintained intact in a diffusion-limited nutrient environment or the develoment of culture devices for perfusion of intact bone tissue.

Cell Cultures

General Considerations

Cell cultures offer a distinctly different approach to *in vitro* biology than organ cultures. Whereas organ cultures depend on maintaining an architectural hierarchy resembling that *in vivo,* the cell culture approach treats mammalian cells virtually like bacterial cultures. This allows the investigator to study the cells' intrinsic functions like proliferation, differentiation, adhesion, migration, matrix synthesis, and death, as well as their interactions with each other ("cellular sociology") and with their environment. Cell culture also allows investigators to expand and select cultures for biochemical analysis, although the development of techniques like RT-PCR (Liu *et al.,* 1994) and laser capture microdissection (Simone *et al.,* 2000) have improved the ability to obtain biochemical or genetic information from small numbers of cells *in situ.*

Cell cultures are basically of two types: primary (or early passage) cultures and permanent cell lines. Like organ cultures, primary cultures are usually heterogeneous; however, also like organ cultures they are less susceptible to the time-dependent changes that occur in culture as cells adapt to the *in vitro* environment, grow at different rates, and accumulate mutations with successive cell divisions. Thus, users of early passage cultures are generally confident that these cells provide a reasonable approximation of the phenotype they exhibited *in vivo.* Consistent differences in the properties of cultured cells from donors differing in age (Ankrom *et al.,* 1998) or genetic background (Bianco *et al.,* 1998) have generally confirmed that *in vivo* phenotypes can be maintained even in heterogeneous cell cultures.

In contrast to short-term cultures, permanent cell lines are generally established with the intent of providing continuing sources of cells with a known and hopefully consistent phenotype. To help ensure cellular uniformity, most permanent lines are clonal by design, although individual sublines of a clonal population exhibit a range of phenotypes (Grigoriadis *et al.,* 1985)! Experience has shown that permanent lines can sustain the expression of a particular trait over extended periods in continuous culture (Majeska *et al.,* 1980); however, phenotypic drift and phenotypic loss are facts of life *in vitro,* and cell lines will undoubtedly need to be recloned and recharacterized.

Permanent cell lines are by definition capable of indefinite (unlimited?) growth *in vitro,* and are therefore described as "immortal." There are several pathways to immortality for a cell, but all likely involve mutation in the genes that regulate initiation or progression through the cell cycle. Spontaneous immortalization *in vivo* usually occurs as a result of neoplastic transformation, and so tumors have been a traditional source of immortalized cells (Rodan *et al.,* 1994). Spontaneous immortalization of cells *in vitro* is rare, but is exemplified by the cells which survive the "crisis" phase of culture during which most cells senesce and die (Freshney, 1987). Direct introduction into target cells of genes responsible for the immortalizing function of tumor viruses like the SV40 T antigen in conjunction with a selectable marker like the neomycin resistance gene (Jat and Sharp, 1986) have been used to generate permanent cell lines *in vitro* (Heath *et al.,* 1989; Keeting *et al.,* 1992). This is probably the method of choice for establishing permanent cell lines from normal human tissues.

Despite the ability to immortalize cells without causing a tumorigenic phenotype, there is in principle some concern about the influence of the immortal phenotype on the normal processes of differentiation. Conditional (temperature-sensitive) mutants have been created (Harris *et al.,* 1995) to eliminate the influence of the immortalization gene during the course of experiments while retaining its function for maintenance of the lines; however, not many investigators have made use of this approach. The development of transgenic mouse strains with defective cell cycle controls necessary to suppress an "immortal" phenotype *in vitro* (Chambers *et al.,* 1993) has also tended to allay fears that immortalization will have adverse effects on the behavior of differentiated cells. The animals have generally normal phenotypes *in vivo* but a propensity to develop tumors; however, *in vitro* the cells derived from normal tissues are capable of indefinite growth.

Osteoblasts and Osteoblast-Like Cells

Cells with an "osteoblast-like" phenotype have probably been the most intensively investigated of all the bone-derived cell types. Isolated from normal bone of humans and all

Table II Examples of Primary Bone and Marrow-Derived Cell Cultures

Major cell type	Tissue source/isolation method	Species	References
Osteoblast-like	Fetal or neonatal Calvaria/Enzyme Digest	Rat Mouse Chick	Grigoriadis *et al.,* 1988; Ganta *et al.,* 1997 Mansukhani *et al.,* 2000 Landis *et al.,* 2000
Osteoblast-like	Trabecular bone/Explant	Human	Robey and Termine, 1985
Osteoblast-like	Trabecular Bone/Enzyme digest	Human	Gundle and Beresford, 1995
Marrow (total)	Extrusion	Mouse Rat	Jilka *et al.,* 1995 Kukita *et al.,* 1997
Marrow Stroma	Total Marrow Nucl. marrow Marrow aspirate	Mouse, rat, human Rat Human	Kuznetsov *et al.,* 1997; Kuznetsov *et al.,* 1997; Muschler *et al.,* 1997
Osteoclasts	 Marrow Marrow Osteoclastoma	Chick Mouse Rat Human	Collin-Osdoby *et al.,* 1991 Wesolowski *et al.,* 1995 Chambers *et al.,* 1987 James *et al.,* 1996
Osteocytes	Calvaria	Chick	van der Plas and Nijweide, 1992
Osteocyte-rich		Mouse	Tanaka-Kamioka *et al.,* 1998

commonly used experimental species, as well as from bone tumors (see Tables II and III), their phenotype has been characterized at the cellular, biochemical, and genetic levels by the matrix macromolecules they produce, by their expression of other functionally relevant markers (e.g., alkaline phosphatase), and by the arrays of regulatory molecules they either recognize or produce (for discussions of bone matrix proteins, see Chapters 12–15) Finally, their phenotype has been established in a functional sense by their ability to produce morphologically characteristic matrix-rich multicellular "nodules" that mineralize, usually in the presence of organic phosphate donors (Owen *et al.,* 1990;Stein *et al.,* 1990). Patterns of gene expression and morphologic changes in cultured osteoblast-like cells appear to correlate well and resemble those seen during bone formation in developing tissues *in vivo* (Weinreb *et al.,* 1990).

While osteoblast-like cell cultures have been used for a multiplicity of purposes, most efforts seem to have been

Table III Examples of Permanent Bone-Derived Cell Lines

Phenotype	Species	Cell line	References	Comments
Osteoblast-like	Rat Osteosarcoma	ROS UMR	Majeska *et al.,* 1980 Martin *et al.,* 1979	
	Human Osteosarcoma	SaOS-2 Mg-63	Rodan *et al.,* 1987 Franceschi *et al.,* 1988	
Osteoblast-like	Rat Mouse	RCJ MC3T3	Grigoriadis *et al.,* 1988 Sudo *et al.,* 1983	Spontaneous Immortalization
Osteoblast-like (Pre-osteoblastic)	Rat	RCT-3 (RCT-1)	Heath *et al.,* 1989 Keeting *et al.,* 1992	SV-40 T *in vitro* immortalization
Osteoblast-like	Human Human Mouse	HOBIT	Harris *et al.,* 1995 Ghosh-Choudhury *et al.,* 1997	SV-40 T *in vitro* (Conditional) BMP-driven SV40T-antigen-expressing mice
Marrow Stroma	Mouse Mouse	MBA-15	Benayahu *et al.,* 1989 Diduch *et al.,* 1993 Matsumoto *et al.,* 1995	 Phenotype variants Support hematopoiesis
	Mouse		Chambers *et al.,* 1993	T-ag-transgenic
Osteocyte-like	Mouse	MLO-Y4	Kato *et al.,* 1997	T-ag-transgenic?
Pre-osteoclast	Human	FLG29.1	Gattei *et al.,* 1992	
Pre-osteoclast	Mouse		Hentunen *et al.,* 1998	

directed toward understanding how these cells respond to an ever-growing list of systemic and local regulatory factors. These studies are reviewed extensively throughout this volume. Of particular interest from a methodologic standpoint, however, are areas of investigation that have gained momentum in recent years, including (i) investigations of cellular mechanosensitivity and (ii) use of cultured osteoblastic cells for tissue engineering applications.

Interest in the mechanisensitivity of bone cells derives largely from extensive observations at the tissue level that bone adapts its shape in response to changes in mechanical loading (Frost, 1987); translating these concepts to the cellular level has been the subject of more recent endeavors (Donahue, 1998). Experiments aimed at defining how cells can sense a mechanical signal has been investigated using osteoblast-like cells in culture as experimental models. In these studies, two approaches have been used to apply mechanical perturbation to cells. The first approach involves deformation of the substrate on which cells are cultured, the second involves fluid flow over the cultured cells. In both cases, the methods used to grow cells and to measure their responses are standard; however, unique culture apparatuses have been developed for experiments of this sort to allow the investigator both to manipulate the mechanical input and to measure it precisely. In these systems, strain application to deformable cellular substrates has been accomplished by use of vacuum (Buckley *et al.,* 1988) or stretching over a template (Schaffer *et al.,* 1994). The first system was shown to be capable of deformations much higher than those which occur in bone, and so has been used with apparent success to study cells which normally experience high strains *in vivo,* such as tendon cells (McNeilly *et al.,* 1996; Tsuzaki *et al.,* 2000). Gerstenfeld and coworkers used the second system to investigate the role of the actin cytoskeleton in the mechanical responses of osteoblasts, and changes in the production of bone matrix proteins (Meazzini *et al.,* 1998; Toma *et al.,* 1997).

The effects of fluid flow on bone cells are of particular interest because a consequence of mechanical stimulation *in vivo* is the flow of fluid through the lacunar–canalicular system (Knothe *et al.,* 1998). A number of fluid flow systems have been used to model this process; some utilize unidirectional pulsatile flow (Ajubi *et al.,* 1999), but a system to deliver bidirectional (reciprocating) flow has also been reported, with some distinct results (Jacobs *et al.,* 1998).

Bone cell culture systems have also been exploited for engineering applications. The overall objective of many bone bioengineering studies is the design of biomaterials that will enhance bone formation, with two goals: improving the interactions of orthopaedic prostheses with bony tissue and repairing osseous defects while providing a weight bearing structure in those defects. Recent knowledge of which materials are biocompatible for placement in human hosts has mainly come from the cocultures of cells and implant materials. These studies have been invaluable for assessing toxicity to osteoblasts, adhesion characteristics, osteoblast phenotypic expression, and mineralization (Davies, 1996). Bioactive bone bonding due to chemical reactions between the bone and materials has led to strong and stable interactions so that breakage due to loading often occurs inside the bone or in the material but not at the interface (Ohgushi and Caplan, 1999). Glass and calcium phosphate ceramics, nonceramic materials such as calcium carbonates, and some organic polymers are capable of bioactive bone bonding. For example, bioactive glass and bioactive glass–ceramics are able to bond bone through the formation of an hydroxyl carbonate apatite layer on their surfaces (Hench and Wilson, 1984). Titanium has also been shown to be a bioreactive material capable of bone-bonding properties under some conditions. The oxide layer of titanium, which naturally forms calcium phosphate similar to apatite, has been hypothesized to be responsible for this bone bonding characteristic (Hanawa, 1989).

Cytotoxicity to particular implant materials has been demonstrated in cocultures of osteoblasts and biomaterials. The methyl-thiazole sulfate test (MTT) measuring dehydrogenase enzymes found in metabolically active cells, has been used extensively to evaluate the effect of numerous materials—nickel, chromium, vanadium, aluminum (McKay *et al.,* 1996), zirconia and alumina ceramics (Josset *et al.,* 1999), polyethereetherketone and epoxy resin polymers with or without carbon fiber reinforcements (Morrison *et al.,* 1995), and others—on immortalized osteoblast cell lines and human bone cell cultures. Assays for alkaline phosphatase, cell growth, leakage of cytosolic lactate dehydrogenase, intracellular reduced glutathione content, protein synthesis, cell morphology, and immunocytochemical staining to detect osteoblast extracellular matrix protein synthesis have often been used in conjunction with the MTT assay to determine osteoblast viability (Josset *et al.,* 1999; McKay *et al.,* 1996; Morrison *et al.,* 1995; Oliva *et al.,* 1996). Interactions of primary rat osteoblasts and macrophages with biodegradable and porous polyesteurethane foam have also been used to assess cytoxicity (Saad *et al.,* 1996).

Enhancing cellular responses to implant materials has led to studies of alterations in the surface characteristics of implant materials. The role of implant surface topography in osteoblast attachment has been reviewed extensively (Chehroudi *et al.,* 1997; Boyan *et al.,* 1996; Larsson *et al.,* 1996) and has shown that rough or textured porous surfaces promote cell attachment (Ahmad *et al.,* 1999; Boyan *et al.,* 1996; Groessner-Schreiber and Tuan, 1992). Attachment to the implant materials is mediated through integrins and focal adhesion proteins (Krause *et al.,* 2000; Schneider and Burridge, 1994).

To improve existing biomaterials, cultures of cells with implant materials, having specific, engineered surface chemistries that stimulate osteoblast function, have been recently developed. Biomimetic surface engineering for bone regeneration has involved tethering integrin binding peptides (Arg-Gly-Asp) and heparin binding domains of bone sialoprotein to stimulate osteoblast attachment and bone formation *in vitro* (Rezania and Healy, 1999; Sofia

et al., 2001). Prefabricated biologic apatite formation on a bioactive apatite–wollastonite (AW) glass ceramic was shown to enhance mineralization more than AW alone in primary rat osteoblast cultures (Loty *et al.,* 2000) and this biomimetic apatite also stimulated the proliferation and differentiation of marrow stem cells (Yoshikawa *et al.,* 1998).

Although the ability of cultured osteoblast-like cells to produce mineralized nodules has provided a convenient *in vitro* correlate of osteogenesis, the generation of extended, higher ordered structures has not been investigated in great detail. Specifically, osteoblastic cells in culture appear to form woven bone like that seen in embryonic bone or in healing fractures, rather than the characteristic lamellar patterns observed in mature bone tissue *in vivo.* The functional requirements of osteoblasts to produce lamellar bone are not known. Some investigators have begun to examine the formation of three-dimensional structures *in vitro* both on surfaces (Castoldi *et al.,* 1997; Manduca *et al.,* 1993; Cooper *et al.,* 1998) and in collagen sponges (Mueller *et al.,* 1999). These and similar approaches may offer a starting point to understand the process of long-range matrix organization.

Finally, despite the usefulness of of osteoblast-like cell cultures for many applications, the question arises of whether the model is sometimes overextended. For example, the fluid flow mechanosensitivity experiments described above will certainly generate useful information about the mechanisms by which these cells recognize and respond to those stimuli. On the other hand, this response *in vivo* is believed to be primarily one of osteocytes. Although osteocytes and osteoblasts represent different stages in the maturation of a single cell type, it remains to be determined how closely an osteoblast-like cell can approximate an osteocyte, even *in vitro.* The ability to isolate and study "authentic" osteocytes (see below) should allow this question to be resolved fairly soon. The same question applies to bone lining cells. A monolayer of quiescent osteoblastic cells appears to be a reasonable model for lining cells, at least as a first approximation. Yet the assumptions underlying this approach remain to be validated; however, part of the problem is that the *in vivo* phenotype of lining cells is poorly understood.

Marrow Stromal Cells

The bone marrow stroma is a loose connective tissue within the marrow containing a heterogeneous population of connective tissue cells whose functions include the production of critical growth factors for hematopoietic cells (Simmons, 1996). The term "marrow stromal cells" has become an operational definition of the adherent population of proliferative cells (i.e., excluding cells like mature macrophages) derived from bone marrow. Marrow stromal cells have become of considerable interest because these populations contain multipotential "mesenchymal stem cells" capable of regenerating the entire repertoire of mesenchymally derived cell types, including osteoblasts (Cassiede *et al.,* 1996; Lazarus *et al.,* 1997).

Culture of marrow stromal cells appears technically straightforward. Total marrow isolates are obtained from "blowouts" of rat or mouse long bones (Zhang *et al.,* 1995) or from human bone biopsies or surgical specimens (Kuznetsov *et al.,* 1997; Muschler *et al.,* 1997). Either the total marrow is cultured or the nucleated cells are isolated first prior to culture. The adherent cells are allowed to grow like typical fibroblastic or osteoblast-like cells until use. Kuznetsov and Robey demonstrated that the requirements for successful initial growth of stromal cells can differ markedly among species (Kuznetsov *et al.,* 1997). Many investigators use primary marrow stromal cultures as their experimental model, but some permanent cell lines derived specifically from marrow stromal cells have been described (Matsumoto *et al.,* 1995; Benayahu *et al.,* 1989; Diduch *et al.,* 1993; Udagawa *et al.,* 1989).

Marrow stromal cell preparations, which contain precursors of all the "mesenchymal" cell lineages (osteoblasts, fibroblasts, chondrocytes, adipocytes, myocytes), have become one of the principal experimental systems used to investigate differentiation and developmental plasticity within these lineages (see Chapter 4 for a detailed review of this area). Because of their heterogeneity, some investigators have preferred clonal cell lines derived from marrow stroma (Udagawa *et al.,* 1989) or from other sources (Katagiri *et al.,* 1990, 1994) to study these differentiation pathways. Yet the heterogeneity of stromal cell preparations makes them especially well suited for *ex vivo* experiments, in which the effects of *in vivo* treatments or genetic differences on the number of osteoblastic progenitor cells in the marrow or their ability to differentiate into osteoblasts can be examined *in vitro.* For example, this approach has been used to study the effects of modulation in gonadal steroid levels (Bellido *et al.,* 1993; Jilka *et al.,* 1998) and on unweighting by hindlimb suspension (Zhang *et al.,* 1995). In studies like these, total marrow is usually isolated by standard techniques (although a novel centrifugation method (Dobson *et al.,* 1999) has been reported to give improved yields and reproducibility), and the number of osteoblastic cells or their progenitors is assesed by a colony or nodule formation assay.

Enumerative assays, which include colony and nodule formation, overcome the two principal disadvantage associated with biochemical techniques to measure a phenotypic trait: first, the outcome is a population average, and second, the assay may not be sufficiently sensitive to detect a rare phenotype. Flow cytometry is perhaps the most powerful tool for enumerative assays, since it measures the expression level of the selected traits in individual cells, and provides complete frequency distributions for them. Multiple properties can be evaluated simultaneously in many cytometers using appropriate fluorescent dyes. The most common flow cytometric assys measure DNA content or damage, including apoptosis (Hamel *et al.,* 1996) and cell surface markers, exemplified by the analysis of STRO-1 expression in human bone and marrow cell preparations (Gronthos and Simmons, 1995; Gronthos *et al.,* 1994; Simmons *et al.,* 1994).

Not all phenotypes are amenable to analysis by flow cytometry. For example, there is no clear marker to identify

a cell capable of replicating effectively at extremely low cell densities, of growing in soft agar, or of differentiating (e.g., into an osteoblast or chondrocyte) under the appropriate permissive conditions. These phenotypes must be assessed functionally. In the case of osteoblasts, cells are plated at a range of densities and grown under conditions permitting expression of an osteoblastic phenotype (ascorbic acid to promote formation of a stable extracellular matrix and an organic phosphate source to promote mineralization). Expression of the osteoblastic phenotype is then assessed, usually by counting the number of mineralized nodules. The number of nodules indicates the number of osteoblastic colony-forming units (CFU-O) present in the original plated population. With increasing dilution, fewer nodules are seen. By relating nodule number to the cells initially plated, the percentage of cells in the original population capable of becoming an osteoblastic colony is determined. Several variations of this general colony formation assay procedure have been reported (Bellows *et al.,* 1990, 1998; Falla *et al.,* 1993; Jilka *et al.,* 1998; Van Vlasselaer *et al.,* 1994); however, in all instances the Poisson distribution governs the probability of colony formation and is the basis for analysis of the data.

An alternative endpoint to mineralized nodule formation for the assessment of osteoblastic differentiation is the expression of alkaline phosphatase, a long-established marker of the osteoblast phenotpye (Majeska *et al.,* 1980; Rodan *et al.,* 1988). Alkaline phosphatase is expressed early in the course of osteoblast maturation, but activity is maintained until the cells mature ultimately into osteocytes (Owen *et al.,* 1990). Although alkaline phosphatase is highly expressed in osteoblasts, it is not completely specific for this cell type; for example, other cells in the marrow stroma probably express this enzyme (Westen and Bainton, 1979). Still, formation of mineralized nodules *in vitro* is virtually always associated with increased alkaline phosphatase expression; however, a systematic evaluation of differences in the outcome of limiting dilution assays using the two endpoints of osteoblastic differentiation have not been carried out.

In addition to its usefulness for investigating the biology of mesenchymal cell differentiation, the marrow stroma is currently the tissue of choice for mesenchymal stem cells to use in tissue engineering applications. Specifically, mesenchymal stem cells that have been expanded in culture and then induced to form bone by dexamethasone, tumor growth factor (TGF)-β, BMP-2, -4, or -7, and growth hormone have been used recently to seed composite grafts for implantation *in vivo* (Ohgushi and Caplan, 1999). Moreover, marrow stromal cells have been investigated as carriers for genes delivered *in vitro,* in the expectation that the cells will populate the host marrow while continuing to deliver the product of the *in vitro*-transfected gene (Oyama *et al.,* 1999; Suzuki *et al.,* 2000).

Although harvesting marrow stromal cells as sources of progenitors of osteoblasts or other cell types is often less involved than obtaining samples of bone or cartilage, it is still not a trivial procedure. Recent reports indicate that cells from more easily attainable sources like muscle (Lee *et al.,* 2000) and fat (Bennett *et al.,* 1991) may be capable of osteogenic differentiation.

OSTEOCYTES

Osteocytes comprise the majority of adult bone cells, but have been difficult to culture for several reasons. First, osteocyte isolation is problematic. They are the only bone cells not found at a surface; rather, they are entombed within mineralized bone matrix. Second, they represent the most highly differentiated state of the osteoblastic cell lineage and are clearly postmitotic *in vivo.* Finally, the distinctive morphology of osteocytes *in vivo* (featuring an extensive array of long, narrow cell processes) is mostly lost when the cells are removed from their matrix, and for a long time there was no other specific marker with which to identify this cell type. There have been two major technical breakthroughs in osteocyte *in vitro* culture. First was the identification by Nijweide and colleagues of a specific osteocyte antigen and the development of an immunoisolation technique to obtain these cells (Nijweide and Mulder, 1986; van der Plas and Nijweide, 1992). Unfortunately, this marker is species-specific for chicken cells. The second was the establishment of the MLO-Y4 osteocytic cell line from a T-antigen-expressing transgenic mouse (Kato *et al.,* 1997). This cell line has been used to identify further phenotypic markers of osteocytes as well as to investigate the biology of these cells. In addition to these systems, other investigators have utilized differential digestion to isolate osteocyte-enriched cell populations and characterized some of their features, particularly the cytoskeleton (Tanaka-Kamioka *et al.,* 1998).

Most studies on osteocytes in culture have focused on their responses to mechanical stimuli, based on the concept that osteocytes are the principal mechanosensitive cells in bone responsible for initiating the tissue's adaptive changes in response to mechanical loading (Donahue, 1998). Fluid flow has been the principal stimulus (Ajubi *et al.,* 1999; Westbroek *et al.,* 2000). These studies are also more fully described in Chapter 6.

The ability to study *in vitro* cells that clearly express an osteocytic, rather than an osteoblastic, phenotype *in vitro* is clearly an expermental advantage. What remains to be established, however, are the importance of the characteristic morphology of osteocytes and the presence of a mineralized matrix surrounding them. How osteocytes generate their characteristic cell processes is not known, although a paper by Reddi and collaborators showed that culture of osteoblastic cells on laminin or matrigel caused them to assume an osteocyte-like morphology in monolayer (Vukicevic *et al.,* 1990). The osteocytic environment *in vivo,* including the nature of its matrix, is poorly understood.

OSTEOCLASTS AND PREOSTEOCLASTS

The isolation and culture of osteoclasts was difficult to achieve, due to their rarity, fragility, and status as terminally differentiated, nonproliferative cells; however, these problems have been overcome, and osteoclasts are now routinely

isolated for use in short-term cultures in much the same way as osteoblastic cells. Mature osteoclasts and late-stage preosteoclasts have been obtained from several species including chick (Collin-Osdoby *et al.,* 1991) and mouse (Wesolowski *et al.,* 1995), and from human giant cell tumors (Drake *et al.,* 1996; James *et al.,* 1991, 1996). Furthermore, immortalization of osteoclast precursors has now led to osteoclastic cell lines (Hentunen *et al.,* 1998, 1999). Osteoclast isolation (Teti *et al.,* 1999) and osteoclast biology (Roodman, 1999) have been recently reviewed and are also discussed further in Chapters 7–9.

The differentiation of osteoclasts from monocytic precursors has also been intensively studied, because this process is crucial to the initiation of bone remodeling. The *in vitro* experimental systems used for such osteoclastogenesis assays are based predominantly on cocultures of hematopoietic osteoclast precursors (of the monocytic line) and mesenchymal cells that stimulate their growth and differentiation. Both cell populations are present in total bone marrow cultures; however, alternative sources of both osteoclast precursors and mesenchymal cells have been used. The common alternative source of monocytic osteoclast precursors is the spleen (Roodman, 1999), while the stromal cell component of osteoclastogenic cocultures has been supplied by a wide range of primary cultures and permanent lines of marrow stroma-derived cells or of osteoblast-like cells. Two of the principal stromal-derived regulators of osteoclastic differentiation appear to be M-CSF and RANK ligand (Itoh *et al.,* 2000).

The criteria for defining an osteoclast, established over several years of investigation, include multinucleation, the expression of tartrate-resistant acid phosphatase activity (TRAP), receptors for (or a proper response to) calcitonin, and a standard *in vitro* functional assay, the ability to create resorption pits when placed on mineralized tissue (see Chapters 7 and 8 for details). Many of these assays, in particular pit formation, are expensive, painstaking, and require specialized equipment and experience to interpret. The simplicity and convenience of counting TRAP-positive multinucleated cells has made this a very popular endpoint for *in vitro* assays of osteoclast formation, and experience has apparently confirmed its validity.

Osteoclast cultures have been used mainly to study the mechanisms that control the function of this cell, and also to identify the factors controlling osteoclast differentiation. These lines of investigation have been enormously productive; however, there are certain areas which have yet to be fully examined, and to do so *in vitro* may require novel methods. For example, we have little knowledge of the factors which control the ongoing activity of osteoclasts during the resorption process. In particular, we do not know the role of nearby cells. Osteoclastic resorption occurs as part of a multicellular unit that forms new osteons in cortical bone or "hemiosteons" on trabecular surfaces (Parfitt, 1994). Among the cells potentially capable of exerting influence on the resorbing osteoclasts are osteocytes, endothelial cells (which remain close behind the osteoclast "cutting cone" and provide the entry point for new monocytes), and the perivascular cells, osteoblasts, and other mesenchymal cells in the vicinity. While pit assays can assess the intrinsic ability of osteoclasts to degrade mineral and osteoid, removal of devitalized tissue does not allow evaluation of the roles played by these cells. Yet measuring osteoclast activity *in vitro* on bone that contains these cellular components will require a substantial development in methodology.

A second area of bone remodeling where *in vitro* systems have yet to fully approximate the *in vivo* situation is the initial stage of osteoclast recruitment. These processes involve two cell types so far underrepresented by *in vitro* models of bone cells: the lining cell and the endothelial cell. Bone remodeling is a targeted process that requires osteoclast progenitor cells to exit the vasculature at specific sites of incipient resorption. How the endothelium changes at those points to allow monocyte penetration is currently unknown. The necessary cell types are available to examine these questions *in vitro,* and mechanistic examples exist in the literature of inflammation (Heil *et al.,* 2000); however, the experimental system needs to be developed.

COCULTURE SYSTEMS

In addition to normal cellular interactions in bone, *in vitro* systems have been used to model pathologic conditions as well. Cancer has been a major focus, in particular breast cancer, prostate cancer, and melanoma. Each of these forms has marked skeletal effects; myeloma and metastatic breast cancer cause dramatic local bone resorption; metastatic prostate cancer produces lesions that are mainly osteogenic (Boyce *et al.,* 1999). In each case tumor cells produce deleterious effects on the skeleton indirectly, by regulating the actions of host osteoblasts or osteoclasts; consequently studies using *in vitro* model systems have been primarily directed toward defining the mechanisms by which the cancer cells signal and modulate host cells. In the case of myeloma (Michigami *et al.,* 2000) and breast cancer (Mbalaviele *et al.,* 1996; Thomas *et al.,* 1999), host– tumor interactions promoting osteoclastogenesis have been examined in marrow cell-based cultures. Prostate cancer cells have also been cocultured with marrow stromal cells (Lang *et al.,* 1997, 1998) and with osteoblast-like cell lines (Koutsilieris *et al.,* 1997; Reyes-Moreno *et al.,* 1995, 1998; Akoum *et al.,* 1996) to identify the putative signaling pathways running in both directions between these cells; i.e., those responsible for stimulating osteoblastic activity and those which promote the growth and survival of the metastatic tumor cells. Finally, *in vitro* systems have been used to investigate the effects of chemotherapeutic drugs (Choki *et al.,* 1998; Koutsilieris *et al.,* 1999a,b, Akoum *et al.,* 1996). Studies showing that bisphosphonates inhibit osteoclast function (Hughes *et al.,* 1995), indicating their potential usefulness to limit tumor-induced osteolysis, have led to direct indications that these compounds may also directly act on tumor cells (Boissier *et al.,* 2000).

Summary

It has been possible to isolate, culture, and study not only bone tissue but also cells representing most of the morphologically distinct cell types associated with bone. The information gathered so far with these techniques has been an invaluable aid to our understanding of skeletal biology. The challenges in methodology now seem to be less of breakthrough than of refinement. Fundamental methods of bone cell and tissue culture, and the means to analyze cell behavior *in vitro,* are largely validated and available to all investigators. These model systems in their current state will continue to be convenient assay systems, useful for characterizing the differences at the cellular level which result from genetics, age or disease. Yet to understand how those cellular differences are translated into alterations at the tissue level will require improvement in the modeling of bone cell interactions with each other and with their environment.

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CHAPTER 90

Biochemical Markers of Bone Metabolism

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Introduction

In order to meet the ever-changing biophysical and chemical needs of the living organism, bone undergoes continuous turnover and remodeling. As discussed elsewhere in this book in more detail, turnover of the skeletal matrix relies on the activity of two major types of cells, namely, bone-forming osteoblasts and bone-resorbing osteoclasts. Accordingly, bone remodeling is achieved by two counteracting processes, i.e., bone formation and bone resorption. Although the various serum and urinary markers of bone turnover include both cell-derived enzymes and nonenzymic peptides, they are usually classified according to the metabolic process they are considered to reflect. For clinical purposes, therefore, markers of bone formation are distinguished from indices of bone resorption (Tables I and II). It should be borne in mind, however, that some of these compounds may reflect, at least to a certain degree, both bone formation and bone resorption (e.g., urinary hydroxyproline) and that most, if not all, of these markers are present in tissues other than bone and may therefore be influenced by nonskeletal processes as well. Third, changes in biochemical markers of bone turnover are usually not disease-specific, but reflect alterations in skeletal metabolism independent of the underlying cause (Tables V–VII). Keeping this in mind and the fact that abnormal biochemical results should always be interpreted within the context of the technical (variablity!) and clinical picture is essential to the meaningful use of biomarkers in the assessment of skeletal disorders.

Under normal conditions, bone formation and bone resorption are coupled to each other, and the long-term maintanance of skeletal balance is achieved through the intricate action of systemic hormones (see Chapters 24–36) and local mediators (see Chapters 37–55). In contrast, metabolic bone diseases, states of increased or decreased mobility, therapeutic interventions, etc., are usually characterized by more or less pronounced and sometimes only transient imbalances in bone turnover. While the long-term results of such imbalances (i.e., changes in bone mass) are assessed with densitometric techniques, the principal domain of bone biomarkers is in the detection of the imbalance itself, and

the classification of low or high, decreased or increased bone turnover.

Basic Aspects

In addition to the cells, bone consists of mineral, proteins, and other macromolecules. The mineral phase of bone is principally composed of crytalline hydroxyapatite and accounts for 60–70% of the weight of bone. The organic phase of bone is principally composed of collagen (90%) and other smaller matrix proteins, glycoproteins, and proteoglycans. The cellular and extracellular components of the skeletal matrix form the basis for the development of biochemical markers that specifically reflect either bone formation or bone resorption.

Throughout life the skeleton undergoes continuous remodeling with removal of old bone and replacement with new bone. Bone turnover is always initiated by osteoclasts eroding a mineralized surface. When osteoclasts resorb bone, they secrete a mixture of acid and neutral proteases which act sequentially to degrade the collagen fibrils into molecular fragments. The biochemical markers of bone resorption therefore include collagen breakdown which include hydroxyproline, hydroxylysine glycosides, and the pyridinoline cross-links. These cross-links range in size from the free amino acids to segments of the N-telopeptide and C-telopeptide domains. Other markers of bone resorption include tartrate-resistant acid phosphatase and bone sialoprotein (Table II). The process of bone resorption is followed by the recruitment of osteoblasts to the outer edge of the erosion cavity. The osteoblasts secrete new bone matrix (osteoid) and gradually fill in the resorption cavity. The biochemical markers of bone formation are products of osteoblastic synthesis. These include N- and C-propeptides of type I collagen, osteocalcin, and bone-specific alkaline phosphatase (Table I).

Markers of Bone Formation

ALKALINE PHOSPHATASE

Alkaline phosphatase (ALP) belongs to a large group of proteins which are on attached to the extracellular surface of cell membranes via a carboxy-terminal glycan-phosphatidylinositol (GPI) anchor (Low and Saltiel, 1988). Four gene loci code for ALP: the three tissue-specific genes encode the intestine, mature placenta, and germ-cell enzymes and the tissue-nonspecific (tns) gene is expressed in numerous tissues (including bone, liver, kidney, and early placenta). Tissue nonspecific ALPs are the products of a single gene, but tissue-specific differences are found in their electrophoretic mobility, stability to heat, and sensitivity to a variety of chemical inhibitors. These differences are due to variations in their carbohydrate side chains (Weiss *et al.,* 1988). Since the two most common sources of elevated alkaline phosphatase levels are liver and bone, a number of techniques have been developed which rely on the differences in the carbohydrate side chains to distinguish between bone and liver isoforms.

Table I Biochemical Markers of Bone Formation[a]

Marker (abbreviation)	Tissue of origin	Analytical specimen	Analytical method	Remarks/specificity
Total alkaline phosphatase (total ALP)	Bone, liver, intestine, kidney, placenta	Serum	Colorimetric	Attached to the extracellular surface of cell membranes; may play a role in matrix mineralization. Specfic for bone formation only in the absence of liver or biliary disease.
Bone-specific alkaline phosphatase (bone ALP)	Bone	Serum	Colorimetric, electrophoretic, precipitation, IRMA, EIA	Attached to the extracellular surface of cell membranes; may play a role in matrix mineralization. Specific product of osteoblasts. Carbohydrate side-chain differences confer bone specificity. Some assays show up to 20% cross-reactivity with liver isoenzyme.
Osteocalcin (OC)	Bone, platelets	Serum	RIA, ELISA (See also Table III)	Contains calcium-binding amino acid, γ-carboxyglutamic acid (Gla), which facilitates interaction with hydroxyapatite. Specific product of osteoblasts; many immunoreactive forms in blood; some may be derived from bone resorption.
Carboxy terminal propeptide of type I procollagen (PICP)	Bone, soft tissue, skin	Serum	RIA, ELISA	Specific product of proliferating osteoblasts and fibroblasts.
Amino-terminal propeptide of type I procollagen (PINP)	Bone, soft tissue, skin	Serum	RIA, ELISA	Specific product of proliferating osteoblast and fibroblasts; partly incorporated into bone extracellular matrix.

[a]Nomenclature and abbreviations follows a recent proposal by a working group of the International Osteoporosis Foundation [Delmas *et al.* (2000) *Osteoporosis Int.* **11**].

Table II Biochemical Markers of Bone Resorption[a]

Marker (Abbreviation)	Tissue of origin	Analytical specimen	Analytical method	Remarks/specificity
Hydroxyproline, total and dialyzable (Hyp)	Bone, cartilage, soft tissue, skin	Urine	Colorimetric HPLC	Present in all fibrillar collagens and partly collagenous proteins, including C1q and elastin. Present in newly synthesized and mature collagen; i.e., both collagen synthesis and tissue breakdown contribute to urinary hydroxyproline.
Pyridinoline (PYD)	Bone, cartilage, tendon, blood vessels	Urine Serum	HPLC ELISA	Collagens, with highest concentrations in cartilage and bone; absent from skin; present in mature collagen only.
Deoxypyridinoline (DPD)	Bone, dentin	Urine Serum	HPLC ELISA	Collagens, with highest concentration in bone; absent from cartilage or skin; present in mature collagen only.
Carboxy terminal cross-linked telopeptide of type I collagen (CTX-MMP)	Bone, skin	Serum	RIA	Collagen type I, with highest contribution probably from bone; may be derived from newly synthesized collagen.
Amino-terminal cross-linked telopeptide of type I collagen (NTX-I)	All tissues containing type I collagen	Urine Serum	ELISA CLIA RIA	Collagen type I, with highest contribution from bone.
Carboxy terminal cross-linked telopeptide of type I collagen (CTX-I)	All tissues containing type I collagen	Urine (α/β) Serum (β only)	ELISA RIA	Collagen type I, with highest contribution probably from bone. Isomerization of aspartyl to β-aspartyl occurs with aging of collagen molecule.
Hydroxylysine glycosides	Bone, soft tissue, skin, serum complement	Urine	HPLC	Hydroxylysine in collagen is glycosylated to varying degrees, depending on tissue type. Glycosylgalactosyl-OHLys in high proportion in collagensof soft tissues, and C1q; Galyctosyl-OHLys in high proportion in skeletal collagens.
Bone sialoprotein (BSP)	Bone, dentin, hypertrophic cartilage	Serum	RIA ELISA	Acidic, phosphorylated glycoprotein, synthesized by osteoblasts and osteoclastic-like cells, laid down in bone extracellular matrix. Appears to be associated with osteoclast function.
Tartrate-resistant acid phosphatase (TRACP)	Bone Blood	Plasma Serum	Colorimetric RIA ELISA	Six isoenzymes found in human tissues (osteoclasts, platelets, erythrocytes). Band 5b predominant in bone (osteoclasts). Enzyme identified in both the ruffled border of the osteoclast membrane and the secretions in the resorptive space.

[a]Nomenclature and abbreviations follows a recent proposal by a working group of the International Osteoporosis Foundation [Delmas *et al.* (2000) *Osteoporosis Int.* **11**].

Early methods included heat denaturation, chemical inhibition of selective activity, gel electrophoresis, and precipitation by wheat germ lectin (Calvo *et al.,* 1966). Bone-specific immunoassays are the most commonly used method today.

Commercial kits are available which utilize monoclonal antibodies with preference for the bone isoform. Alkphase-B (METRA Biosystems, Mountain View, CA) uses a single monoclonal anti-body which is bound to a microtiter plate and captures alkaline phosphatase from the sample and activity of the bound enzyme is measured directly (Gomes *et al.,* 1995). A two-site immunoassay (Tandem-R-Ostase, Hybritech, San Diego, CA) relies on the use of two monoclonal antibodies, both of which react preferentially with the bone isoform. This assay measures mass of the enzyme (Garnero and Delmas, 1993). A second kit from Hybritech, Tandem-MP-Ostase, is an EIA which measures the activity of antibody-bound enzyme. Comparisons of mass and activity-based immunoassays show that two methods generally provide similar clinical information. However, demonstrated variations in glycosylation patterns raise the question of immunological heterogeneity in the bone isoform which may affect the mass to activity ratio (Kress, 1998).

There is a great deal of inter-individual variation in ALP levels, but for any one individual, values change little with time. ALP is cleared from the blood very slowly; the half-life varies from 40 hr for bone to 7 days for placental isoforms (Crofton, 1982). Biological within day variation of total alkaline phosphatase is estimated to be less than 4%. There is some evidence of a slight diurnal variation in ALP activity that may derive from the bone (Nielsen *et al.,* 1990).

OSTEOCALCIN

Osteocalcin is a small protein synthesized by mature osteoblasts, odontoblasts, and hypertrophic chondrocytes. It is characterized by the presence of three residues of the calcium-binding amino acid, γ-carboxyglutamic acid (Gla). Osteocalcin is thought to interact directly with hydroxyapatite in bone through its Gla residue (Hauschka *et al.,* 1989).

Osteocalcin is primarily deposited in the extracellular matrix of bone, but a small amount enters the blood. Serum osteocalcin is a sensitive and specific marker of osteoblastic activity and its serum level reflects the rate of bone formation. There are several commercial kits for human osteocalcin (Table III), although considerable inconsistency results when comparing values from these various assays and wide variations are reported in control and patient populations (Gundberg, 1998; Gundberg and Nishimoto, 1999). Various fragments of osteocalcin can circulate (Gundberg and Weinstein, 1986; Garnero *et al.,* 1994a; Taylor *et al.* 1990). Commercial and research assay variability is thought to be the result of differences in the ability of the various antibodies to recognize these fragments. Several laboratories have developed two-site immunoassays with the intention of measuring only the intact molecule. However, rapid loss of immunoreactivity is observed with these assays when samples are left at room temperature for a few hours (Colford *et al.,* 1999; Garnero *et al.,* 1992; Monaghan *et al.,* 1993; Parvianen *et al.,* 1994).

A new generation of assays is based upon studies which suggest that intact osteocalcin is degraded to a large N-terminal- mid-molecule fragment in the range of residues 1–43 (OC1–43). The large N-terminal mid-molecule fragment is thought to be generated by proteolysis in the circulation or during sample processing and storage, but there is some evidence that the osteoblast is an additional source of the fragment (Gundberg *et al.,* 2000). One study found that the intact molecule represents only about one-third of the circulating osteocalcin immunoreactivity. One third is comprised of a large N-terminal mid-molecule fragment and another third by several other smaller fragments (Garnero *et al.,* 1994a). Osteocalcin levels decrease with incubation at room temperature when measured by conventional radioimmunoassays (RIAs) or by intact assays, but values are stable with an assay that recognizes both the intact and large N-terminal mid-molecule fragment. With this assay apparent instability of osteocalcin in the circulation and during sample handling is eliminated and the correlation with

Table III Commercially Available Kits for Osteocalcin

Name	Company	Type
ELSA-OST-NAT	CIS-bio International, Gif-sur-Yvette, France	IRMA
Intact Osteocalcin	Biomedical Technologies, Stoughton, MA	ELISA
Human Osteocalcin	Quest, San Juan Capistrano, CA	IRMA
N-tact Osteo- SP	Incstar Corporation Stillwater, MN	IRMA
ELSA-OSTEO	CIS-bio International Gif-sur-Yvette, France	IRMA capture: 25–37 detection: 5–13
Active Human Osteocalcin	Diagnostic Systems Laboratories, Webster, TX	IRMA capture:? detection: 30–49
N-Mid Osteocalcin	Osteometer Biotech, Rodovre, Denmark	IRMA capture: 20–43 detection: 7–19
Mid-tact OC	Biomedical Technologies, Stoughton, MA	ELISA capture:5–19 detection: 21–43
BGP	Mitsubishi Yuka IDS, Boldon, Tyne and Wear, UK	IRMA capture: 12–33 detection: 30–49
Ostk-PR	CIS-bio International, Gif-sur-Yvette, France	RIA; polyclonal antibody to bovine OC
Osteocalcin	Diagnostic Systems Laboratories, Webster, TX	RIA; polyclonal antibody to human OC
OSCAtest	Brahms- Henning, Berlin, Germany	RIA; polyclonal antibody to human OC
NovoCalcin	METRA Biosystems, Mountain View, CA	ELISA; single monoclonal antibody to bovine OC

bone density is improved (Minisola *et al.,* 1997; Dumon *et al.,* 1996; Rosenquist *et al.,* 1995).

The chief route of circulating osteocalcin catabolism is renal glomerular filtration and degradation. The plasma half-life is about 20 min in humans. Immunoreactive fragments of osteocalcin have also been reported in the urine (Taylor *et al.,* 1990). Osteocalcin levels follow a circadian rhythm characterized by a decline during the morning to a low around noon, followed by a gradual rise which peaks after midnight (Gundberg *et al.,* 1985). The difference between the peak and nadir in a 24 hr period can range from 10 to 20% depending upon the assay used.

PROCOLLAGEN PEPTIDES

All collagens contain molecular domains of triple-helical conformation (see Chapter 12). The newly translated polypeptide, a pre-pro-α chain, includes a signal sequence and amino (N)- and carboxyl (C)-terminal propeptide extensions. During collagen synthesis, the C-propeptides guide the selection and association of the individual pro-α chains and prevent premature intracellular fiber formation. Specific extracellular endoproteinases cleave the procollagen molecule at precise sites in each chain. (Fig. 1) The C-terminal endoproteinase is identical to bone morphogenic protein (BMP) (Kessler *et al.,* 1996). The C-propeptide (PICP), which is a trimeric globular glycoprotein, is stabilized by disulfide bonds and circulates as a single molecule (Olsen *et al.,* 1977). It has a serum half-life of 6–8 min and is cleared in the liver endothelial cells by the mannose-6-phosphate receptor (Smedsrod *et al.,* 1990). The procollagen type I aminoterminal propeptide (PINP) is a partly globular, partly helical (collagenous) 35-kDa protein. PINP circulates mainly as the intact trimeric molecule but monomers are also found (Brandt *et al.,* 1999). The intact molecule is cleared from the circulation by the scavenger receptor of liver endothelial cells (Kivirikko and Myllyla, 1980). There have been suggestions that a low-molecular-weight, degraded form of PINP monomer also circulates (Melkko *et al.,* 1996) .

The procollagen extension peptides are cleaved from the newly formed molecule in a stoichiometric relationship with collagen biosynthesis. Therefore, they should reflect bone formation in a manner analogous to the assessment of C-peptide for endogenous insulin production. However, because type I collagen is also a component of several soft tissues (fibrocartilage, tendon, skin, gingiva, intestine, heart valve, and large vessels) there is a potential contribution to circulating procollagens from soft tissue synthesis of type I collagen. Nevertheless, because the rate of turnover of collagen in bone is faster than in other tissues, changes in procollagen concentrations are assumed to reflect changes primarily in bone collagen synthesis. Similar to other markers, PICP and PINP demonstrate a circadian rhythm with peak values occurring in the early morning hours and nadirs in the afternoon (Hassager *et al.,* 1992; Saggese *et al.,* 1994).

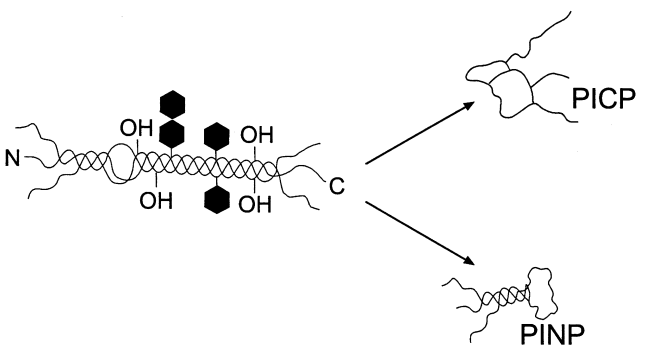

Figure 1 Procollagen Peptides. Type I collagen polypeptides are synthesized as pre-pro-α chains which contain a peptide secretory sequence and amino (N)- and carboxyl (C)-terminal extension peptides. These extension peptides guide helical folding of the molecule. After secretion, the N- and C-terminal extension peptides are cleaved by specific peptidases and the extension peptides can enter the circulation.

Assays for the measurement of PICP are based on polyclonal antisera made against purified collagen isolated from human skin or lung fibroblast cultures (PICP, Orion Diagnostics, Espoo, Finland, distributed in the United States by Diasorin, INCStar, Stillwater, MN). Another assay (Metra BioSystems, Mountain View, CA) uses an enzyme-linked immrosorbant assay (ELISA) format. Several assays have also been developed to measure PINP in blood. Early research assays used synthetic peptides for antibody production and assay reagents and results with these assays are variable. Meanwhile, antibodies have been made to authentic PINP. Depending upon the assay format, these measure either the the intact trimeric form of PINP (intact PINP) only or both the intact trimeric plus the monomeric forms (total PINP)(Melkko *et al.,* 1996; Orum *et al.,* 1996). One assay which measures only intact PINP is in commercial format (PINP, Orion Diagnostics, distributed in the United States by Diasorin, INCStar).

Studies indicate that PINP is a better measure of changes in rate of bone collagen synthesis than PICP. In contrast to other measures of bone turnover, there are only slight increases in PICP after the menopause and PICP correlates only weakly with spinal bone mineral density (Charles *et al.,*1994; Minisola *et al.,* 1994). Measurement of intact PINP appears to be more sensitive than total PINP and may provide more specific clinical information (Brandt *et al.,* 1999; Orum *et al.,* 1996; Chandani *et al.,* 2000; Dominguez *et al.,* 1998).

Markers of Bone Resorption

HYDROXYPROLINE

Hydroxyproline (Hyp) is present in essentially all tissues and all genetic types of collagen (Chapter 72). Hydroxyproline is derived from the breakdown of collagen. The majority of the the breakdown products are reabsorbed by the renal tubules and broken down in the liver while only about 10% of is excreted in the urine. Most are contained in di- and tripeptides (Prockop and Kivirikko, 1967; Smith, 1980). The remaining peptides in the urine are of approximately 5 kDa. There is a small amount of the free imino acid in urine. Hydroxyproline can never be reincorporated into newly synthesized collagen (Prockop, 1964). However, both collagen synthesis and tissue breakdown con-

tribute to urinary hydroxyproline. Colorimetric methods or high-performance liquid chromatography (HPLC) are commonly used to measure urinary hydroxyproline.

Although, urinary Hyp has the longest history of use as a resorption, it has now been largely replaced by other bone resorption markers. The main reasons are its lack of specificity for bone, the need to restrict intake of gelatin and other collagen-rich foods to avoid the contributions of exogenous Hyp to urinary measurments, and the contributions of Hyp from the degradation of newly synthesized collagen (Calvo *et al.,*1996).

COLLAGEN CROSS-LINKS

Pyridinoline and Deoxypyridinoline (Chapter 13) Newly deposited collagen fibrils in the extracellular matrix are stabilized by intramolecular and intermolecular cross-links. The main cross-links in skeletal tissues are the trivalent structures, deoxypyridinoline (DPD) and pyridinoline (PYD). The pyridinoline cross-links occur at two sites placed symmetrically at about 90 residues from the ends of the 1000-residue helical domain in the collagen fibril (Fig. 2). Pyridinolines act as mature cross-links in types I, II, and III collagens of all major connective tissues other than skin (Eyre *et al.,* 1984, 1988). This includes type I collagen of bone, dentin, ligaments, fascia, tendon, vascular walls, muscle, and intestine. In all tissues PYD predominates, with Dpy being the minor component. However, DPD is most abundant in bone and is considered the more bone-specific marker since bone represents the major reservoir of total collagen in the body and turns over faster than most major connective tissues. This conclusion is supported by the strong correlations between DPD and resorption rates as determined by radio tracer kinetics (Eastell *et al.,* 1997).

Since only 40–50% of the cross-links are free, many investigators have measured total pyridinolines (peptide bound plus free). These analyses are performed by acid hydrolysis of the urine sample to liberate peptide-bound and conjugated forms, followed by solid-phase extraction, separation by HPLC, and quantitation by fluorescence (Eyre *et al.,* 1984; Black *et al.,* 1988, 1989). Recent advances in chromatographic methods have allowed direct analysis of urine without acid hydrolysis. Good correlations are found between free and total cross-links in normal subjects and in patients with disorders associated with increased bone resorption.

The cross-link markers provide distinct advantages over urinary Hyp. First, they are not influenced by dietary intake (Colwell *et al.,* 1993). Second, they are formed only at the final stages of fibril formation and are therefore unaffected by degradation of newly synthesized collagen. Finally, they are not further metabolized nor are they reused in collagen biosynthesis.

Several direct immunoassays for the cross-links and a number of ELISA systems have been reported (Seyedin *et al.,*1993; Robins *et al.,* 1994; Gomez *et al.,* 1996). Commercial kits are available which measure both DPD and PYD in combination (Pyrilinks II, METRA Biosystems) or DPD alone (Pyrilinks D, METRA Biosystems). Direct comparison of the immunoassays with HPLC shows a high correlation between the methods.

Cross-Linked Telopeptides There are several assays that are based on measurement of peptides associated with the cross-link regions in collagen. These assays are derived from peptides at both ends of the collagen type I molecule. An assay based on a cross-linked peptide from the N-terminal telopeptide of collagen type I, referred to as NTX-I, is based on a monoclonal antibody raised against a peptide isolated from urine of a patient with Paget's disease of bone (Hanson *et al.,* 1992). The antibody specifically recognizes the cross-linked α2(I)N-telopeptide sequence, QYDGKGVG, and in which K (lysine) is involved in the trivalent cross-linkage (Eyre, 1995). *In vitro* studies show that NTX-I can be quantitatively released from bone by the action of cathepsin K, a specific protease of the osteolast. (Atley *et al.,* 2000). The NTX-I ELISA is available in a commercial, microtiter-plate format (Osteomark, Ostex International Inc., Seattle, WA). The assay measures the NTX-I peptide in spot urines,

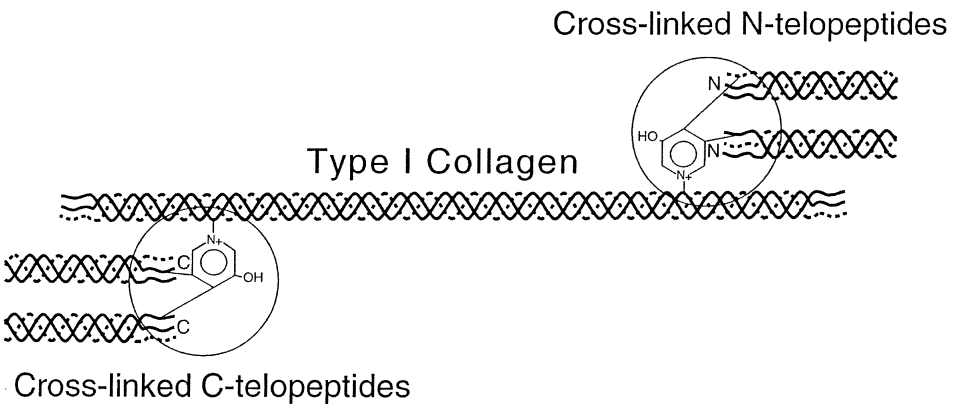

Figure 2 Cross-linked N- and C-telopeptides of type I collagen. The pyridinoline cross-links occur at two intermolecular sites in the collagen fibril: the aminoterminal telopeptide (NTX-I) and the carboxyterminal telopeptide (CTX-I; CTX-MMP).

calibrated in molar equivalents of type I collagen and results are normalized to creatinine. Recently the assay has been developed for use in serum (Clemens *et al.,* 1997).

Several assays for the measurement of the C-terminal telopeptide of type I collagen (CTX-I) in urine have been developed. One assay measures the C-terminal type I collagen telopeptide in serum. The antigenic determinant requires a trivalent cross-link and is generated by matrix metalloproteinase digestion of type I collagen (Risteli and Risteli, 1999). The assay is therefore termed CTX-MMP (previously ICTP). Another assay is based on a polyclonal antibody raised against a synthetic eight-amino-acid peptide (EKAHDGGR) derived from a segment of the C-telopeptide of the collagen α1(I) chain. This assay recognises only a form of the peptide containing an β-aspartyl peptide bond (AHD-β-GGR). The isomerization of aspartyl to a-aspartyl residues occurs over extended periods of time and is associated with the aging of proteins and peptides (Bonde *et al.,* 1997) . The isomerization process reaches a maximum 3 years after the bone is mineralized and thus this assay measures the degradation of relatively old bone (Fledelius *et al.,* 1997) Another CTX-I assay measures only the nonisomerized form of this octapeptide and presumably measures the degradation of relatively young bone (α-Ctx) (Bonde *et al.,* 1997). Both of these assays and a recently developed serum CTX-I assay are commercially available (Osteometer BioTech, Denmark).

Galactosyl Hydroxylysine

Hydroxylysine, another modified amino acid particular to collagens is glycosylated to varying degrees depending on the tissue type (Segrest and Cunningham, 1970). Two glycosides are formed, galactolysyl hydroxylysine (Gal-Hyl) and glucosyl galactosyl hydroxylysine (Glc-Gal-Hyl). Because of tissue-specific differences, Gal-Hyl is considered to be relatively specific to bone collagen degradation (Krane *et al.,* 1977). Glycosylated hydroxylysine residues appear not to be reused or catabolized when collagen is degraded. Furthermore, they do no appear to be absorbed in significant levels from a normal diet (Segrest and Cunningham, 1970). An HPLC method has been employed for measuring Gal-Hyl involving dansylation of the lysine, resolution by reversed-phase HPLC and detection by fluorescence (Moro *et al.,* 1984; Yoshihara *et al.,* 1993). This assay generally shows good correlations with other resorption markers (Moro *et al.,* 1997).

Tartrate-Resistant Acid Phosphatase

During bone resorption, osteoclasts secrete acid and enzymes into the space between the ruffled border of the osteoclast and the surface of the bone. The enzyme, tartrate-resistant acid phosphatase (TRACP) has been identified in both the ruffled border of the osteoclast membrane and the secretions in the resorptive space (Minkin, 1982). Six isoenzymes (Types 0–5) of acid phosphatase have been identified by electrophoresis of human tissues (Yam, 1974). Band 5 is expressed by osteoclasts, alveolar and monocyte-derived macrophages, and the placenta (Cheung *et al.,* 1995). On the basis of the catalytic and ionic properties and carbohydrate content, two isoenzymes of band 5 TRACP have been identified. Both band-5 acid phosphatases are resistant to tartrate inhibition. Band 5a TRACP is sialylated, whereas band 5b, which is predominant in bone, lacks sialic acid and pH optima of the two isoforms differ (Whitaker *et al.,*1989; Lam *et al.,* 1978, 1980; Schiele *et al.,* 1988).

TRACP activity in serum reflects bone resorption rates (Yam, 1974). However, difficulty in distinguishing the osteoclastic enzyme in serum from other tissue acid phosphatases and instability of the enzyme in serum have presented the greatest technical problems. TRACP activity in serum has been measured kinetically (Whitaker *et al.,*1989; Lam *et al.,* 1978, 1980; Schiele *et al.,* 1988; Lau *et al.,* 1987), and more recently by immunoassay (Cheung *et al.,* 1995; Kraezlin *et al.,* 1990; Halleen *et al.,* 2000), but these methods vary in specificity for the osteoclast-derived isoenzyme. Recent assays employed an antibody produced against TRACP from a bone extract. This method showed good correlation between immunoreactivity and enzyme activity, little cross-reaction with acid phosphatases from nonosteoclastic sources, and sensitivity to changes in bone turnover after 6 months of estrogen replacement therapy (Halleen *et al.,* 2000).

Bone Sialoprotein

Bone sialoprotein (BSP) is an acidic, phosphorylated glycoprotein that is synthesized by osteoblasts and osteoclastic-like cell in culture. It has an unglycosylated mass of 33 kDa (glycosylated: 70–80 kDa). Although the function of BSP is still not fully understood, BSP stimulates hydroxyapatite formation *in vitro* and appears to mediate cell–cell interactions via an integrin binding site (Chapter 17). BSP is relatively restricted to bone but it is also expressed by trophoblasts and is strongly upregulated by many tumors. Recently it has been suggested that BSP may play a role in angiogenesis associated with bone formation, tumor growth, and metastasis (Bellahcene *et al.,* 2000). A small amount of BSP is found in the circulation and as such is a potential marker of bone turnover (Seibel *et al.,* 1996).

Variability

Variability can have a serious impact on the clinical interpretation of levels of markers of bone turnover in metabolic bone disease, particularly in diseases such as osteoporosis where only subtle differences in markers are found. The analytical precision of both the manual and the automated assays is minimal. Thus the major component of variability is preanalytical variability. The sources of preanalytical variability can be considered to fall into broad categories: (1) uncontrollable sources such as age, gender, menopausal status, drugs, or recent fracture and (2) controllable sources such as circadian, menstrual, or exercise effects.

Uncontrollable Sources of Variability

Age and Gender

Levels of markers of bone turnover are highest in the first 2 years of life (Szulc *et al.,* 2000). They decrease during childhood but increase again during puberty to 4–10 times the adult level (Blumsohn *et al.,* 1994a) and then decrease to adult levels. The pattern of change in markers with age is different in men and women. The pubertal increase occurs later in boys than in girls and levels of markers do not reach a nadir until the fifth or sixth decade, after which they may increase slightly in eighth and ninth decades (Fatayerji and Eastell, 1999). In women, marker levels reach a nadir in the fourth and fifth decades and then increase rapidly (Garnero *et al.,* 1996a) at the menopause and remain elevated into the eighth and ninth decades. Mean concentrations of urinary telopeptides are up to 100% higher and serum telopeptides 55–70% higher in postmenopausal women than in premenopausal women, whereas bone formation markers are between 20 and 50% higher (Clemens *et al.,* 1997; Ravn *et al.,* 1996; Woitge *et al.,* 1996). However, there is considerable overlap between the two groups with only 25% of postmenopausal women having levels of markers above the premenopausal reference range (Garnero *et al.,* 1996b).

Ethnicity

There are no consistent differences in bone turnover markers in young blacks and whites (Henry and Eastell, 2000) although in peri- and postmenopausal women there is a trend for levels to be 5–15% lower in black women than in white women (Bikle *et al.,* 1999). In Asian women, uNTX tends to be higher than in white women, but bone formation markers are similar (Alekel *et al.,* 1999).

Oral contraception

Oral contraception may decrease bone turnover slightly in late premenopausal women but in general has little impact on the levels of bone turnover markers. In women ages 20 to 30 years uDPD and uPYD excretion is lowered (Paoletti *et al.,* 2000), while in women in the fifth decade there is a consistent 15–30% decrease in total bone turnover (Garnero *et al.,* 1995).

Fracture

Fracture healing has a significant effect on levels of markers of bone turnover. In the first and second week after fracture they increase rapidly and reach a peak within 8 weeks (Ingle *et al.,* 1999; Ohishi *et al.,* 1998). Markers of bone formation tend to increase more (20–55%) and remain elevated for longer (6–12 months) than markers of bone resorption (6 months).

Pregnancy

Large increases in markers of bone turnover are observed during pregnancy and lactation. Bone resorption, followed by bone formation, starts to increase in the second trimester. At term, urinary NTX-I, CTX-I and pyridinolines are two- to threefold higher than before pregnancy (Naylor *et al.,* 2000). Bone formation markers, with the exception of osteocalcin, are increased by 35–100% in the third trimester (Cross *et al.,* 1995; Naylor *et al.,* 2000; Rodin *et al.,* 1989). The lack of change in osteocalcin may be due to degradation by the placenta and the increase in renal function during pregnancy. Markers decrease gradually in the first 6 to 12 months postpartum in all mothers, but in lactating mothers the decrease is less rapid (Kent *et al.,* 1990; Yamaga *et al.,* 1996). Once lactation ceases, markers gradually return to the levels found in the nonlactating premenopausal women (Kalkwarf *et al.,* 1999).

Immobilization

Immobilization leads to an increase in bone resorption which is proportional to the degree of immobilization (Lips *et al.,* 1990). During complete bedrest, uNTX, uDPD, uPYD, and sCTX-MMP increase by 30–80%. The increase in bone resorption occurs rapidly: NTX-I is increased by up to 50% within the first week (Inoue *et al.,* 2000; Zerwekh *et al.,* 1998). During remobilization marker levels gradually return to normal.

Drugs and Diseases

Several types of drugs prescribed for conditions other than metabolic bone disease can substantially affect levels of biochemical markers of bone turnover. Corticosteroids suppress levels of markers of bone formation, particularly osteocalcin which may be depressed by 50% (Oikarinen *et al.,* 1992). Markers of bone resorption are generally unaffected (Ebeling *et al.,* 1998). Short-term high-dose heparin treatment and thiazide both supress bone turnover, osteocalcin being reduced by 40 and 25% respectively (Cantini *et al.,* 1995; Perry *et al.,* 1993). Anticonvulsant treatment leads to a threefold increase in urinary pyridinoline (Ohishi *et al.,* 1996).

Variability of markers of bone turnover may also be affected by concomitant diseases which affect the synthesis of nonosseous sources of markers and/or clearance of specific markers. For instance in liver disease, collagen-derived markers are increased, probably due to the increase in fibrotic tissue and impaired clearance of procollagens (Guanabens *et al.,* 1998); in renal impairment osteocalcin is increased due to decreased clearance by the kidney (Delmas *et al.,* 1983) and in rheumatoid arthritis increased levels of pyridinoline cross-links are derived from inflamed synovium and increased in degradation of cartilage (Gough *et al.,* 1994).

Controllable Sources of Variability

Circadian

Circadian variability probably has more impact on preanalytical variability of markers of bone turnover than any other factor. Both resorption and formation markers exhibit significant circadian rhythms increasing at night, reaching a peak between 01.30 and 08.00 and falling during the day to

a nadir in the afternoon (Blumsohn *et al.,* 1994b; Eastell *et al.,* 1992). The amplitude of the rhythm is greater for resorption markers than for formation markers. For serum CTX-I and urinary DPD the amplitude of the rhythm may be as much as 80% of the 24 hr mean, whereas for PICP and osteocalcin it is 5–20% of the 24-hr mean (Blumsohn *et al.,* 1994b; Greenspan *et al.,* 1997; Wichers *et al.,* 1999). The importance of precise timing of sample collection cannot be overemphasised. For example if urinary NTX-I is measured in samples collected at 07.00 and at 15.00, there will be a 50% difference which is comparable to the change in NTX-I expected after 6 months of HRT.

MENSTRUAL

The variation in levels of markers of bone turnover during the menstrual cycle is small, less than 20% (Gorai *et al.,* 1998), and may be regarded as insignificant.

SEASONAL

Woigte *et al.* (1998a, 2000a) have demonstrated that there is seasonal variability in markers of bone turnover of up to 12% with levels being 20% higher in winter than in summer. Seasonal changes depend on the latitude and possibly diet of the population (Rosen *et al.,* 1994).

EXERCISE

The effect of exercise on levels of bone markers depends on several factors including the age of the subject, the type and intensity of the exercise, and whether it is aerobic or anaerobic (Woitge *et al.,* 1998b). The acute effect of exercise may be to increase collagen turnover (PICP, CTX-MMP, and urinary pyridinolines) by up to 20% (Wallace *et al.,* 2000) either transiently or for as much as 72 hr depending on the type of exercise (Landberg *et al.,* 1999). Although it is difficult to define the precise effect of exercise on bone turnover it is advisable to that subjects do not exercise for 24 hr before samples are collected for the measurements of markers of bone turnover.

Within Subject Variability

In the interpretation of markers of bone turnover, within-subject variability should be kept to a minimum. Table IV summarizes the published data on the short (<8 weeks)- and long-term (>8 weeks) within-subject variability of markers of bone turnover. The greatest variability is found in the urinary markers of bone resorption. The variability of the newer serum markers of bone resorption is smaller and similar to that of the formation markers which are also measured in serum (Eastell *et al.,* 2000). It should be borne in mind that these values were derived from well controlled research studies and that in clinical practice within-subject variability will be greater. It should also be noted that published values for the variability of a marker depend on the subjects, the length of study and on the method of calculation of the coefficient of variation. Uncontrollable sources of variability can be accounted for by using the appropriate age- and gender-specific reference ranges and by using published data to account for other sources such as drugs and fracture healing. Controllable sources of variability can be minimized by standardizing the timing and conditions of sample collections. The within subject variability of markers can also be minimized by measuring multiple samples or by making a single measurement of pooled samples (Popp-Snijders *et al.,* 1996).

Clinical Use

Metabolic Bone Diseases

PAGET'S DISEASE OF BONE

Paget's disease of bone is the paradigm of high turnover skeletal disorders. The active disease is characterized by a brisk increase in both osteoblast and osteoclast function (see Chapter 71). Not surprisingly, changes in bone metabolic markers are usually very pronounced during active states. While the diagnosis of Paget's disease of bone and the extent of skeletal involvement is assessed primarily through radiographic analysis and bone scans, biochemical markers of bone metabolism are used to define the activity of the disease and its response to treatment.

Alkaline phosphatase activity is the classical biochemical marker of activity in Paget's disease of bone. Serum levels of both the total (total ALP) and bone-specific (bone ALP) enzymes are markedly increased in the active state, whereas normal or only slightly elevated levels may be seen in patients with mono-ostotic or inactive disease (Adami *et al.,* 1993; Alvarez *et al.,* 1995, 2000; Braga *et al.,* 1993; Deftos *et al.,* 1991; Duda *et al.,* 1988; Hyldstrup *et al.,* 1988; Garnero and Delmas, 1993; Moss, 1982, 1984a,b; Panigrahi *et al.,* 1994). Particularly high levels of serum bone ALP are typically seen in patients with involvement of the skull, and in rare cases of sarcomatous degeneration of Pagetic bone. In most cases, serum levels of both total and bone ALP are highly correlated with measurements of serum procollagen propeptides (but not of osteocalcin) and with the urinary concentrations of various bone resorption markers (Alvarez *et al.,* 1995; Deftos *et al.,* 1991; Hyldstrup *et al.,* 1988; Kaddam *et al.,* 1994; Torres *et al.,* 1989). These observations strongly indicate that even in the active stage of Paget's disease, coupling of anabolic and catabolic processes is still preserved.

Serial measurements of serum total or bone ALP may be employed to monitor the therapeutic response (Garnero and Delmas, 1993, Alvarez *et al.,* 1995, 2000; Woitge *et al.,* 2000) and also to detect a recrudescence of disease activity in the stable patient (Fig. 3). However, although posttherapeutic changes of bone ALP are somewhat more pronounced than those of total ALP, its longitudinal diagnostic sensitivity is rarely superior to that of total ALP.

Although osteocalcin is considered to be a specific osteoblast product, its serum concentrations are often low in pagetic patients, even if high levels of serum total or bone

Table IV Total within Subject Variability of Markers of Bone Turnover

Marker	Short-term CVti (%)	Study period	References	Long-term CVti (%)	Study period	References
Formation						
OC	6.5–12.7	1–4 Weeks	Jensen *et al.,* 1994[a]; Panteghini *et al.,* 1995[a]; Braga de Castro *et al.,* 1999	7.7–27.3	6–9 Months	Hannon *et al.,* 1998[b]; Gertz *et al.,* 1994
Total ALP	5.0–6.3	4 Weeks	Jensen *et al.,* 1994[a]; Panteghini *et al.,* 1995[a]	5.5–13.6	6–20 Months	Alvarez *et al.,* 2000[a]; Morris *et al.,* 1990[b]; Hannon *et al.,* 1998[b]
Bone ALP	7.1–12.8	1–4 Weeks	Panteghini *et al.,* 1995[a]; Braga de Castro *et al.,* 1999	7.3–9.3	6–12 Months	Alvarez *et al.,* 2000[a]; Rosen *et al.,* 2000[b]; Hannon *et al.,* 1998[b]
PINP				7.5–8	6–12 Months	Alvarez *et al.,* 2000[a]; Hannon *et al.,* 1998[b]
PICP	10.6	1 Week	Braga de Castro *et al.,* 1999	8.6–9	3–6 Months	Stevenson *et al.,* 1997; Hannon *et al.,* 1998[b]
Resorption						
UHyp	17.3–40.6	2 Days–5 Weeks	Jensen *et al.,* 1994[a]; Vasikaran *et al.,* 1994; Beck Jensen *et al.,* 1997[b]	19.0–53.0	6–20 Months	Alvarez *et al.,* 2000[a]; Hannon *et al.,* 1998[b]; Morris *et al.,* 1990[b]; Gertz *et al.,* 1994
UPYD (HPLC)	10.3–26.0	5 Days–5 Weeks	Jensen *et al.,* 1994[a]; Colwell *et al.,* 1993; Beck Jensen *et al.,* 1997[b]; McLaren *et al.,* 1993; Ginty *et al.,* 1998			
UDPD (HPLC)	12.2–26.0	5 Days–5 Weeks	Jensen *et al.,* 1994[a]; Colwell *et al.,* 1993; Beck Jensen *et al.,* 1997[b]; McLaren *et al.,* 1993; Ginty *et al.,* 1998	17.1–69.8	6–9 Months	Hannon *et al.,* 1998[b]; Gertz *et al.,* 1994
UfDPD	10.3–11.6	5 Days–1 Week	Popp-Snijders *et al.,* 1996; Braga de Castro *et al.,* 1999	9.3–16.3	5–12 Months	Ju *et al.,* 1997; Rosen *et al.,* 2000[b]; Hannon *et al.,* 1998[b]
UfPYD	15.0–19.1	1–4 Weeks	Panteghini *et al.,* 1996[a]; Seyedin *et al.,* 1993; Braga de Castro *et al.,* 1999	13.2	6 Months	Hannon *et al.,* 1998[b]
UNTX-I	10.0–33.1	3 Days–5 Weeks	Orwoll *et al.,* 1998; Popp-Snijders *et al.,* 1996; Beck Jensen *et al.,* 1997[b]; Gertz *et al.,* 1994; Braga de Castro *et al.,* 1999	15.6–27.0	3–12 Months	Alvarez *et al.,* 2000[a]; Ju *et al.,* 1997; Rosen *et al.,* 2000[b]; Orwoll *et al.,* 1998; Hannon *et al.,* 1998[b]; Gertz *et al.,* 1994
SNTX-I	6.3	3 Days	Eastell *et al.,* 2000	7.5	2 Months	Eastell *et al.,* 2000
UCTX-I				26.3–47.9	5–36 Months	Alvarez *et al.,* 2000[a]; Ju *et al.,* 1997; Hannon *et al.,* 1998[b]; Garnero *et al.,* 1999
SCTX-I	7.9–14.3	2 Weeks	Christgau *et al.,* 2000	13.1–15.1	1 year	Alvarez *et al.,* 2000[a]; Rosen *et al.,* 2000[b]; Christgau *et al.,* 2000
UCTX-MMP				8.2–10.0	3–6 Months	Stevenson *et al.,* 1997; Hannon *et al.,* 1998[b]
TRACP	12.7	4 Weeks	Panteghini *et al.,* 1995[a]	6.2	6 Months	Hannon *et al.,* 1998[b]

[a]Total within subject CV (CV_{ti}) calculated from published data $CV_{ti} = \sqrt{(CV_i^2 + CV_a^2)}$.
[b]Total within subject CV (CV_{ti}) calculated from published value of least significant change (LSC).

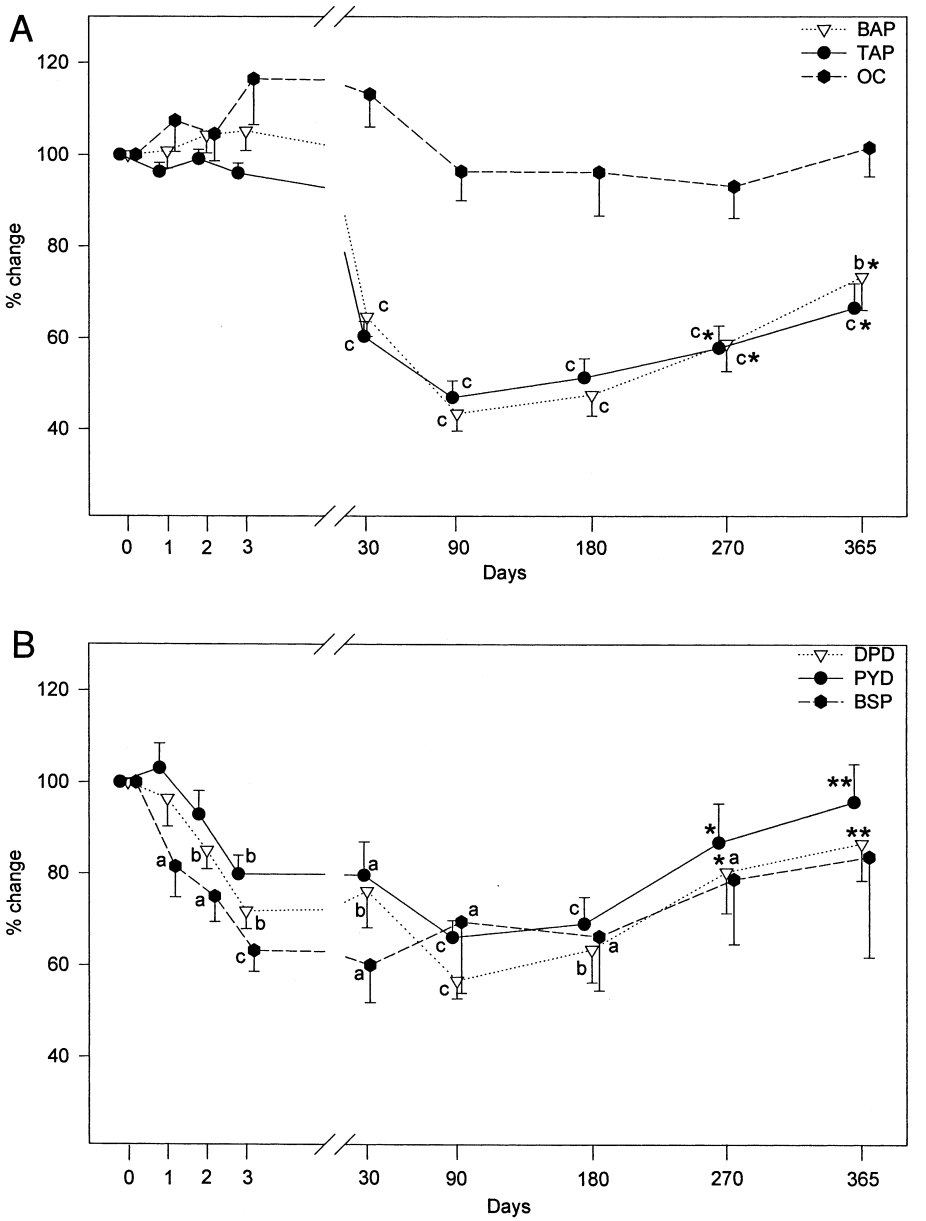

Figure 3 Effect of ibandronate treatment on markers of bone turnover in patients with Paget's disease of bone. Patients were treated with a single dose of 2 mg Ibandronate at day 0 and followed for 1 year. Results are mean ± SE. (A) Bone formation markers. Note that total ALP (TAP) and bone-specific ALP (BAP) show almost identical curves during the entire study period. (B) Bone resorption markers. Reprinted with permission from Woitge *et al.* (2000).

ALP, and urinary pyridinium cross-links are observed. Consequently, correlations of osteocalcin with other markers of bone formation or resorption are usually weak (Duda *et al.,* 1988; Ebeling *et al.,* 1992; Kaddam *et al.,* 1994; Papapoulos *et al.,* 1987), and unlike other markers of bone turnover, serum osteocalcin levels tend to rise after bisphosphonate treatment (Papapoulos *et al.,* 1987). Although the reasons for these discrepancies are currently not well understood, most authors seem to agree that osteocalcin measurements are of no particular value in Paget's disease of bone.

Serum levels of the carboxyterminal procollagen type I propeptide (PICP) are usually elevated in Paget's disease of bone. While earlier reports show changes in PICP concentrations comparable to those of serum total ALP (Simon *et al.,* 1984), newer studies utilizing different antibodies observed only minor variations in PICP levels with *Z* scores 15 times lower than those of serum bone ALP (Ebeling *et al.,* 1992). Interestingly, in the latter study, no changes are seen in the serum levels of the aminoterminal propeptide of procollagen type I (PINP), whereas in contrast, significantly elevated levels of the aminoterminal propeptide of type III procollagen (PIIINP) and declining levels following bisphosphonate treatment have been observed (Simon *et al.,* 1984).

Table V Factors Affecting the Serum Levels of Total Alkaline Phosphatase

Increase	Decrease	Increase or decrease
Somatic growth	Growth hormone deficiency	
Paget's disease of bone	Hypoparathyroidism	
Rickets and osteomalacia	Hypothyroidism	
Renal osteodystrophy	Familial or sporadic hypophosphatasemia	
Hyperparathyroidism (severe)	Achrondroplasia	
Thyrotoxicosis		
Acromegaly (active)		
Skeletal malignancies (osteosarcoma)		
Multiple myoloma		
Metastatic bone disease (osteoblastic)		
Osteogenesis imperfecta		
Fibrous dysplasia		
Familial hyperphosphatasemia		
Idiopathic hyperphosphatasemia		
Hyperostosis frontalis interna		
Recent fractures		
Age	Anemia (severe)	Race
Hepatic and biliary diseases, incl.		Diurnal rhythms
Hepatotoxicity and malignancies		Seasonal rhythms
Other malignancies (production of Ectopic AP, e.g., Regan-, Nagao-AP)		Rheumatoid arthritis
Late pregnancy (placental AP)		
Treatment with	*Treatment with*	
Corticosteroids	Bisphosphonates	
Anticonvulsants	Calcitonin	
Fluorides	Estrogen	
Growth hormones		
Vitamin D_3		

In untreated patients with active Paget's disease of bone, a marked elevation is generally observed in the urinary excretion of pyridinium cross-links and cross-linked collagen telopeptides (Robins, 1982; Robins *et al.,* 1991, 1994; Woitge *et al.,* 1999) (Figs. 3 and 4). These changes are usually more pronounced than those seen with urinary Hyp, hydroxylysine glycosides, and serum TRACP (Colson *et al.,* 1990; Michalsky *et al.,* 1995; Patel *et al.,* 1995; Scarnecchia *et al.,* 1991), suggesting that the cross-link-related markers provide the most sensitive index of bone resorption in Paget's disease of bone. Longitudinal studies monitoring the effect of intravenous bisphosphonates on bone turnover in pagetic patients generally demonstrate a rapid decrease in the urinary excretion of cross-link components (Robins *et al.,* 1991; Knaus *et al.,* 1996; Woitge *et al.,* 2000) (Fig. 3). Bisphosphonate treatment appears to have more pronounced effects on the excretion of the cross-linked telopeptide (peptide-bound) components than on the peptide-free pyridinium derivates (Garnero *et al.,* 1995a). However, it is neither clear whether the differences between cross-link markers observed after bisphosphonate therapy are due to changes in skeletal or extraskeletal metabolism, nor whether these differences are indicative of the clinical sensitivity and usefulness of the various markers. (Woitge *et al.,* 1996; Woitge and Seibel, 1997). In contrast, much evidence has accrued that posttherapeutic changes in urinary Hyp, hydroxylysine glycosides, serum bone ALP, and TRACP occur either later or are less pronounced than those of the cross-link derivatives (Garnero *et al.,* 1995a; Knaus *et al.,* 1996; Robins *et al.,* 1994; Woitge *et al.,* 2000).

Serum total TRACP and the osteoblast-specific TRACP5b are both highly elevated in active Paget's disease of bone (Halleen *et al.,* 2000). Similarly, BSP is found in high concentrations in patients with active disease (Seibel *et al.,* 1996; Woitge *et al.,* 1999). After treatment with bisphosphonates, a rapid and sustained fall in sBSP levels is seen (Woitge *et al.,* 2000).

When the various biochemical markers of bone turnover are compared with regard to their discriminative power, serum total and bone ALP activities and the urinary pyridinoline cross-links appear to provide the most useful information (Alvarez *et al.,* 1995). However, as pagetic bone lesions

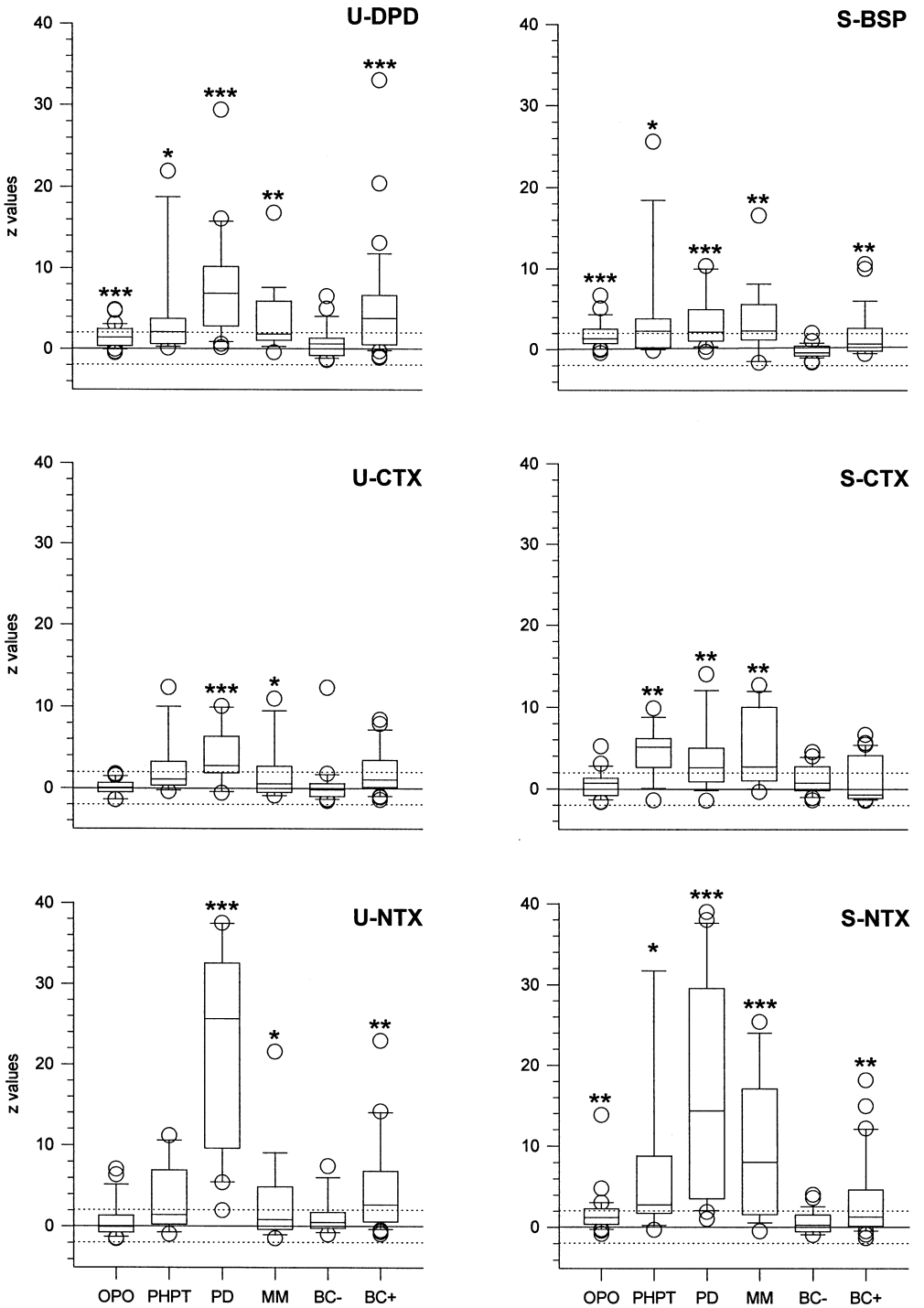

Figure 4 Serum and urinary markers of bone resorption in metabolic bone diseases. OPO, osteoporosis; PHPT, primary hyperparathyroidism; PD, Paget's disease of bone; MM, multiple myeloma; BC-, breast cancer without bone metastases; BC+, breast cancer with bone metastases. DPD, deoxypyridinoline; BSP, bone sialoprotein; CTX-I, C-terminal telopeptide of type I collagen; NTX-I, N-terminal telopeptide of type I collagen; S, serum; U, urine. Shown are box-and-wisker plots, where the horizontal line within the box represents the group median. Circles are individual values above and below the 95th percentile, respectively. All values are *z* values as compared to a healthy, age-matched population. The zero (solid) line represents the mean of the control group, the dotted lines the upper and lower limit of normal, respectively ($\pm$ 2 SD). Level of significance: *P* versus controls $^{*}<0.05$; $^{**}<0.01$; $^{***}<0.001$. Reprinted with permission from Woitge *et al.* (1999).

Table VI Factors Affecting the Serum Levels of Osteocalcin

Increase	Decrease	Increase or decrease
Somatic growth	Growth hormone deficiency	Metastatic bone disease
Acromegaly	Hypoparathyroidism	Hypercalcemia of malignancy
Hyperparathyroidism	Hypothyroidism	Osteomalacia
Hyperthyroidism	Senile osteoporosis (intact OC)	Paget's disease of bone
Malignancies		Postmenopausal osteoporosis
Renal osteodystrophy		
Osteogenesis imperfecta		
Recent fractures		
Senile osteoporosis (undercarboxylated OC)		
Age beyond 50 years	Alcoholism	Race
Female sex	Anorexia nervosa	Diurnal rhythms
Chronic renal failure	Liver disease	Menstrual cycle
Exercise	Microprolactinoma	Seasonal rhythms
Lactation	Pregnancy	Osteoarthritis
Obesity		Rheumatoid arthritis
Treatment with	*Treatment with*	
Fluorides	Bisphosphonates	
Growth hormones	Calcitonin	
Thyroid hormones (TSH-suppressive)	Corticosteroids	
Vitamin D_3	Estrogen	
Anticonvulsants		

usually represent a severe perturbation of bone metabolism, the diagnosis does not require extremely sensitive methods. Metabolic changes might therefore be easily monitored by markers such as serum total ALP or urinary Hyp. At least in active Paget's disease of bone, most of the newer metabolic markers do not extend any particular benefit over the conventionally used indices.

Primary Hyperparathyroidism

Primary hyperparathyroidism (PHPT) is characterized by overproduction of parathyroid hormone which usually results in hypercalcemia, hypophosphatemia, and hypercalciuria. While in years past, PHPT was associated with gross bone involvement, symptomatic bone pain and significantly elevated levels of serum bone ALP, the clinical profile of the disorder has now changed toward a predominance of asymptomatic cases. Nonetheless, accelerated bone turnover and loss of predominantly cortical bone is seen in many patients with PHPT (Silverberg *et al.,* 1989; Cortet *et al.,* 2000).

In asymptomatic PHPT, serum total bone ALP is usually not significantly elevated, although in active disease, levels tend to be found in the upper third of the normal range (Silverberg *et al.,* 1995). In contrast, symptomatic patients often show elevated levels of both serum total bone ALP and osteocalcin (OC) (Minisola *et al.,* 1989). Both serum total bone ALP and OC correlate positively with serum calcium concentrations (Charles *et al.,* 1986; Hyldstrup *et al.,* 1988; Parfitt *et al.,* 1987; Yoneda *et al.,* 1986), other markers of bone formation and resorption (Arbault *et al.,* 1995; Minisola *et al.,* 1989; Stepan *et al.,* 1983; Torres *et al.,* 1989; Yoneda *et al.,* 1986), but inversively with bone mineral density measurements (Minisola *et al.,* 1989; Pfeilschifter *et al.,* 1992; Yoneda *et al.,* 1986). Following successful parathyroidectomy, serum concentrations of total bone ALP and OC usually decline within several weeks to months (Deftos *et al.,* 1982; Seibel *et al.,* 1992; Silverberg *et al.,* 1999).

Due to other sources such as the liver, serum total bone ALP is considered somewhat unspecific for bone. Efforts have therefore been made to develop assays for the measurement of the bone-specific isoenzyme of bone ALP. Among the numerous methods described, electrophoretic separation, lectin precipitation, and immunoassay techniques have now reached routine clinical applicability. So far, clinical studies have confirmed a higher specificity (i.e., a lower number of false positive results) for the bone-specific assays, but sensitivity seems to remain a controversial issue. While some studies found the bone-specific isoenzyme to be a more sensitive parameter of bone involvement than total bone ALP (Braga *et al.,* 1993; Garnero and Delmas, 1993), other reports found no improvement in the discrimination between normals and patients with PHPT (Stepan *et al.,* 1987a; Silverberg *et al.,* 1991; Woitge *et al.,* 1996; Woitge and Seibel, 1997). For clinical purposes, neither serum total or bone-specific bone ALP nor serum osteocalcin seems to possess distinct diagnostic value in PHPT, but serial measurements may be useful in the follow-up and monitoring of individual patients.

Table VII Expected Changes in Markers of Bone Turnover in Various Clinical Situations

	AP	OC	PICP	OHP	OHLyG	XL	ICTP	TRACP
Postmenopausal OP	⇔	↑[a]	⇔	↑[a]	↑[a]	↑[a]	↑[a]	↑[a]
Senile OP	⇔	↑[b]	⇔	⇔	⇔	⇔	⇔	⇔
Primary HPT	↑[c]	↑[c]	↑[c]	↑[c]	↑[c]	↑	↑	↑[c]
Osteomalacia[d]	⇑	Variable	↑	↑	↑	⇑	⇑	↑
Paget's disease	⇑	Variable	↑[c]	⇑	↑	⇑	⇑	↑
Hyperthyroidism	↑[c]	↑[c]	⇔	⇔	↑	↑[c]	↑[c]	↑[c]
Hypoparathyroidism	⇔	↓	⇔	⇔	⇔	↓	↓	↓
Multiple myeloma	↑[c]	⇑[e]	Variable	⇑	⇑	⇑	⇑ ⇑	↑
Skeletal metastases	↑[cf]	↑[cf]	⇔[g] ↑[f]	⇑[c9]	↑[c9]	⇑[9]	⇑[9]	↑[c9]
Immobilization	⇔/↑	↑	⇔/↑	⇔/↑	⇔/↑	↑	↑	⇔/↑
Glucocorticoid excess	Variable	⇓	↓	⇔/↑	↑	↑	↑	↑
Rheumatoid arthritis	⇔	Variable	↑[c]	⇔/↑	↑	⇑	⇑	⇔/↑
Rec. fractures w/o OP	↑	↑	⇔/↑	⇔/↑	↑	↑	↑	↑
Post-TPX (early)	↓	⇓	⇓	↑	↑	⇑	↑	↑
Renal failure w/o BD	⇔	⇑ ⇑	⇔	⇔	⇔	⇔	⇑ ⇑	⇔
Hepatic failure w/o BD	⇑ ⇑	⇔	⇑ ⇑	⇔/↑	⇔/↑	⇔/↑	⇑	⇔

Note. OP, osteoporosis; HPT, hyperparathyroidism; BD, bone disease; TPX, transplantation; AP, serum alkaline phosphatase; OC, serum osteocalcin; CICP, serum carboxyterminal propeptide of type I procollagen; OHP, urinary hydroxyproline; OHLyG, urinary hydroxylysine glycosides; XL, pyridinium cross-links; ICTP, cross-linked telopeptide of type I collagen; TRACP, tartrate-resistant acid phosphatase. ↑, Elevated; ⇑, strongly elevated; ⇑ ⇑,invariably elevated; ↓, decreased; ⇓, strongly decreased; ⇔, no change.

[a]Only in high turnover disease.
[b]Undercarboxylated OC only.
[c]Only in extensive or active disease.
[d]Due to malabsorption, Vitamin D deficiency and/or chronic hypocalcemia.
[e]Inversely correlated with stage of disease.
[f]Osteoblastic metastases.
[g]Osteolytic metastases.

The amino- and carboxyterminal propeptides of procollagen are considered markers of osteoblast activation and of skeletal collagen synthesis. Significant correlations with cancellous bone formation have been demonstrated for the carboxyterminal propeptide (Parfitt *et al.,* 1987), and at least the amino-terminal propeptide seems to be a useful marker of bone metabolism in PHPT (Ebeling *et al.,* 1992; Coen *et al.,* 1994; Minisola *et al.,* 1994).

As PTH is known to stimulate osteoclast activity indirectly, changes in bone resorption markers are to be expected. However, in most patients with asymptomatic or mild PHPT, urinary Hyp is normal or only slightly elevated (Hyldstrup *et al.,* 1984, Silverberg *et al.,* 1989; Seibel *et al.,* 1992). This observation can be largely attributed to the low specificity of Hyp to skeletal tissues. In contrast, urinary concentrations of the pyridinium cross-links of collagen, pyridinoline and deoxypyridinoline, are elevated in approximately two-thirds of patients with asymptomatic or mild PHPT (Seibel *et al.,* 1992; Silverberg *et al.,* 1999). For obvious reasons, the changes seen in these patients are of lesser magnitude than those observed in Paget's disease of bone, and the individual values tend to vary within a wide range (Robins *et al.,* 1991; Seibel *et al.,* 1992; Minisola *et al.,* 1997; Woitge *et al.,* 1999, 2000) (Fig. 4). This variation is explained, at least in part, by the fact that the urinary concentrations of PYD and DPD closely correlate with the disease activity. After successful parathyroidectomy, urinary levels of both cross-linked compounds fall quickly. These changes precede those of urinary Hyp and serum alkaline phosphatase by approximately 6 months. Pyridinium cross-links thus may be good markers for monitoring the postsurgical correction of elevated bone remodeling (Seibel *et al.,* 1992; LoCascio *et al.,* 1994; Guo *et al.,* 1996).

Total serum TRACP is often elevated in patients with PHPT (Kraenzlin *et al.,* 1990). A correlation with the extent of bone involvement (Stepan *et al.,* 1987a), other markers of bone metabolism (Scarnecchia *et al.,* 1991) and histomorphometric parameters of bone resorption (Colson *et al.,* 1990) has been described. Remarkably, elevated serum TRACP values decline to the normal range within days after successful parathyroidectomy, preceding changes in serum bone ALP or OC (Scarnecchia *et al.,* 1991; Stepan *et al.,* 1987a, de la Piedra *et al.,* 1994). More recently, osteoclast-specific TRACP5b has been studied and significantly elevated serum levels have been reported (Halleen *et al.,* 2000).

Although very few studies are presently available, serum BSP also seems to be a valid marker of bone turnover in patients with PHPT. In a cross-sectional study, serum BSP

levels were significantly elevated over normal, and a positive correlation was observed with both urinary PYD and DPD (Seibel *et al.,* 1996; Woitge *et al.,* 1999) (Fig. 4).

Postmenopausal and Senile Osteoporosis

In contrast to metabolic bone diseases such as Paget's disease and renal osteodystrophy, osteoporosis is a condition where subtle modifications of bone turnover can lead to a substantial loss of bone mass after a long period of time (Chapter 73). The slight abnormalities explain why most conventional markers, like serum total ALP and urinary hyp, are in the normal range in most patients with osteoporosis.

Prediction of Bone Loss A single measurement of bone mineral density can provide a useful estimate of fracture risk. However, besides bone mass level, bone loss is also regarded as a potential predictor of fracture risk (Kanis, 1994). Longitudinal studies of early postmenopausal women suggest that about 35% lose significant amounts of bone mineral rapidly (the so-called "fast losers"), whereas 65% lose bone mineral more slowly ("slow losers") (Christiansen *et al.,* 1987, 1990). The rate of bone loss in postmenopausal women may be assessed indirectly by the use of biochemical markers. Several studies have shown a sharp increase by about 30 to 100% of both formation and resorption markers at the menopause and a return to premenopausal levels after a few months of hormone replacement therapy (Christiansen *et al.,* 1985; Crilly *et al.,* 1980; Seibel *et al.,* 1993; Stepan *et al.,* 1987b). In addition, fast losers appear to have elevated concentrations of these markers compared to slow losers (Christiansen *et al.,* 1987, 1990; Dresner-Pollak *et al.,* 1996). These early studies mostly examined bone loss from the forearm and are in keeping with the results using recently developed markers to predict forearm bone loss over 4 years (Garnero *et al.,* 1999a). These new markers have similar predictive value for forearm, hip, and spine bone loss (Rogers *et al.,* 2000; Marcus *et al.,* 1999; Ross and Knowlton, 1998) (Fig. 5).

Despite these encouraging results, no consensus has been reached about whether biochemical markers provide a significant benefit in the context of identifying fast bone losers. First of all, more data are needed to show that fast losers identified soon after the menopause will remain fast losers. Hui *et al.,* (1990) found that bone mass measurements at the forearm showed poor correlations between rates of loss in sequential 5-year periods. In other words, the predictive relationship between biochemical measurements and the rate of bone loss could likewise vary with time (Blumsohn and Eastell, 1992). Furthermore, biochemical markers reflect the rate of turnover in the whole skeleton, instead of the turnover at a particular skeletal site. In fact, in the studies of Christiansen *et al.,* (Christiansen *et al.,* 1987, 1990; Hansen *et al.,* 1991), the panel of biochemical markers predicts bone loss only in the proximal forearm. Finally, the interpretation of biochemical markers clearly depends upon the timing of the sample with respect to the interval since menopause, since rates of loss are greatest in the immediate postmenopausal period. More long-term prospective studies are needed to assess the accuracy of biochemical markers in the prediction of bone loss at different skeletal sites.

Bone Turnover in Subjects with Osteoporotic Fractures In patients with untreated osteoporosis, biochemical markers of bone turnover may vary over a wide range of values and may often exceed the normal range. Despite the somewhat heterogenous results of the various assays for markers of bone turnover, most studies in postmenopausal osteoporotic women indicate a higher rate of both resorption and formation (Seibel *et al.,* 1993; Eastell *et al.,* 1993; Garnero *et al.,*

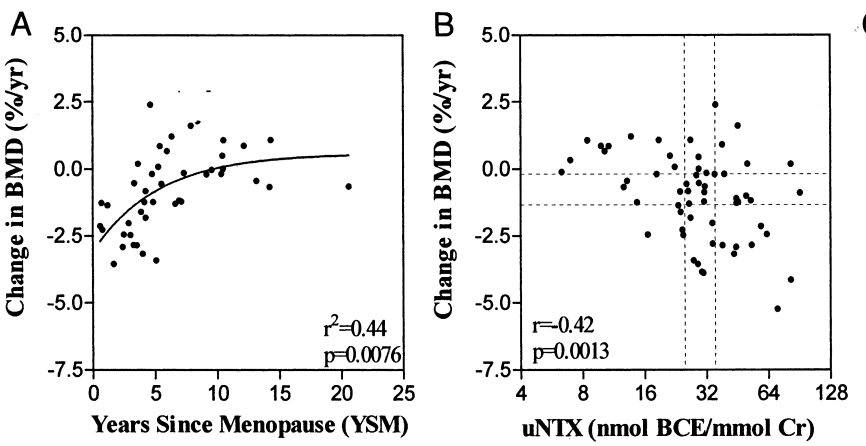

Figure 5 The relationship between change in bone mineral density of the spine and bone turnover. (A) Note how the bone loss is most rapid in the first 5 years after the menopause. (B) The higher the rate of bone resorption (measured by NTX-I in a second morning void urine sample and expressed as a ratio to creatinine) the greater the rate of bone loss. (C) The ability of bone resorption markers to classify individuals into tertile of bone loss is poor with a kappa score of less than 0.2, making this approach unsuitable for use in the individual. Adapted from Rogers *et al.* (2000).

1994a; Kushida *et al.,* 1995). Nevertheless, none of the currently available biochemical markers provides sufficient diagnostic information to be useful in the screening for the presence or likelihood of vertebral fractures (Seibel *et al.,* 1994).

In hip fracture patients studied immediately after the fracture occurred, resorption markers appear to be increased to a greater extent than formation markers (Akesson *et al.,* 1993, 1995a,b; Cheung *et al.,* 1995a), suggesting an imbalance between bone formation and bone resorption. It remains possible that this potential uncoupling of formation and resorption partly reflects the effect of age or also events associated with hip fracture. In postmenopausal women, Kelly *et al.* (1989) provided evidence for a decline in formation markers in elderly postmenopausal women, whereas resorption markers remain at the same level. The observed abnormalities in bone turnover in patients with vertebral and hip fractures may play a role in the decrease of bone mass and consequently the increased bone fragility that characterizes the osteoporotic fracture.

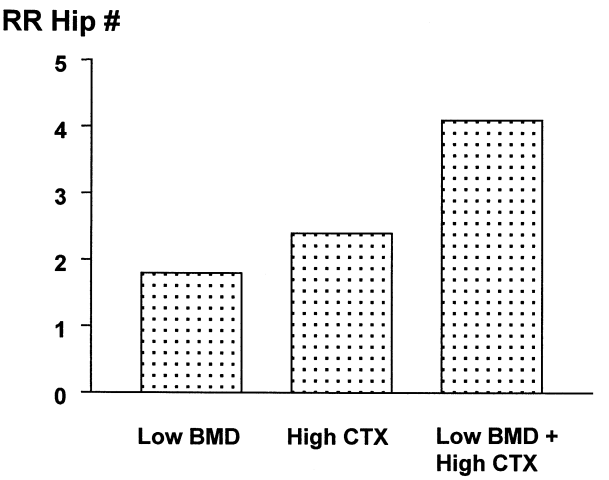

Figure 6 Predictive value of bone mineral density and bone turnover. The risk of hip fracture is higher in women with low hip bone mineral density ($T < -2.9$), high urinary CTX-I ($T > 2$), and even higher with both low bone mineral density ($T < -2.5$) and high urinary CTX-I ($T > 2$). Figure produced from data based on Garnero *et al.* (1998).

Prediction of Osteoporotic Fractures (Chapter 74) High rates of bone turnover may be associated not only with bone loss but also with a disruption of the trabecular network that gives bone its structural integrity. Loss of connectivity through disruption of the trabecular network is not necessarily reflected in a decrease of bone mass. In other words, biochemical markers may be independent predictors of fracture by providing insight into this qualitative feature of bone.

In a 12-year follow-up study, vertebral fractures occurred in those subjects with the highest rates of loss, as assessed by a panel of biochemical markers (Hansen *et al.,* 1991). In a prospective study (including 17 incident hip fractures), we obtained evidence that both urinary pyridinoline cross-links (as determined by HPLC) and free deoxypyridinoline (as determined by ELISA) are associated with an increased hip fracture risk (van Daele *et al.,* 1996). Similar results have been obtained in a larger prospective study of 120 elderly women who sustained a hip fracture during a 22-month follow-up study (Garnero *et al.,* 1996). In both studies, markers of formation (i.e., serum osteocalcin and bone alkaline phosphatase) were not associated with an increased hip fracture risk. However, in two recent studies high levels of bone alkaline phosphatase were associated with increased fracture risk (Ross *et al.,* 2000; Garnero *et al.,* 2000).

A different situation may be present in elderly subjects with osteoporosis. In this population, the effect of aging per se and possibly also a deficiency in vitamins K and D may lead to an impairment in the γ-carboxylation of osteocalcin and ultimately to an increase in the proportion of partially undercarboxylated circulating osteocalcin. Certain studies indicate that the proportion of undercarboxylated osteocalcin in serum may be an important determinant of femoral bone mineral density in elderly women, as high serum levels of undercarboxylated osteocalcin were associated with a low bone density at the hip and an increased risk of hip fractures (Szulc *et al.,* 1993).

Taken together, present evidence indicates that older individuals with increased bone resorption and perhaps increased bone formation are at increased risk of hip fracture. Indeed, the information is complementary to that provided by bone mass measurements. Thus, those women with high bone resorption and low bone density or low bone mineral density or ultrasound values are at high risk of hip fracture (Garnero *et al.,* 1998) (Fig. 6). These observations, together with the increasing rate of bone loss with age (Ensrud *et al.,* 1995; Jones *et al.,* 1994), provide a rationale for designing intervention strategies to reduce bone turnover in these elderly subjects to levels found in premenopausal women.

Selection and Follow-up of Treatment Biochemical markers may be helpful in choosing therapies that will yield the best results. A retrospective analysis of a group of elderly osteoporotic women treated with salmon calcitonin showed that the women with baseline high bone turnover had the best response to treatment in terms of an increase of bone mineral density (Civitelli *et al.,* 1988). Similar results have been obtained by other investigators (Garnero *et al.,* 1994b; Overgaard *et al.,* 1990; Ravn *et al.,* 1999). In fact, these results show that high bone turnover and consequently a larger remodeling space may permit a larger gain of bone mass in response to treatment with an antiresorptive agent. Nevertheless, the association is not sufficiently strong in the individual to predict who will have a good bone densitometric response to therapy.

Bone mass measurements are the accepted approach for monitoring treatment. Serial measurements can be used to identify patients who do not respond to treatment and may well improve their compliance with treatment. In addition, the larger the increase in bone mass at the hip, the greater the reduction in the risk of spine fracture (Hochberg *et al.,* 1999). However, the relatively small changes in bone mass

measurements observed in relation to their precision error precludes an effect of treatment to be observed after a short therapeutic period (Eastell, 1996). Furthermore, the spine is the most responsive site but it is often affected by degenerative changes especially in older individuals.

Biochemical markers have several properties that make them suitable for treatment monitoring. Significant changes in biochemical markers are usually seen within a few months of treatment. In response to hormone replacement therapy, changes of 50 to 100% have been reported, and in postmenopausal osteoporotic women, treatment with bisphosphonates led to decreased levels of most resorption and formation markers to a similar extent (Garnero *et al.,* 1994b; Braga de Castro *et al.,* 1999) (Fig. 7). Smaller changes in bone turnover markers have been reported for intranasal calcitonin (Chesnut *et al.,* 2000; Kraenzlin *et al.,* 1996) and for raloxifene (Delmas *et al.,* 1997). Therefore, despite biological variability of biochemical markers (see Chapter 93), the ratio of change to variability (the "signal-to-noise ratio") for the biochemical markers appears to be better at 3 to 6 months than at bone mass measurements made at 1 to 2 years.

There have been several methodological approaches to assessing the response to therapy. In the least significant change approach (Braga de Castro *et al.,* 1999; Hannon *et al.,* 1998; Rosen *et al.,* 1998) (Fig. 7), the variability of the marker is calculated as a coefficient of variation and this estimate is multiplied by a constant (2.77) to obtain the change (two-tailed) that can be considered significant (at $P = 0.05$) based on single measurements at baseline and follow-up. Changes less than this should be interpreted with caution; small decreases in bone markers may be subsequently followed by increases, the so-called "regression toward the mean" phenomenon (Cummings *et al.,* 2000). An alternative approach is to estimate the change in the marker that is associated with a 90% specificity of response as defined by bone density response (Garnero *et al.,* 1999b). This approach results in cutoffs that are similar to the least significant change approach, e.g., a decrease in bone alkaline phosphatase of 40%.

With respect to the response to antiresorptive agents, Garnero *et al.* [1995] showed that estrogens and bisphosphonates may affect the pattern of bone collagen degradation differently. Bisphosphonate treatment produced a marked decrease in the urinary excretion of high-molecular-weight ("peptide bound") collagen degradation products (e.g., the aminoterminal collagen type I telopeptide) without significant changes in the excretion of free pyridinolines. This behavior is in sharp contrast with the effects of estrogen therapy, which results in a decrease of both free and peptide-bound cross-links.

There have been no studies to examine the relationship between the change in bone turnover marker and the fracture risk during treatment with antiresorptive therapy. However, there are significant inverse relationships between the change in bone turnover markers and change in bone mineral density. When these relationships are examined in a population treated with a single dose of drug the correlation coefficient with spine and hip bone mineral density is in the order of 0.3 to 0.4 [Greenspan *et al.,* 1998]. This relatively low correlation is likely to be a consequence of the difficulty in measuring small change in bone mineral density.

Taken together, the correlation between the early changes in biochemical markers and the subsequent changes in bone mass suggest that markers of bone turnover are helpful in monitoring therapy. At present, the markers with the highest clinical utility are deoxypyridinoline measured by HPLC, the peptide-bound cross-links measured in urine or serum (for bone resorption), and serum bone-specific alkaline phosphatase and osteocalcin (for bone formation). Whether combinations of biochemical markers will add to the usefulness of single measurements remains to be elucidated.

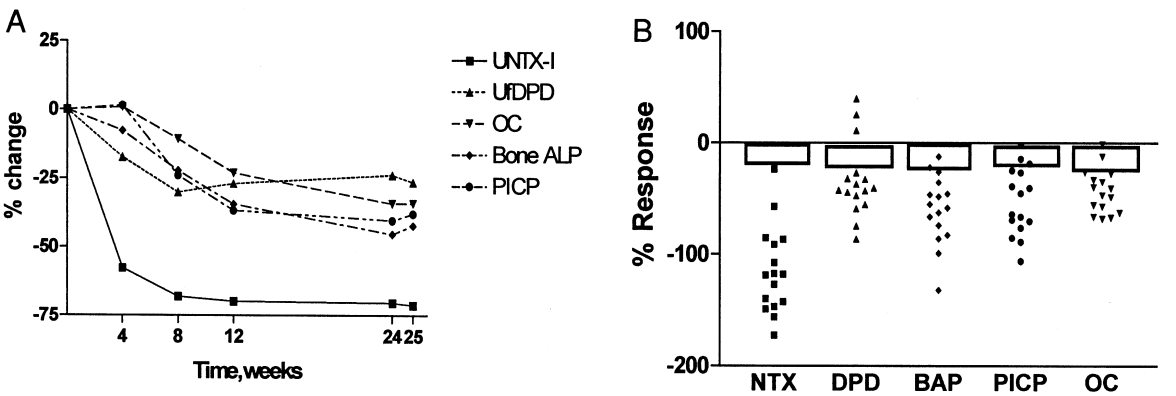

Figure 7 Effect of alendronate therapy on bone turnover markers over 6 Months. (A) Note how the bone resorption markers are suppressed within 4 to 8 Weeks of starting therapy; the decrease in bone formation markers is somewhat slower. (B) The boxes show the least significant change bounds for each marker. Note that most patients show a response to treatment at 6 months. Patients entering studies such as this are highly selected; in clinical practice, patients may have secondary osteoporosis that prevents them responding to therapy, or they may not comply with instructions. Adapted from Braga de Castro *et al.* (1999).

Changes in Bone Metabolism Induced by Immunosuppressive Agents

GLUCOCORTICOSTEROIDS

Prolonged administration of high-dose glucocorticosteroids (GCs) leads to bone loss (Chapter 41). Although the exact mechanisms of glucocorticoid-induced bone loss are still obscure, both osteoblast and osteoclast function are affected and an uncoupling of bone formation and resorption may be present.

It has been long appreciated that GCs inhibit osteoblastic collagen synthesis and matrix formation (Lukert and Raisz, 1990; Lukert *et al.,* 1986). This is usually reflected by a significant fall in serum OC, total and bone-specific ALP, and the procollagen propeptides (Ekenstam *et al.,* 1988; Prummel *et al.,* 1991; Reid *et al.,* 1986). Therefore, GCs appear to affect osteoblast function on several levels, including collagen synthesis, matrix maturation, and matrix mineralization. Other studies indicate that the inhibition of cellular mediators such as insulin-like growth factor 1 are of pivotal importance in GC-induced changes of bone cell function (Okazaki *et al.,* 1994).

In contrast to the changes seen in markers of bone formation, GC treatment can lead sometimes to rapid and persistent increments in bone resorption markers such as urinary pyridinium cross-links. Again, interference of GCs with osteoclast function and calcium metabolism seem to be rather complex and not well understood. Intestinal calcium absorption is usually inhibited, while renal calcium excretion may be greatly increased. The resulting, often transient, hypocalcemia possibly leads to secondary hyperparathyroidism, which in turn may be responsible for subsequent osteoclast activation (Libanati *et al.,* 1992). Additionally, GCs may exhibit direct effects on both PTH secretion and osteoclast function, leading to the observed changes in bone resorption. However, there is also now abundand evidence to argue against an important role for secondary hyperparathyroidism in GC-induced osteoporosis.

CYCLOSPORINES

Experimental and clinical investigations indicate that cyclosporines (CSs) and related immunosuppressants can also cause bone loss. In transplant recipients, CSs are rarely used as a single drug, so data from clinical studies are difficult to interpret. The complex but additive effects of diuretics, GCs, and CS on bone metabolism always have to be taken into account. Therefore, the most substantial data on the effect of CS alone on bone turnover are available from experimental studies.

Intravenous administration of cyclosporines A and G (CSA, CSG) to rats at doses comparable to those used in human transplant recipients leads to rapid bone loss (Movsowitz *et al.,* 1988; Schlosberg *et al.,* 1989). In these *in vivo* models, increased serum levels of calcitriol, osteocalcin, and alkaline phosphatase indicate stimulation of osteoblast function, whereas higher urinary concentrations of calcium, hydrodioxyproline, and pyridinium cross-links point also to enhanced bone resorption (Joffe *et al.,* 1992; Movsowitz *et al.,* 1988; Orcel *et al.,* 1989; Schlosberg *et al.,* 1989; Stein *et al.,* 1991). The latter effect may be attenuated by simultaneous administration of estrogens or calcitonin, suggesting that CS-induced *in vivo* bone resorption appears to be mediated by osteoclasts (Joffe *et al.,* 1992; Stein *et al.,* 1991). *In vitro,* CSA seems to inhibit not only osteoclast recruitment and maturation (Orcel *et al.,* 1991), but also basic and PTH-stimulated bone resorption (Stewart *et al.,* 1986, 1989). These observations indicate that the *in vivo* effects of CS on bone turnover may be mediated by and dependent on the function of immunocompetent cells and their specific mediators (Kahan, 1989; Stewart and Stern, 1989). Furthermore, direct effects of CS on renal and gonadal function (nephrotoxicity, hypogonadism) may also influence calcium balance and bone turnover.

Posttransplantation Bone Loss

Changes in bone metabolism following organ transplantation are complex. Preexisting diseases, the sequelae of long-standing hepatic, renal, cardiac, and/or pulmonary failure, immobilization, hypogonadism, and the (side) effects of various therapeutic agents may all have their own negative effects on bone metabolism. Changes in biochemical markers after organ transplantation are therefore influenced by a multitude of factors and do not indicate a specific etiological basis.

HEART TRANSPLANTATION

Prior to heart transplantation, bone mass is usually somewhat lower than normal, and accelerated bone turnover may be seen (Shane *et al.,* 1995). In these patients, elevated serum levels of osteocalcin and calcitriol seem to be associated with a higher risk of posttransplantation fractures, whereas hyperparathyroidism appears to act as a protective factor (Shane *et al.,* 1996).

The development of osteoporosis following heart transplantation has been recognized as a major problem (Shane *et al.,* 1996; Glendenning *et al.,* 1999; Leidig-Bruckner, 2001). Bone loss is most pronounced during the first 6 months after transplantation and may be as much as 20% from baseline (Shane and Epstein, 1994; Epstein *et al.,* 1995; Shane *et al.,* 1993; Valimaki *et al.,* 1999; Stempfle *et al.,* 1999). At this time, serum osteocalcin values are almost invariably suppressed, probably due to high doses of glucocorticosteroids given during the first months after transplantation. This suppression of osteoblast function is accompanied by a significant rise in the excretion of Hyp and pyridinium cross-links, which is usually attributed to the synergistic effects of glucocorticoids, cyclosporins, and diuretics, but also to the development of toxic kidney damage and hypogonadism (Stempfle *et al.,* 1995).

Approximately 6 months after transplantation and probably due to the reduction of oral glucocorticoids, serum osteocalcin and urinary cross-link concentrations return to baseline values, indicating normalization of bone turnover and the end of the high-risk period. Interestingly, an inverse correlation between serum osteocalcin levels and the rate of bone loss

has been reported for this time point (i.e., 6 months post-TPX), and serum osteocalcin levels were shown to be a predictor of the prospective bone loss at the lumbar spine of these patients (Sambrook *et al.,* 1994a,b). Given these and other observations, measurements of serum osteocalcin appear to be of some clinical relevance in the management and follow-up of cardiac transplant recipients. However, one should be aware of the fact that in some patients, a rise in serum osteocalcin levels may reflect the development of cyclosporine-associated renal insufficiency rather than normalization of bone turnover.

KIDNEY TRANSPLANTATION

All patients with long-standing renal failure show more or less pronounced signs of skeletal involvement, the most common being secondary hyperparathyroidism, osteomalacia, osteoporosis and adynamic bone pathology (for review, see Hruska and Teitelbaum, 1995). In these patients, biochemical markers of bone metabolism may vary considerably, depending on renal function, type of bone involvement, and treatment. Serum calcium levels are usually low, but may increase with the use of calcitriol. Alkaline phosphatase activities are high in secondary hyperparathyroidism and osteomalacia, but may be low in adynamic bone disease or aluminum intoxication. Serum osteocalcin levels are almost always elevated due to the accumulation of osteocalcin fragments detected in most assays. Therefore, serum osteocalcin is not a valuable marker of bone formation in patients with renal failure (Gundberg and Weinstein, 1986). The urinary excretion of PYD and DPD decrease below a glomular filtration rate of 25 ml/min. Anuric patients usually show greatly elevated serum concentrations of both free and peptide-bound cross-link components (Urena *et al.,* 1994).

Following renal transplantation, most patients sustain bone loss, although changes are usually not as pronounced as in cardiac or hepatic transplantation. The biochemical profile is often variable (Reusz *et al.,* 2000). Immediately after transplantation, most patients exhibit a dramatic fall in serum osteocalcin values, which may in fact decrease to subnormal levels. This observation is obviously not explained by the mere improvement of peptide clearance, but rather probably reflects the deleterious effects of glucocorticoids on bone formation. A suppression of osteoblast activity may also result in a transient decrease of serum alkaline phosphatase activity (Boiskin *et al.,* 1989; Julian *et al.,* 1991; Schmidt *et al.,* 1989). However, persistent elevations of the enzyme have been reported after transplantation, possibly due to the stimulating effects of cyclosporine (Loertscher *et al.,* 1983; Withold *et al.,* 1995). In most patients, a slow rise in the urinary excretion of pyridinium cross-links reflects increased bone resorption. As in cardiac patients, serum OC levels tend to return to the normal range within 6 to 12 months after transplantation (Boiskin *et al.,* 1989; Schmidt *et al.,* 1989). In some patients, an increase in parathyroid hormone or procollagen type I propeptide may be observed several months after renal transplantation (Degenhardt *et al.,* 1995; Schmidt *et al.,* 1989).

Metastatic Bone Disease

The occurrence of bone metastases is usually associated with a profound disruption in skeletal equilibrium and an uncoupling of bone formation and resorption processes (see Chapters 60 and 61). While widespread metastatic disease is difficult to treat, antiresorptive agents such as bisphosphonates may be helpful during earlier stages of skeletal involvement. Early diagnosis and monitoring of bone metastases are therefore of major clinical and therapeutic importance.

The diagnostic workup of the patient with suspected bone metastases usually includes bone scanning and radiographic evaluation, biochemical, and sometimes histological techniques. Although serological tumor markers such as CEA, PSA, or CA 15-3 are valuable tools in monitoring the course of primary and secondary malignancies, they do not reflect the changes in skeletal metabolism associated with the occurrence of bone metastases. This aspect of malignant bone disease may be revealed by the use of biochemical markers of bone metabolism (for current review, see Demers *et al.,* 2000).

Depending on the extent and the progression of the lesion, osteoblastic bone metastases of primary breast or prostate cancer are often associated with more or less pronounced changes in serum total and/or bone ALP (Epstein, 1988; Francini *et al.,* 1990). During early stages, when bone formation and bone resorption are still coupled to each other, a correlation between serum total and bone ALP and other markers of bone formation (OC, PINP) or of bone resorption (urinary calcium or Hyp) is occasionally observed (Blomqvist *et al.,* 1987). In breast cancer, local progression of bone metastases appears to be assessed more accurately by monitoring serum total ALP than by CEA or CA 15-3 (Francini *et al.,* 1990). In hypercalcemia of malignancy (HOM), however, serum total ALP and urinary Hyp excretion may diverge, thus reflecting an uncoupling of bone formation and resorption (Torres *et al.,* 1989). If the lytic component predominates, a dissociation even between formation markers such as bone ALP and OC may be observed (Duda *et al.,* 1988). This deviation is usually attributed to the fact that OC is associated with mineralization processes, whereas serum bone ALP is considered to reflect the synthesis and maturation of the organic matrix itself. In these cases, normal serum bone ALP levels may be misinterpreted as a sign of intact bone metabolism.

Normocalcemic patients with metastatic osteopathy often show elevated serum OC levels (Slovik *et al.,* 1984; Stracke *et al.,* 1985). However, with the development of hypercalcemia due to skeletal metastases, osteocalcin values may decrease into or below the lower range of normal (de la Piedra *et al.,* 1987; Torres *et al.,* 1989). In humoral hypercalcemia of malignancy, variable serum OC levels are seen (Body *et al.,* 1986). Interestingly, metastastic spread to bone appears to be associated with a loss of otherwise detectable correlations between serum OC and other markers of bone turnover (Carlson *et al.,* 1992; de la Piedra *et al.,* 1987; Duda *et al.,* 1988; Torres *et al.,* 1989). In contrast, soft-tissue metastases

apparently have no influence on serum OC whatsoever (Stracke *et al.,* 1985).

A reduction in serum OC values is usually seen after chemotherapy (Slovik *et al.,* 1984) and in hypercalcemic patients following bisphosphonate treatment (Body *et al.,* 1986). Serum OC may therefore be used for therapeutic monitoring.

In multiple myeloma, osteoblast-inhibiting factors and low rates of bone formation are detectable in about 80% of all patients (Evans *et al.,* 1989), while the remaining 20% show biochemical signs of accelerated bone turnover. Interestingly, an inverse correlation between serum OC levels and the disease stage (Durie-Salmon) has been shown in patients with multiple myeloma, a low serum OC typically being associated with a significantly shorter survival time. Also, patients with hypercalcemia due to extensive osteolytic lesions exhibit lower OC levels than the total population of hypercalcemic myeloma patients (Bataille *et al.,* 1987). Following chemotherapy-induced remission of the disease, serum OC values generally tend to return to the normal range (Carlson *et al.,* 1992). Thus, serum OC appears to have greater prognostic value than conventional markers such as hemoglobin, β2-microglobulin, and lactate dehydrogenase (Carlson *et al.,* 1992).

In normocalcemic patients with metastatic breast cancer, a close correlation between serum levels of the aminoterminal propeptide of type III procollagen (NPIIIP, a marker of collagen synthesis in connective tissues), total ALP, OC, and urinary Hyp has been described (Blomqvist *et al.,* 1987). Serum NPIIIP appears to be a sensitive marker that accurately reflects therapeutically induced remission of skeletal metastases. This high sensitivity of NPIIIP may be due to the fact that the metastatic process originates in the bone marrow (which is rich in type III collagen) and only later spreads to mineralized bone.

Tumor-induced release of calcium from mineralized tissues is usually compensated by an increased renal excretion, and hypercalciuria is a feature often seen in patients with metastatic spread to bone. However, one should bear in mind that the urinary excretion of calcium is also influenced by renal function and by dietary intake. In most tumor patients, calciuria does not correlate well with other markers of bone resorption or of bone formation (Blomqvist *et al.,* 1987; Coombes *et al.,* 1983). However, at comparable serum calcium levels, patients with breast cancer and skeletal metastates show significantly higher urinary calcium concentrations than women in whom the malignant disease is restricted to soft tissues only. Although under certain conditions hypercalciuria may precede radiographic detection of osteolytic lesions, no relationship is generally observed with the extent of skeletal involvement (Campbell *et al.,* 1983). Following bisphosphonate therapy, a significant decrease in hypercalciuria is associated with a normalization of initially increased erosion surfaces and reduced trabecular volume (Body and Delmas, 1992; Clarke *et al.,* 1992).

In general, urinary calcium excretion appears to be a sensitive but nonspecific marker of skeletal metabolism. Nonetheless, its role in the therapeutic monitoring and clinical follow-up of metastatic bone disease is undisputed. It should be noted, however, that in addition to the factors mentioned earlier, urinary calcium excretion in tumor patients may be influenced by tumor-induced mediators such as parathyroid hormone-related peptide. Paraneoplastic phenomena of this kind could also explain the discrepancy observed between the excretion of calcium and hydroxypyridinium crosslinks in humoral hypercalcemia of malignancy (Body and Delmas, 1992).

The urinary excretion of Hyp is significantly higher in breast cancer with overt metastatic bone disease than in subjects with tumors that have not metastasized (Powles *et al.,* 1976). At the same time, urinary Hyp seems to be a sensitive marker to predict the therapeutic response to antineoplastic therapy (Blomqvist *et al.,* 1987). In prostate cancer metastatic to bone, a rapid fall in urinary Hyp values is usually seen during biphosphonate treatment (Clarke *et al.,* 1992). Patients with hypercalcemia of malignancy have consistently elevated urinary Hyp concentrations (Blomqvist *et al.,* 1987).

The urinary excretion of pyridinium cross-links is increased in approximately 80–90% of all tumor patients with bone metastases (Paterson *et al.,* 1991; Body and Delmas, 1992; Coleman *et al.,* 1992; Pecherstorfer *et al.,* 1995) (Fig. 8). Furthermore, preliminary data suggest that cross-link excretion may rise even before skeletal metastases are evident by conventional imaging techniques (Paterson *et al.,* 1991; Pecherstorfer *et al.,* 1995). However, given the high sensitivity of pyridinium cross-links for collagen breakdown, systemic effects of tumor growth on bone metabolism and humoral PTHrP-mediated bone resorption may contribute to elevated urinary cross-link concentrations without evidence of skeletal involvement (Pecherstorfer *et al.,* 1995). Serum CTX-MMP, a high-molecular-weight component released during collagen breakdown, has also been shown to be a sensitive marker of osteolytic bone destruction in multiple myeloma, and appears to predict disease progression independently from immunoglobulin production (Risteli and Risteli, 1999; Horiguchi *et al.,* 2000).

Following intravenous bisphosphonate treatment of hypercalcemic patients, regression of hypercalciuria (Coleman *et al.,* 1992) and elevated Hyp excretion (Body and Delmas, 1992) appear to be more pronounced than the decrease in hydroxypyridinium cross-links. Interestingly, in our own experience, a renewed increase in urinary cross-links is observed only 10–14 days after pamidronate therapy. No correlation is detectable between calcium and cross-link excretion (Coleman *et al.,* 1992; Pecherstorfer *et al.,* 1995), a finding that again suggests that demineralization and destruction of the intercellular matrix are distinct processes in the course of skeletal metastases. Local radiotherapy of painful lesions leads to a reduction in bone resorption markers (Hoskin *et al.,* 2000).

Compared to healthy subjects or tumor patients at the premetastatic stage, patients with bone metastases usually show elevated serum TRACP levels (Scarnecchia *et al.,* 1991; Tavassoli *et al.,* 1980; Wada *et al.,* 1999), which respond to

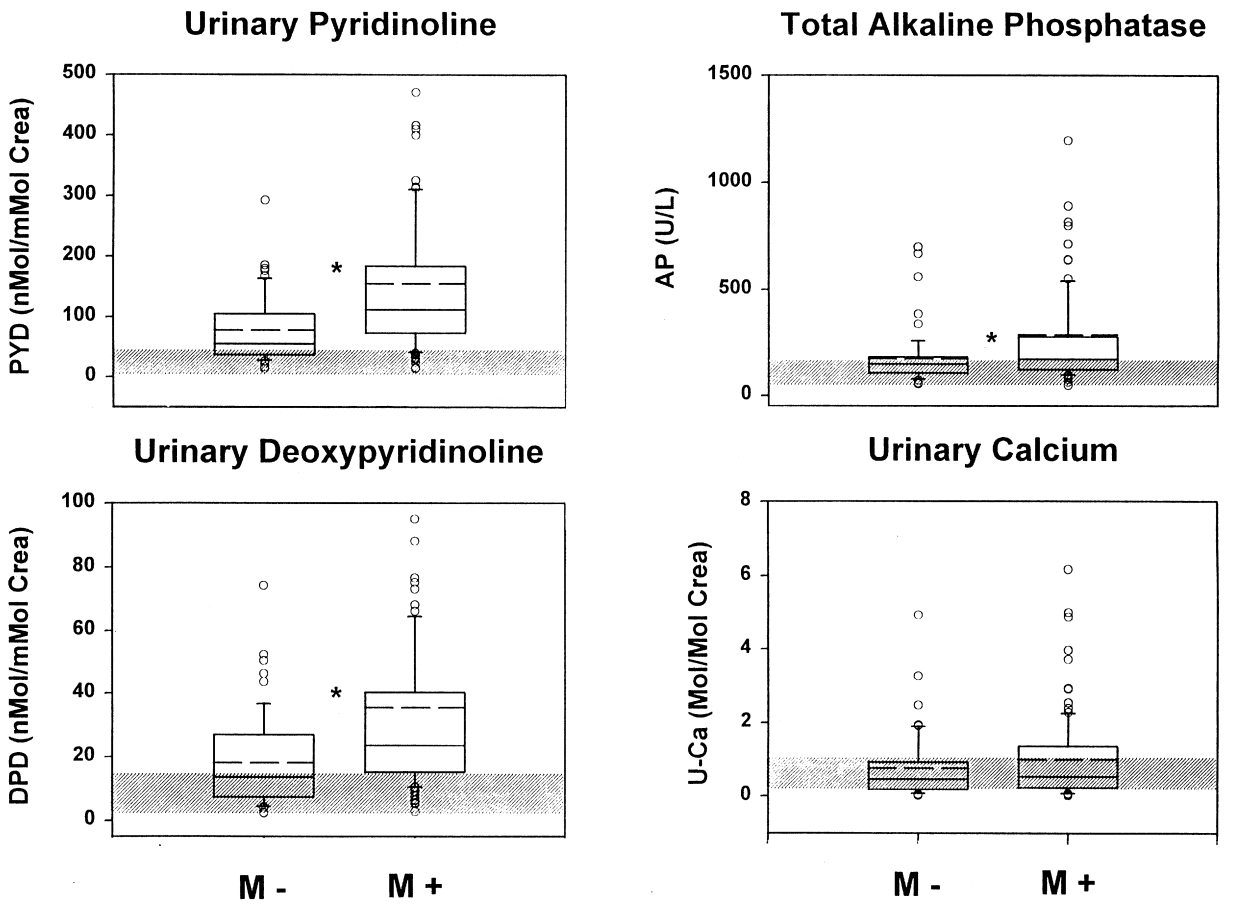

Figure 8 Markers of bone turnover in metastatic bone disease. Urinary pyridinium cross-links, calcium and serum alkaline phosphatase in tumor patients with (M+) and without (M−) skeletal metastases. The shaded area indicates the normal range. Reprinted with permission from Pecherstorfer *et al.* (1995).

bisphosphonate treatment (Lau *et al.,* 1987). Differentiation of serum total acid phosphatases can be of particular interest in patients with metastatic prostate cancer: while the fraction sensitive to tartrate inhibition is produced by the tumor itself, elevated serum levels of tartrate-resistant acid phosphatase are of skeletal origin and indicate the presence of osteoclast activation (Tavassoli *et al.,* 1980).

Serum bone sialoprotein (BSP) are significantly increased in patients with metastatic bone disease compared ot healthy controls (Seibel *et al.,* 1996; Karmatschek *et al.,* 1997; Woitge *et al.,*1999) (Fig. 2). Also, serum BSP has also been shown to be a strong indicator of future metastases in breast cancer patients (Diel *et al.,* 1999).

In summary, biochemical markers of bone turnover may be useful tools in the diagnosis, monitoring, and follow-up of malignant diseases and their metastatic spread to bone. Markers of bone resorption appear to be of greater clinical value than formation markers, as they reflect the destructive behavior of invasive skeletal metastases. Presently, most clinical experience is available for urinary Hyp, which generally allows a relatively accurate assessment of osteolytic activity. However, the development of convenient immunoassays for the determination of pyridinium cross-links is likely to supplant Hyp in the foreseeable future, as they have for other clinical uses of bone marker.

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CHAPTER 91

Methods and Clinical Issues in Bone Densitometry and Quantitative Ultrasonometry

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Introduction

Measurement of bone mineral density (BMD) has become an essential element in the evaluation of patients at risk for osteoporosis (Kanis *et al.,* 1997). Growing awareness of the impact of osteoporosis on the elderly population (Jacobson *et al.,* 1992; Koval *et al.,* 1995) and the consequent costs of healthcare (Ray *et al.,* 1997), together with the development of new treatments to prevent fractures (Black *et al.,* 1996; Cummings *et al.,* 1998; Ettinger *et al.,* 1999; Reginster *et al.,* 2000), have led to a rapid increase in the demand for bone densitometry services. Since its introduction in 1987, dual X-ray absorptiometry (DXA) has become the measurement technique most associated with the rapid growth in the clinical applications of bone densitometry (Genant *et al.,* 1996; Blake and Fogelman, 1997a). With its high precision, DXA is well suited to be used in the diagnosis of osteoporosis, to aid decisions about treatment and to monitor patients.

The Definition of Osteoporosis

In the early 1990s, a Consensus Meeting defined osteoporosis as "a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture" (Consensus Development Conference, 1993). It should be noted that this definition does not require an individual to have sustained a fracture before a diagnosis of osteoporosis is made, but introduces the concept of low bone mass and its relationship to increased fracture risk. While it could be argued that it is wrong to define a disease on the basis of what is essentially a risk factor, i.e., low bone density, there is nevertheless some logic to this as fractures tend to occur late in the disease process, when skeletal integrity is already severely compromised. It is therefore desirable to identify those individuals at high risk with a view to instituting treatment to prevent fractures from occurring.

Today, there is general agreement that BMD measurements are the most effective way of identifying these patients. Indeed, the widespread availability of bone densitometry systems has led to working definitions of osteoporosis that are increasingly based on measurements of BMD. In particular, in 1994 a World Health Organization (WHO) Study Group recommended a clinical definition of osteoporosis based on expressing BMD measurements in standard deviation (SD) units called *T* scores (WHO, 1994; Kanis *et al.,* 1994). The *T* score is calculated by taking the difference between a patient's measured BMD and the mean BMD of healthy young adults matched for gender and ethnic group, and expressing the difference relative to the young adult population SD:

$$T\ \text{score} = \frac{\text{measured BMD} - \text{young adult mean BMD}}{\text{young adult standard deviation}}. \quad [1]$$

A *T* score result therefore indicates the difference between the patient's BMD and the ideal peak bone mass achieved by a young adult.

Table I The WHO Study Group Recommendations for the Definitions of Osteoporosis and Osteopenia (WHO, 1994; Kanis *et al.,* 1994)

Terminology	*T* Score definition
Normal	$T \geq -1.0$
Osteopenia	$-2.5 < T < -1.0$
Osteoporosis	$T \leq -2.5$
Established osteoporosis	$T \leq -2.5$ in the presence of one or more fragility fractures

The WHO report recommended that a woman should be classified as having osteoporosis if she has a *T* score ≤ -2.5 (Table I). An intermediate state of low bone mass (osteopenia) was also proposed, defined by a *T* score between -2.5 and -1. A *T* score ≥ -1 was taken to be normal. Finally, a fourth state, established osteoporosis, denoted osteoporosis as defined above, but in the presence of one or more fragility fractures.

The WHO classification of BMD measurements to show osteoporosis, osteopenia, or normal skeletal status was intended to identify patients with high, intermediate, and low risk of fracture, respectively. However, it is important to recognize that the specific *T* score thresholds listed in Table I refer only to X-ray absorptiometry measurements of the spine, hip, or forearm. As will be discussed later, these definitions cannot automatically be applied to other BMD measurement sites, or to other methodologies such as quantitative computed tomography or quantitative ultrasonometry, without careful evaluation.

The rationale for the WHO definition of osteoporosis is that it captures around 30% of all white postmenopausal women, a figure that approximates to the lifetime risk of fracture for a 50-year-old woman. In comparison, it can be argued that the WHO definition of osteopenia captures too high a percentage of women to be clinically useful, and nowadays this term is being used less often, particularly in the context of therapeutic decision making. In contrast, the WHO definition of osteoporosis has had a major influence on clinical practice, to the extent that if the question is: "Does this patient have osteoporosis, yes or no?", this is now regarded as a *T* score issue.

Alongside the *T* score, another useful way of expressing BMD measurements is in *Z* score units (Blake and Fogelman, 1997b). Like the *T* score, the *Z* score is expressed in units of the population SD. However, instead of comparing the patient's BMD with the young adult mean, it is compared with the mean BMD expected for a healthy normal subject matched for age, gender, and ethnic origin:

$$Z \text{ score} = \frac{\text{measured BMD} - \text{age-matched mean BMD}}{\text{age-matched standard deviation}}. \quad [2]$$

Although not as widely used as *T* scores, the *Z* score nevertheless remains a useful concept because it expresses the patient's risk of sustaining an osteoporotic fracture relative to their peers. Typically, every reduction of 1 SD in BMD equates to an approximately twofold increase in the likelihood of fracture (Marshall *et al.,* 1996). It follows that patients with a *Z* score <-1 are at a significantly increased risk of fracture compared with individuals with a normal bone density for their age.

Techniques Available for Bone Densitometry Studies

Table II lists the methods currently available for the noninvasive assessment of the skeleton for the diagnosis of osteoporosis and/or the evaluation of an increased risk of fracture. These include dual X-ray absorptiometry (DXA), spinal quantitative computed tomography (QCT), peripheral DXA (pDXA), peripheral QCT (pQCT), radiographic absorptiometry (RA), and quantitative ultrasonometry (QUS). These techniques differ substantially in physical principles, in clinical discrimination and utility, and in general availability and cost. Each is reviewed briefly below. The reader can find further information about these techniques in several comprehensive reviews (Cann, 1988; Genant *et al.,* 1996; Grampp *et al.,* 1997; Glüer *et al.,* 1997; Blake *et al.,* 1999; Njeh *et al.,* 1999a).

Dual X-ray Absorptiometry

Over the past decade, DXA has established itself as the most widely used method of measuring BMD because of its advantages of high precision, short scan times, low radiation dose and stable calibration. DXA equipment (Fig. 1) allows scanning of the spine (Fig. 2A) and hip (Fig. 2B), usually regarded as the most important measurement sites because they are frequent sites of fractures that cause substantial impairment of quality of life, morbidity, and mortality. A measurement of hip BMD has been shown to be the most reliable way of evaluating the risk of hip fracture (Cummings *et al.,* 1993; Marshall *et al.,* 1996; Black *et al.,* 2000). Also, because of the metabolically active trabecular bone in the vertebral bodies, the spine is regarded as the optimum site for monitoring response to treatment (Eastell, 1998).

The fundamental principle behind DXA is the measurement of the transmission through the body of X-rays of two different photon energies. Because of the dependence of the attenuation coefficient on atomic number and photon energy, measurement of the transmission factors at two energies enables the "areal" densities (i.e., the mass per unit projected area) of two different types of tissue to be inferred (Blake and Fogelman, 1997a). In DXA scans these are taken to be bone mineral (hydroxyapatite) and soft tissue, respectively. Radiation dose to the patient is very low (1 to 10 μSv) (Njeh *et al.,* 1999b) and is comparable to the average daily dose from natural background radiation of 7 μSv.

Table II Characteristics of Different Bone Densitometry Techniques

Technique[a]	Regions of interest	Units reported	Precision (%CV)	Effective dose (μSv)
DXA	PA spine	BMD (g/cm^2)	1	1–10
	Proximal femur		1–2	1–10
	Total body		1	3
QCT	Spine	BMD (g/cm^3)	3	50–500
pDXA	Forearm	BMD (g/cm^2)	1–2	0.1
	Calcaneus		1–2	0.1
pQCT	Forearm	BMD (g/cm^3)	1–2	1–3
RA	Phalanx	BMD (g/cm^2)	1–2	10
QUS	Calcaneus	BUA (dB/MHz)	2–5	None
	Calcaneus	SOS (m/s)	0.1–1	None
	Tibia	SOS (m/s)	1–2	None
	Multi-site	SOS (m/s)	1–2	None

Note. [a] DXA, dual-energy X-ray absorptiometry; QCT, quantitative computed tomography; pDXA, peripheral pDXA; QCT, peripheral QCT; RA, radiographic absorptiometry; QUS, quantitative ultrasonometry.

It is widely recognized that the accuracy of DXA scans is limited by the variable composition of soft tissue. Because of its higher hydrogen content, the attenuation coefficient of fat is different from that of lean tissue. Differences in the soft tissue composition in the path of the X-ray beam through bone compared with the adjacent soft tissue reference area will cause errors in the BMD measurements (Tothill and Pye, 1992). Svendsen *et al.* (1995) reported a cadaver study in which the effect of fat inhomogeneity on the random accuracy errors for BMD measurements in the spine, hip, and forearm were examined. The root mean square accuracy errors were reported to be 3% for forearm, 5% for spine, and 6% for femoral neck and total hip BMD. The population SD (Eq. (1)), which is typically about 10% of the mean young adult BMD, includes the random errors due to the effects of these variations in body composition.

The first generation of DXA scanners used a pinhole collimator, producing a pencil beam coupled to a single scintillation detector in the scanning arm. Since then the most significant development has been the introduction of new systems that use a slit collimator to generate a fan beam coupled to a linear array of solid-state detectors. As a result, image resolution has improved, and scan times have shortened from around 5–10 min for the early pencil beam models to

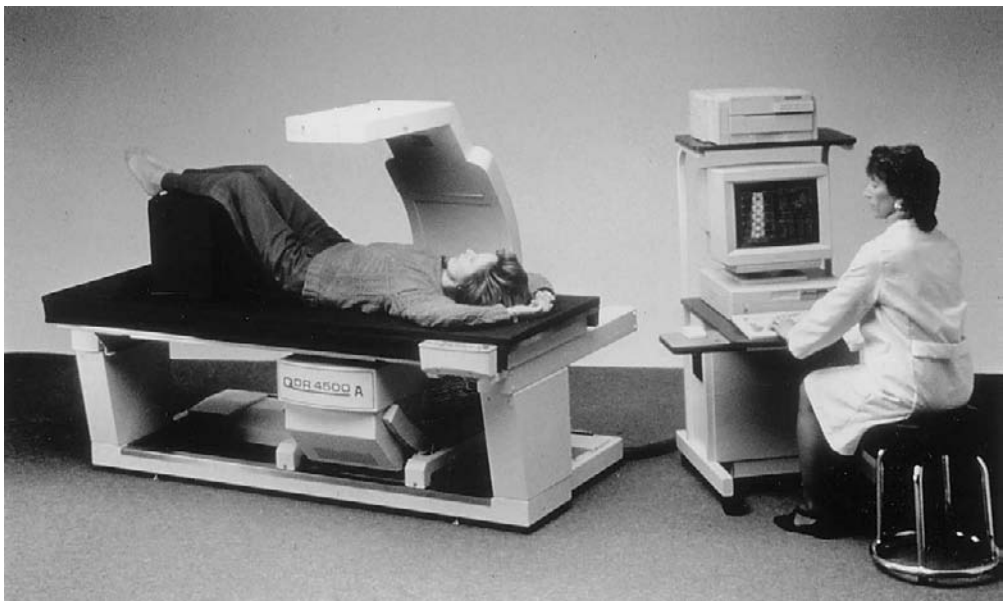

Figure 1 The QDR4500 fan-beam DXA scanner (Hologic Inc., Bedford, MA). Densitometers such as this are most frequently used for measuring spine and hip BMD, but can also be used for total body, forearm, and lateral projection studies of the spine.

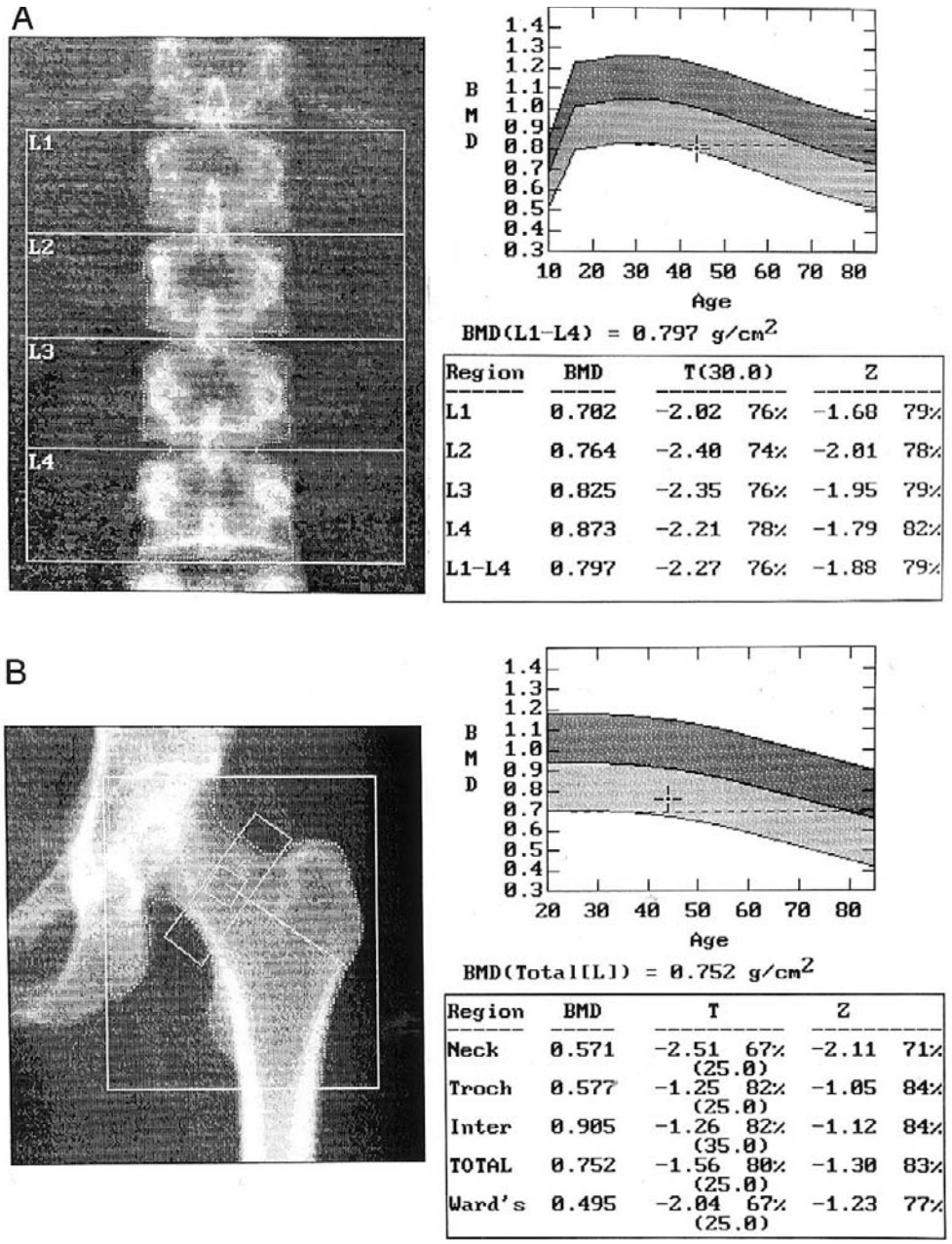

Figure 2 (A) Part of a computer printout from a DXA scan of the spine. The printout shows (clockwise from left): (1) scan image of lumbar spine, (2) patient's age and BMD plotted with respect to the reference range, (3) BMD figures for individual vertebrae and total spine (L1–L4) together with interpretation in terms of *T* scores and *Z* scores. (B) Part of a computer printout from a DXA scan of the hip. The printout shows (clockwise from left): (1) scan image of proximal femur; (2) patient's age and BMD for the total femur ROI plotted with respect to the NHANES III reference range; (3) BMD figures for five different ROIs in the hip (femoral neck, greater trochanter, intertrochanteric, total femur, and Ward's triangle) together with interpretation in terms of *T* scores and *Z* scores using the NHANES III reference range.

10–30s for the latest fan beam systems. Radiation dose to patients is generally higher for fan-beam systems compared with pencil beam, and the resulting increased scatter dose to technologists may require precautions to limit exposure such as placing the operator's console further away from the scanning table (Patel *et al.,* 1996).

Quantitative Computed Tomography

QCT has the advantage that it determines the true three-dimensional ("volumetric") bone density (units: mg/cm^3) compared with the two-dimensional areal density measured by DXA. QCT is usually applied to measure the trabecular bone in the vertebral bodies (Fig. 3) (Cann *et al.,* 1988).

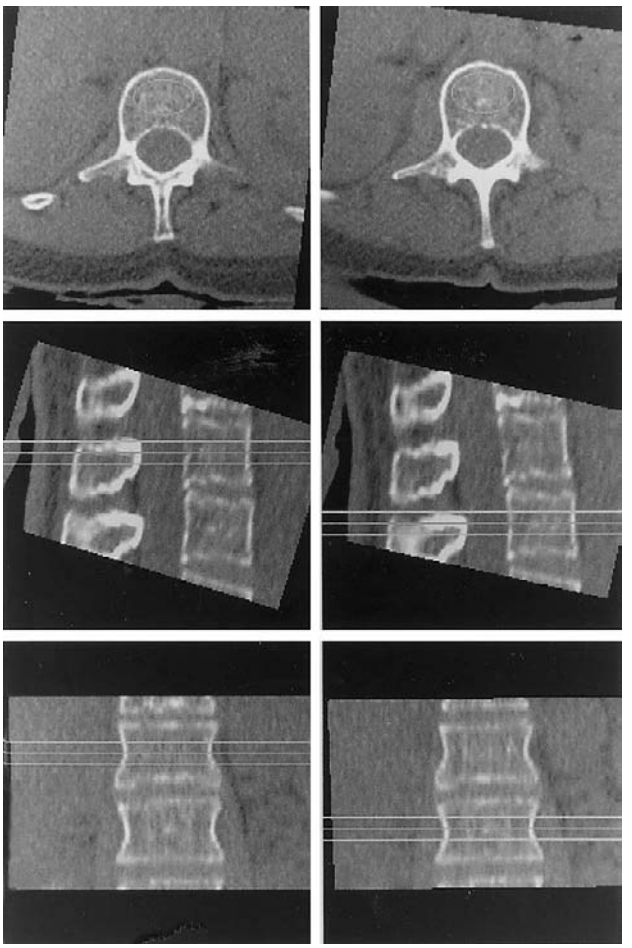

Figure 3 Part of a computer printout from a QCT scan of the spine showing transverse, sagittal, and coronal images of two lumbar vertebrae. The study was analyzed using a commercially available QCT software package (Mindways Software Inc, San Francisco, CA).

The measurement can be performed on any clinical CT scanner, provided that the patient is scanned with an external reference phantom to calibrate the CT numbers to bone equivalent values. Most CT manufacturers provide a software package to automate the placement of the regions of interest (ROIs) within the vertebral bodies. Patient dose is much lower than for standard CT scans, provided that the examination is performed correctly (Kalender, 1992). QCT studies are generally performed using a single kilovolt setting (single-energy QCT), when the principal source of error is the variable composition of the bone marrow. However, a dual-kilovolt scan (dual energy QCT) is also possible. This reduces the accuracy errors, but at the price of poorer precision and higher radiation dose. The advantage of spinal QCT is the high responsiveness of the vertebral trabecular bone to aging and disease (Cann, 1988; Grampp *et al.,* 1997). The principal disadvantage is the cost of the equipment.

Peripheral DXA, Peripheral QCT, and Radiographic Absorptiometry

Despite the expansion in the number of osteoporosis clinics, in most countries there are inadequate resources to meet demand, and DXA scans are not available to all patients who could conceivably benefit. Furthermore, conventional DXA scanning is perceived as costly, not least because of the need to refer patients to hospital-based facilities. Thus, despite the widespread popularity of BMD studies of the spine and femur, recent years have seen a revival of interest in small, low-cost, X-ray absorptiometry devices dedicated to scanning the peripheral skeleton (Glüer *et al.,* 1997). The first bone densitometers were forearm scanners that used the technique of single photon absorptiometry (SPA) based on a ^{125}I radionuclide source (Cameron and Sorensen, 1963). Follow-up of patients after SPA studies has shown that forearm bone density measurements can predict fracture risk over 25 years (Düppe *et al.,* 1997). In recent years the technology has been updated by replacing the radionuclide source with a low-voltage X-ray tube ($40-60\ kV_p$) and using the principles of DXA to perform BMD scans of the distal radius (Fig. 4) and the calcaneus. The advantages of pDXA systems include the small footprint of the devices, relatively low cost, and an extremely low radiation dose of around 0.1 μSv (Patel *et al.,* 1999).

Just as pDXA devices were developed as an alternative to DXA scanning of the central skeleton, small dedicated pQCT systems are also available for measuring the forearm (Glüer *et al.,* 1997). These devices have the advantage of separating the trabecular and cortical bone of the ultradistal radius and of reporting volumetric density. Although widely used in some countries in Europe, they have been primarily limited to research studies in the United States (Baran *et al.,* 1997).

Radiographic absorptiometry (RA) is a technique that was developed many years ago for assessing bone density in the hand, but has recently attracted renewed interest (Glüer *et al.,* 1997). It has the advantage of using conventional X-ray equipment with the addition of a small aluminum wedge in the image field for calibration. The radiographic image is captured on a PC and then processed automatically using a specially developed software application to measure BMD in the phalanges. The main advantage of RA is its potential for general use based on the widespread availability of conventional film radiography.

Peripheral X-ray absorptiometry methods such as those described above have obvious advantages when selecting bone densitometry methodologies suitable for use in physicians' offices or in primary care. However, epidemiological studies suggest that the discriminatory ability of peripheral BMD measurements to predict spine and hip fractures is rather lower than for spine and hip BMD measurements (Marshall *et al.,* 1996). Also, changes in forearm BMD in response to estrogen and other treatments for osteoporosis are relatively small, making such measurements less suitable than spine BMD for monitoring patients' response

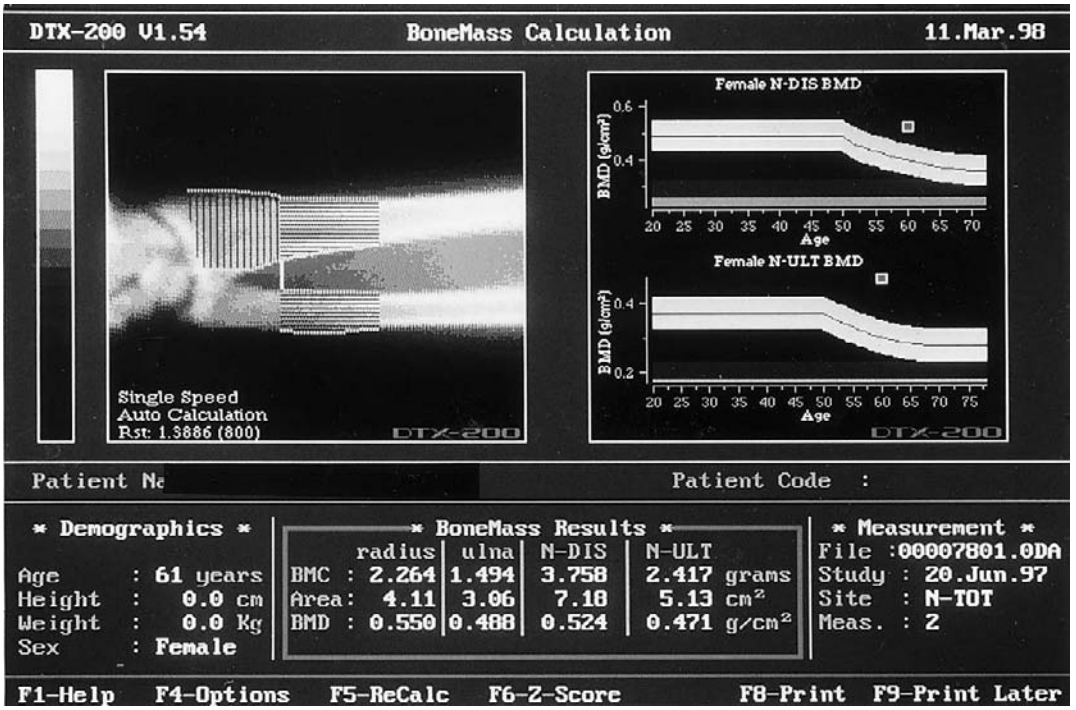

Figure 4 Computer printout from a pDXA scan of the distal forearm. The scan was performed on a DTX-200 system (Osteometer Meditech, Hawthorne, CA).

(Faulkner, 1998). Finally, although the radiation doses to patient and operator are both extremely small, pDXA and pQCT devices are subject to government regulatory requirements controlling the use of X-ray equipment, including the training of technologists and physicians in the principles of radiation safety.

Quantitative Ultrasonometry

QUS for measuring the peripheral skeleton has raised considerable interest in recent years (Njeh *et al.,* 1997, 1999a). There is a wide variety of equipment available, with most devices using the heel as the measurement site (Fig. 5). The calcaneus is chosen because it encompasses a large volume of trabecular bone between relatively flat faces and is readily accessible for transmission measurements. The physical principles of QUS measurements are outlined in Fig. 6. An ultrasound pulse passing through cancellous bone is strongly attenuated as the signal is scattered and absorbed by trabeculae (Fig. 6A). The power spectrum of the pulse transmitted through the patient's heel is compared to a reference trace measured through water alone (Fig. 6B). The additional attenuation measured through the patient's heel (measured in decibels (dB)) increases linearly with frequency, and the slope of the relationship is referred to as the broadband ultrasonic attenuation (BUA: units dB/MHz) (Fig. 6C). BUA is reduced in patients with osteoporosis, because there are fewer trabeculae in the calcaneus to attenuate the signal. As well as BUA, most QUS systems also measure the speed of sound (SOS) in the heel by dividing the distance between the ultrasound transducers by the propagation time (units: m/s) (Fig. 6A). SOS values are reduced in patients with osteoporosis because, with the loss of mineralized bone, the elastic modulus of the bone is decreased. Some manufacturers combine the BUA and SOS values into a single parameter referred to as "stiffness" or the "quantitative ultrasound index" (QUI). These combinations have no particular physical meaning, but may improve precision and

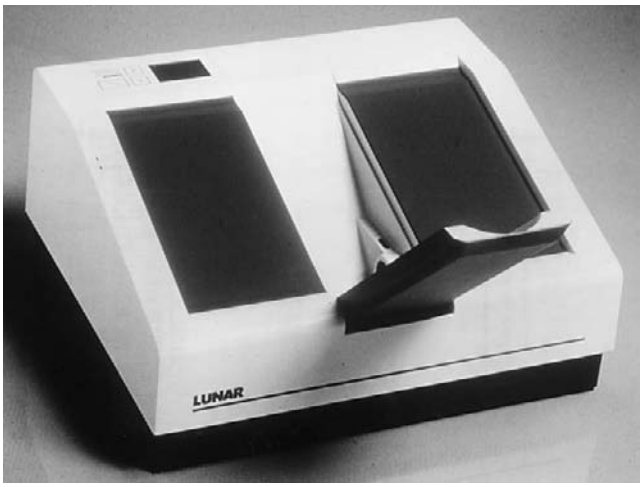

Figure 5 The Achilles system for performing QUS measurements in the heel (Lunar Corp., Madison, WI). Devices such as this measure BUA and SOS in the calcaneus. The two measurements are combined into a single index ("stiffness") which is claimed to improve discrimination compared with BUA or SOS alone.

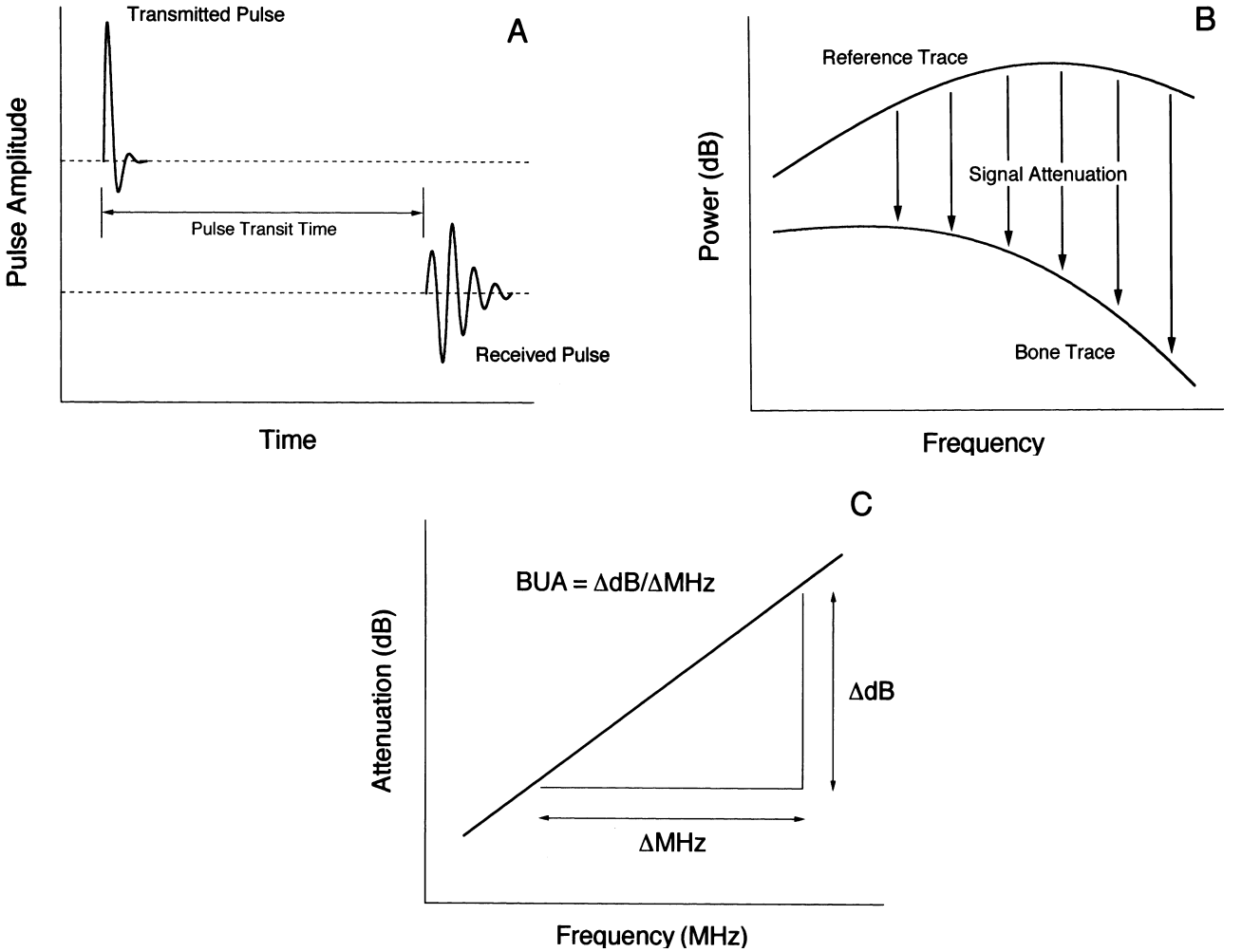

Figure 6 The physical principles behind the measurement of BUA and SOS. (A) The received pulse is digitized and Fourier analysis used to determine the power spectrum. The pulse transit time is used for the SOS measurement. (B) The power spectrum of the signal transmitted through the patient's heel is compared with a reference trace from a signal transmitted through water. The difference between the two traces is the attenuation due to the patient's heel. (C) When the attenuation through the patient's heel is plotted against frequency, a linear relationship is found at frequencies below 1 MHz. BUA is defined as the slope of the regression line and is measured in units of dB/MHz.

discrimination by averaging out errors such as those caused by temperature variations (Nicholson and Bouxsein, 1999). With most early-generation QUS devices, the patient's foot was placed in a water bath to couple the ultrasound signal to the heel. However, most later devices are dry contact systems in which rubber pads covered with ultrasound gel are pressed against the patient's heel.

A major attraction of bone ultrasound devices is that they do not use ionizing radiation, and therefore avoid the regulatory requirements for X-ray systems mentioned above. Also, the instrumentation is relatively inexpensive and several devices, especially among the dry systems, are designed to be portable. Therefore ultrasound systems could be made much more widely available than conventional DXA scanners, which are largely restricted to hospital-based osteoporosis clinics. Moreover, recent evidence from several large prospective studies confirm that QUS measurements are predictive of hip fracture risk (Hans *et al.,* 1996; Bauer *et al.,* 1997; Pluijm *et al.,* 1999).

There remain, however, a number of difficulties in the interpretation of QUS measurements. In general, the fracture studies mentioned above were conducted in elderly populations aged $\geq$70 years, examined only hip fracture risk, and used the earlier generation of water-based calcaneal QUS systems. Another difficulty is that QUS measurements are not readily encompassed within the WHO definitions of osteoporosis and osteopenia (Faulkner *et al.,* 1999; Delmas, 2000). Recently, Kanis and Glüer (2000) proposed that hip BMD should be the "gold standard" for the definition of osteoporosis and that in the case of peripheral methodologies such as QUS, intervention thresholds should be devised so that measurements are interpreted in terms of a fracture risk equivalent to that determined by hip DXA.

There are also a number of technical limitations to QUS. Due to the wave physics of the signal, measurements may be affected by errors due to diffraction and phase cancellation (Petley *et al.,* 1995). Also, many devices use a foot support that positions the patient's heel between fixed transducers.

Thus the measurement site is not readily adapted to different sizes and shapes of the calcaneus, and the exact anatomical site of the measurement varies from patient to patient. Furthermore, as a measurement site the calcaneus has the disadvantage of being sensitive to the amount of exercise the patient takes. The problem of ensuring an anatomically consistent measurement site may be solved by imaging QUS systems that perform a faster scan of the heel and enable a more consistent placement of the ROI (Fournier *et al.,* 1997). Finally, it is widely accepted that the relatively poor precision of QUS measurements makes most devices unsuitable for monitoring patients' response to treatment (Glüer, 1997). In general, the phantoms provided with QUS devices are not particularly anthropomorphic, and thus the problem of poor precision may be compounded by limitations in instrument quality control.

The Relationship between Different Types of Measurement

The spine and femur are generally regarded as the most important DXA measurement sites because they are the sites of the osteoporotic fractures that cause the greatest impairment of quality of life, morbidity, and mortality. Many would still consider spine BMD the optimum measurement because of its sensitivity to the changes associated with aging, disease, and therapy. However, it has the disadvantage that, with advancing age, measurements are often affected by the presence of degenerative changes that lead to the artificial elevation of BMD values. This becomes an increasing problem after the age of 70, but can occur earlier. Others would argue that hip BMD is the most useful measurement, since it is the most predictive of hip fracture (Cummings *et al.,* 1993; Marshall *et al.,* 1996; Black *et al.,* 2000), which is clinically the most important fracture. In the research community, a consensus is developing that the total femur should be the "gold standard" for bone densitometry measurements (Kanis and Glüer, 2000; Black, 2000). In practice, when DXA measurements are carried out, spine and hip BMD are usually both available for evaluation.

As osteoporosis is common and is a primary care disease, there is a need for a "simpler" evaluation of BMD than DXA, which is generally found only in large hospitals. There is therefore considerable interest in pDXA and QUS devices, as such systems are smaller and cheaper than DXA. Since osteoporosis is a systemic disease, bone loss is not limited to the axial skeleton. However, correlation coefficients between BMD measurements at different skeletal sites are typically around $r \approx 0.6–0.7$, and thus a measurement at one site is far from being a perfect predictor of that at any other. Correlations between QUS and BMD measurements are even poorer, with coefficients of around $r \approx 0.4–0.5$. Thus, whatever intervention threshold is chosen as the basis for recommending treatment, different groups of patients will be selected depending on the measurement site and technology in use. This raises the question of whether there is any one type of measurement that performs better than the others at identifying the patients most at risk of an osteoporotic fracture.

Which Measurement Is Best?

The most rigorous and convincing method of comparing the ability of different measurement sites and methodologies to predict osteoporotic fractures is through a prospective study of incident fractures. One of the largest and most ambitious of these studies is the Study of Osteoporotic Fractures (SOF), which involved more than 9000 women age 65 years and over recruited in four regions of the United States. A commonly used descriptive approach to presenting data from such studies is to divide subjects into four quartiles based on their baseline BMD values and plot the fracture risk for each quartile. Fig. 7 shows SOF data for hip fracture risk plotted in this way (Cummings *et al.,* 1993). The data show that patients in the lowest quartile of hip BMD have approximately 10 times the risk of hip fracture of patients in the highest quartile.

It is conventional to perform a statistical analysis of epidemiological studies of fracture risk using a "gradient of risk" model in which fracture risk increases exponentially with decreasing BMD (Jergas and Glüer, 1997). Mathematically, this can be done using either logistic regression or proportional hazard models (Hui *et al.,* 1995). The results are expressed in terms of the relative risk (RR), which is the increased risk factor for each 1 SD decrease in BMD. Fig. 8 shows SOF data presented by Black *et al.* (2000) for the RR values for hip fracture risk for different BMD sites. With around 250 fracture cases included in this analysis, the study presents highly statistically significant evidence that hip fracture risk is best predicted by a measurement of femur BMD.

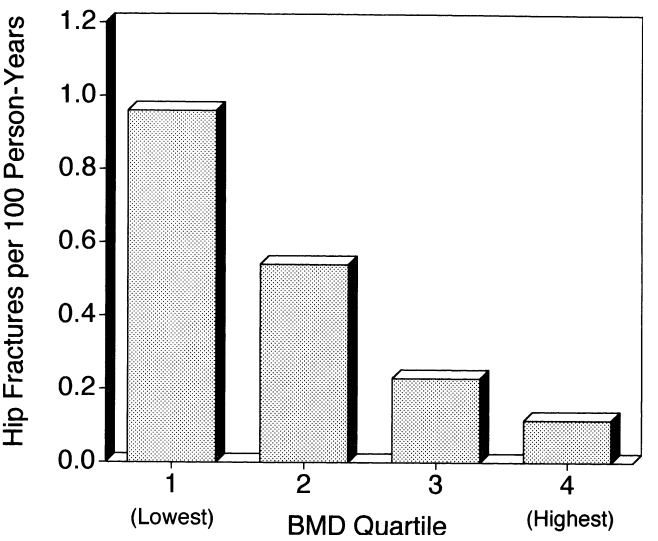

Figure 7 Incidence of hip fracture by BMD quartile for femoral neck BMD. Data from Cummings *et al.* (1993).

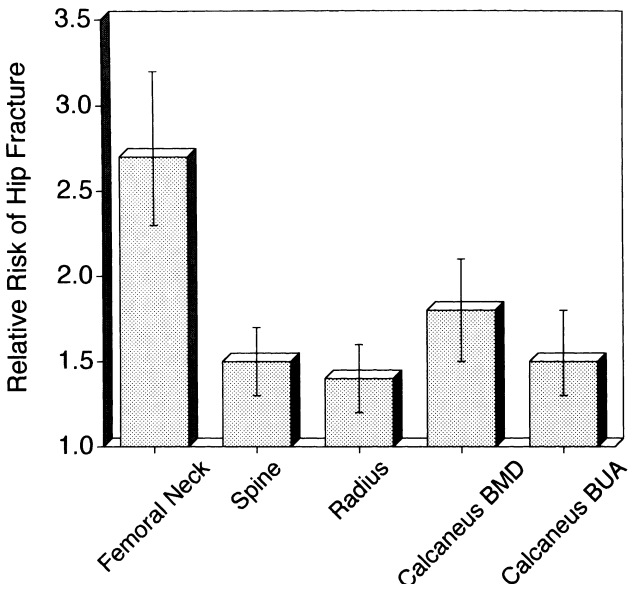

Figure 8 Relative risk (RR) values for 5-year hip fracture incidence for BMD measurements of the femoral neck, lumbar spine, forearm, and calcaneus and BUA measurements of the calcaneus. RR is the increased risk of fracture for a 1 SD decrease in BMD or BUA. The figure is drawn from SOF study data reported by Black *et al.* (2000) and is based on approximately 250 cases of hip fracture recorded in the study population of 9000 white women age 65 years and over.

For a wider view of the merits of measurement sites other than the hip, a metaanalysis of fracture studies is required. Such an analysis was published by Marshall *et al.* (1996), and their collated data for the prediction of different types of fracture from BMD measurements at different skeletal sites are shown in Fig. 9. While these results confirm that hip BMD is best for predicting hip fracture, the degree to which spine BMD best predicts vertebral fracture or a radius BMD forearm fracture is less conclusive. Interestingly, when assessed by the ability to predict fractures occurring at any site, risk ratio figures for different measurement sites are closely comparable.

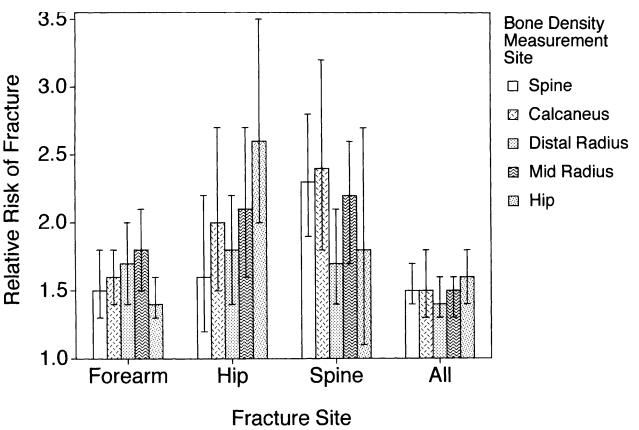

Figure 9 Relative risk (RR) values for fractures at different skeletal sites for bone density measurements in the spine, calcaneus, distal radius, mid-radius, and hip. Data are taken from the metaanalysis of prospective studies collated by Marshall *et al.* (1996).

In summary, there is now compelling evidence that a measurement of hip BMD is the most reliable way of predicting hip fracture risk. However, for other fracture sites, or for all fractures taken together, any differences between the various types of BMD measurement have yet to be conclusively established. Recent studies extend these conclusions to include QUS measurements of the calcaneus. Hans *et al.* (1996) reported RR results for hip fracture of 2.0 for BUA and 1.7 for SOS based on women age 75 years and over enrolled in the EPIDOS study, while Bauer *et al.* (1997) reported similar findings from the SOF study. Following these reports, there was general agreement that QUS devices could have an important role in the assessment of fracture risk, particularly in elderly women (Glüer, 1997). The most recent data from the SOF study give a rather lower RR value of 1.5 for heel BUA, a figure comparable to forearm BMD (Fig. 8) (Black *et al.,* 2000), and the clinical implications of this result with regard to the more widespread application of QUS is not yet clear.

The Interpretation of BMD Results: Reference Ranges

If the WHO criterion of a T score ≤ -2.5 is used to define osteoporosis, then it is apparent that any errors in the mean BMD or population SD of the reference group could lead to significant differences in the apparent incidence of osteoporosis when applied to other populations. The majority of centers providing a scanning service use reference ranges provided by the equipment manufacturers, and issues over the accuracy of these ranges have caused controversy in the past (Faulkner *et al.,* 1996). This continues to be a problematic area in view of the large number of new devices which are being introduced for the assessment of the skeleton. However, for DXA the problem is now largely resolved after a report by the International Committee for Standards in Bone Measurement (ICSBM) (Hanson, 1997), which recommended that hip BMD measurements should be interpreted using the total femur region of interest and the hip BMD reference ranges derived from the US NHANES III study (Looker *et al.,* 1998). The NHANES III project studied a nationally representative sample of over 14,000 men and women with approximately equal numbers of non-Hispanic white, non-Hispanic black, and Mexican Americans. The ICSBM report recommends use of the total femur ROI instead of the previously widely used femoral neck site because of its improved precision and the fact that it is the hip region most readily implemented on all DXA manufacturers' systems.

Many centers have already acted upon these recommendations, and they are increasingly being used for scan reporting. It is important to note that these changes affect the percentage of patients who are diagnosed as having osteoporosis at the hip. Using the total femur ROI and the NHANES III

reference range, rather fewer patients will be diagnosed as having osteoporosis than using the femoral neck ROI and the manufacturer's original reference range (Chen *et al.,* 1998). There is no definite right or wrong answer in this situation. What is more important is to have a consistent approach, and it is certainly highly desirable to have universally accepted BMD criteria for the diagnosis of osteoporosis.

One advantage of presenting bone densitometry results in terms of *T* scores is that they avoid the confusion caused by the raw BMD figures that differ for different manufacturers' equipment (Genant *et al.,* 1994). The ICSBM Committee has addressed this issue by publishing equations which allow each manufacturer to express their BMD values on a consistent scale in standardized units (sBMD: units mg/cm^2) (Steiger, 1995; Hanson, 1997). In practice sBMD units are seldom used in the clinical management of patients but can be useful in some research applications.

The Clinical Use of Bone Densitometry

With the development of new treatments for preventing osteoporosis and the wider availability of bone densitometry equipment, much debate has centered on the issues of the clinical indications for the diagnostic use of bone densitometry and recommendations for the initiation of treatment based on the findings. In the United States, an influential report was published by the National Osteoporosis Foundation (NOF, 1998a). In Europe similar reports have been issued by the European Foundation for Osteoporosis (EFFO) (Kanis *et al.,* 1997; Kanis and Glüer, 2000), and in the United Kingdom by the Royal College of Physicians (RCP, 1999).

The NOF report included a sophisticated set of guidelines for therapeutic intervention. Various nomograms were developed that incorporate age, BMD, and four other risk factors for osteoporosis (Table III). An interesting aspect of the NOF approach is that the calculations for therapeutic intervention are based on the concept of a quality adjusted life year (QALY) valued at $30,000. This is a relatively high value, and one that would not be considered appropriate for application in Europe. This implies that there may have to be different BMD criteria for therapeutic intervention in different countries throughout the world. It also follows from the NOF approach that there will be different thresholds for intervention depending on the cost of treatment. While the NOF report is an extremely important document, with an extensive review of the relevant background information, it is nevertheless complex, and it is unlikely that primary care physicians will instigate treatment based on such a scheme. The NOF subsequently published a physicians' handbook with simplified recommendations which included the availability of BMD measurements for all women over the age of 65 years and in all postmenopausal women under the age of 65 in whom clinical risk factors are present (NOF, 1998b).

Clinical guidelines for the prevention and treatment of osteoporosis in the United Kingdom were recently published by the Royal College of Physicians (RCP, 1999). These concluded that at present there is no consensus for a policy of population screening using BMD scans. Instead, a case-finding strategy is recommended for referring patients for bone densitometry based on a list of widely accepted clinical risk factors for osteoporosis (Table IV). The list is identical to that published in the EFFO report (Kanis *et al.,* 1997). The RCP report also recommended *T* score of ≤ -2.5 at the spine or hip as the basis for instigating therapy.

It is important to emphasize that the WHO definition of osteopenia ($-2.5 < T < -1$) is not useful in isolation with regard to decisions about treatment, since it captures too high a percentage of postmenopausal women and, in fairness, was never intended to be used in this way. A considerable body of evidence indicates that it is the patients with the most severe

Table III Risk Factors for Osteoporosis Additional to Age and BMD Incorporated in the NOF Guidelines for Therapeutic Intervention

- History of fracture after age 40
- History of hip, wrist, or vertebral fracture in a first-degree relative
- Being in lowest quartile for body weight (≤57.8 kg (127 lb))
- Current cigarette smoking habit

Note. Reproduced from National Osteoporosis Foundation guidelines (NOF, 1998a,b).

Table IV Risk Factors Providing Indications for the Diagnostic Use of Bone Densitometry

1. Presence of strong risk factors
   - Estrogen deficiency
     - Premature menopause (age <45 years)
     - Prolonged secondary amenorrhoea (>1 year)
     - Primary hypogonadism
   - Corticosteroid therapy
     - Prednisolone >7.5 mg/day for 1 year or more
   - Maternal family history of hip fracture
   - Low body mass index (<19 kg/m^2)
   - Other disorders associated with osteoporosis
     - Anorexia nervosa
     - Malabsorption syndrome
     - Primary hyperparathyroidism
     - Posttransplantation
     - Chronic renal failure
     - Hyperthyroidism
     - Prolonged immobilization
     - Cushing's syndrome
2. Radiographic evidence of osteopenia and/or vertebral deformity
3. Previous fragility fracture, especially of the hip, spine, or wrist
4. Loss of height, thoracic kyphosis (after radiographic confirmation of vertebral deformities)

Note. Table reproduced from the Royal College of Physicians guidelines (RCP, 1999)

disease who benefit most from anti-resorptive therapies such as bisphosphonates (Cummings *et al.,* 1998). Thus there seems to be a consensus supporting the use of a spine or hip *T* score of ≤ -2.5 as the appropriate intervention threshold for instigating treatment in white women. However, it is important to take all the other relevant clinical factors into account such as those listed in Tables III and IV. In particular, the age of the patient and whether there is a history of previous fragility fractures are important independent predictors of future fracture risk. No consensus has yet emerged on what intervention thresholds are appropriate in men and other ethnic groups. However, the revised guidelines recently published by Kanis and Glüer (2000) suggest that the same absolute BMD thresholds applied to white women may also be applicable to these other groups.

The Interpretation of Peripheral Measurements

In recent years the WHO definition of osteoporosis as a *T* score ≤ -2.5 has been widely adopted as a basis for therapeutic decision making. However, a disadvantage is that due to different age-related changes in *T* score for different technologies and measurement sites, patients will cross the $T = -2.5$ threshold at different ages depending on what type of measurement is made (Fig. 10). To understand the clinical consequences of this, one needs to convert the threshold of $T = -2.5$ to an equivalent *Z* score value. It is clear from Fig. 10 that methodologies for which the mean *T* score falls more slowly with age will require patients to achieve a more negative *Z* score before the $T = -2.5$ threshold is triggered. With this information, and assuming that the distribution of *Z* score values is gaussian, it can be estimated that at age 65 the percentage of patients with a *T* score ≤ -2.5 for the Hologic Sahara QUS device, DXA femoral neck BMD, DXA spine BMD, and spine QCT are 3, 16, 24, and 60%, respectively (Blake and Fogelman, 2001). It is therefore clear that large differences in the numbers of patients recommended for treatment could arise depending on the type of bone densitometry equipment used. To be fair to the WHO report, its recommendation for defining osteoporosis was only intended to apply to BMD measurements of the spine, femoral neck, or forearm. However, only recently has it become widely appreciated that the WHO criteria cannot be applied uncritically to other types of measurement (Faulkner *et al.,* 1999; Delmas, 2000; Miller, 2000). Given the need to make greater use of the small, low-cost devices for scanning the peripheral skeleton if the many millions of women most at risk are to be identified and treated, it is important to consider what approaches to the interpretation of bone density measurements can be adopted to ensure the greatest degree of consistency between the different methodologies.

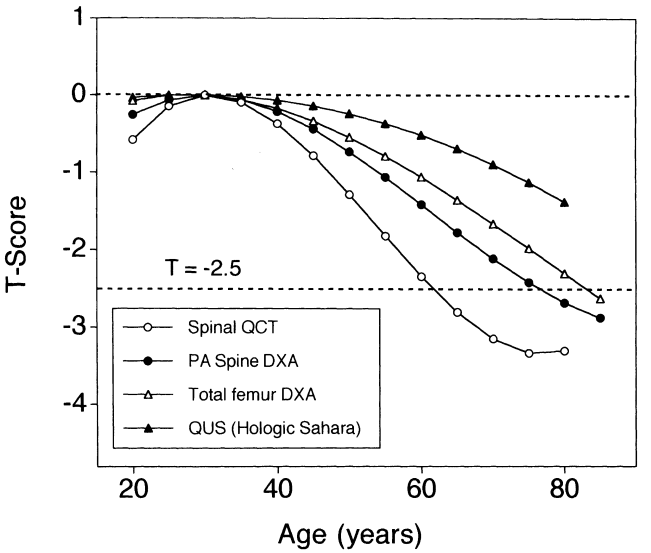

Figure 10 Age-related decline in mean *T* score for white female subjects for DXA of the lumbar spine (Hologic manufacturer's reference range); DXA of the femoral neck (NHANES III reference range (Looker *et al.,* 1998)); QCT of the spine (reference data from Cann *et al.* (1985)); Quantitative ultrasound measurements of the calcaneus. Reference data from *Frost et al.* (1999).

The inconsistency between different *T* score figures, taken with confirmation from fracture studies that strengthens evidence that femur BMD is the best measurement for predicting hip fracture (Black *et al.,* 2000), have led to recommendations that the femur should be adopted as the gold standard site for the diagnosis of osteoporosis (Kanis and Glüer, 2000; Black, 2000). However, a way is needed to ensure that broadly comparable treatment decisions can be made using other methodologies such as pDXA and QUS. Recently, several reports have proposed that other measurement modalities such as pDXA and QUS should be utilized by establishing device specific thresholds that correspond to a 5-year hip fracture risk equal to that set by a femoral neck *T* score of -2.5 (Kanis and Glüer, 2000; Black, 2000). Although no formal recommendations have yet been publicized, it is clear that some such approach is required to ensure that proper use can be made of the many different types of peripheral device now available.

The Problem of Identifying Different Patients for Treatment

Given the range of different types of measurement, there is clearly cause for concern about the potential for conflicting findings between different techniques. If two types of measurement were to correlate perfectly ($r = 1.0$), they would identify exactly the same patients in the at-risk group. However, in practice different types of measurement often correlate poorly, with $r \approx 0.6-0.7$ between BMD results from different sites and $r \approx 0.4-0.5$ between QUS and BMD measurements. This raises the issue of how concerned clinicians should be that, due to the imperfect correlations, different patients are selected for treatment on the basis of different sites and techniques.

To understand this problem, it should be borne in mind that there is no such thing as an absolute fracture threshold. As demonstrated by the quartile plot of fracture incidence (Fig. 7), there will always be a substantial overlap between measurements from fracture and nonfracture patients, and absolute discrimination between these groups is not possible using any type of BMD measurement. Depending on the rules agreed for the clinical interpretation of BMD measurements, only a certain percentage of all those patients scanned who later go on to sustain a fracture will be included in the at-risk group. It is therefore clear that, depending on the correlation coefficient between them, different types of measurement are identifying different individuals from this fracture group. More detailed analysis shows that the most successful technique (i.e., the measurement that captures the largest percentage of future fracture cases) is the one with the highest RR value (Blake and Fogelman, 2001).

It is clear that bone densitometry studies provide a measure of fracture risk that is analogous to assessment of blood pressure with regard to the risk of stroke or measurement of cholesterol with regard to the risk of developing ischemic heart disease (Marshall *et al.,* 1996). It is important to distinguish the concepts of risk as applied to an individual and to a population. Although BMD may be the most important single risk factor for fracture, osteoporotic fractures are nevertheless multifactorial and, in addition to low bone density, will depend upon other issues such as accidents and the propensity to fall. BMD measurements are well suited to the study of populations, where they are effective in identifying patients who have a higher than average risk of fracture, but are less good at identifying those specific individuals who will later sustain a fracture.

Summary and Conclusions

During the past decade the pace of technological innovation has been rapid, with the introduction of many new or improved radiological devices for the noninvasive assessment of patients' skeletal status. DXA scanning of the hip and spine remains the "gold standard," although there is now a wider appreciation of the need for smaller, cheaper devices for scanning the peripheral skeleton if the many millions of women most at risk of a fragility fracture are to be identified and treated. Several sets of guidelines for the clinical use of bone densitometry have been published, and most have included recommendations for intervention thresholds for initiating treatment in white women. The WHO criterion of a T score ≤ -2.5 has been especially influential, although cannot automatically be applied to the newer peripheral techniques such as QUS or in men and patients from other ethnic groups.

At the present time, most experts do not advocate mass screening of the population for osteoporosis, and instead the guidelines recommend a case-finding strategy based on identifying patients with generally accepted clinical risk factors. However, with the widespread availability of QUS systems, this view may change. The advantages of QUS outlined above mean that it may have a role in many specialist departments and primary care facilities. However, in view of the large number of commercial devices available, there are concerns about whether all the reference ranges are accurate and appropriate. As emphasized above, the WHO definition of a T score of ≤ -2.5 cannot automatically be applied to QUS, and there is a consensus emerging toward defining intervention thresholds for peripheral devices based on estimates of absolute fracture risk. It seems premature to advocate the routine use of QUS until these issues have been resolved and appropriate clinical strategies have been agreed upon. Nevertheless, it is probable that ultrasound will be widely used for the assessment of the skeleton within the next 5 to 10 years, and at that point there would effectively be screening for osteoporosis.

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CHAPTER 92

Controversial Issues in Bone Densitometry

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Bone densitometry is an extremely valuable tool for the diagnosis of osteoporosis, assessment of fracture risk, tracking the course of disease states that alter bone remodeling, and monitoring the response to treatment for osteoporosis and other metabolic bone diseases (Miller *et al.,* 1999; Miller and Bonnick, 1999). As clinical approaches to osteoporosis have expanded, with more diagnostic and therapeutic agents, questions regarding the utility of bone mass measurements have also increased. This chapter will discuss key controversial issues that have recently become prominent. The issues to be addressed are:

1. discrepancies in *T*-score determination among bone densitometers;
2. prevalence versus risk in reporting bone mineral density device results;
3. differences in ethnic- and gender-specific databases among different bone density devices;
4. value and limitations of serial bone mineral density measurements.

Discrepancies in *T* Score Determination among Bone Densitometers

In 1994, a working group of the World Health Organization (WHO) established levels of bone mineral density (BMD) that could be used for the diagnosis of osteoporosis. The basis for the well-known cutoff for the diagnosis of osteoporosis (*T* score less than −2.5) was data examining both prevalence and risk of fragility fractures in elderly postmenopausal Caucasian women (WHO Study Group, 1994). This *T* score value was intended to be used as an epidemiological cutoff to describe a BMD level which could diagnose osteoporosis in the absence of a fragility fracture. In practice, this *T* score has been applied broadly to patients of both genders and various ethnicities. In addition, it has been used for the assessment of premenopausal women and patients receiving chronic glucocorticoid therapy. The appropriateness of extrapolating the WHO cutoff levels to men, various ethnic groups, patients with secondary causes of osteoporosis, and premenopausal women will be discussed later.

The WHO established the BMD-cutoffs for the diagnosis of osteoporosis, using the *T*-score, which is the number of standard deviations (SD) a patient's BMD is from the young normal mean BMD, rather than the absolute BMD value, to reduce the effect of measured differences in BMD produced by different equipment at identical skeletal sites. These differences are due to the different ways that manufacturers calibrate BMD with their devices and to different regions of interest (ROI) assessed by the different scanners. The WHO used the comparison of a patient's BMD to the young normal reference population rather than an age-matched reference population because defining osteoporosis on the basis of age-matched data would not detect the true prevalence of low bone mass in the elderly population since bone loss is a universal process of aging. The prevalence of osteoporosis would not increase with age if age-matched comparisons were used, and that is not logical. At the time of the WHO deliberations, there were very few bone densitometer devices available. Therefore, there were a limited number of reference databases from which the *T* score could be derived.

Each manufacturer of densitometry equipment has an adequate database, especially for the Caucasian female population. Yet, because the young normal reference populations are not consistent among the manufacturers, a patient's *T* score at

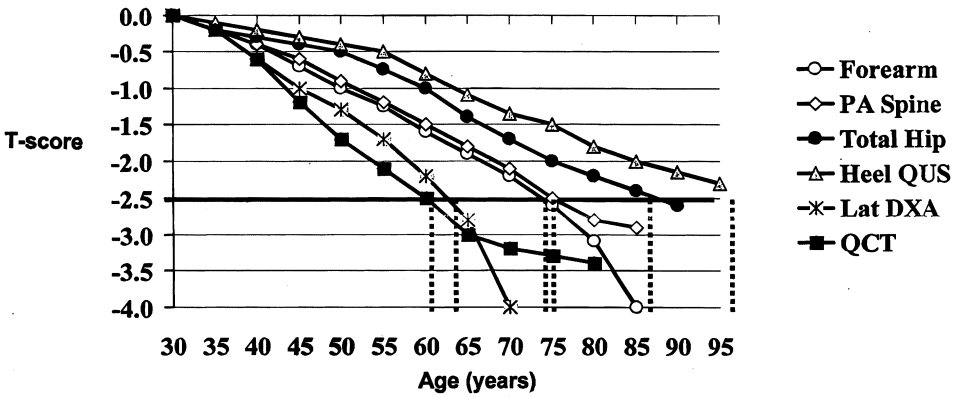

Figure 1 Age-related decline in mean Caucasian female *T* scores for different BMD technologies based on manufacturer reference ranges. The hip DXA reference data are from the NHANES study. The DXA normative data for the PA spine (L1–L4), lateral spine (L2–L4), and forearm (one-third region) were obtained from the Hologic QDR-4500 densitometer. Heel normative data were taken from the estimated BMD for the Hologic Sahara ultrasound unit. Spinal QCTs are those used by the Image Analysis reference system. Reproduced from Faulkner *et al.* (1999).

a specific skeletal site may differ depending on the equipment used. This may lead to different WHO classifications, based solely on inconsistent young normal databases (Ahmed *et al.,* 1997; Simmons *et al.,* 1997). This *T*-score discrepancy is compounded if a patient's BMD is measured at different skeletal sites using different technologies (e.g., dual-energy X-ray absorptiometry (DXA) of the hip vs ultrasound of the heel) (Faulkner *et al.,* 1999). Figure 1 demonstrates this discrepancy, which may lead to different diagnostic conclusions. For example, using heel ultrasonometry, patients, on average, must be 107 years of age before they fall, on average, below the WHO diagnostic threshold of −2.5. In contrast, using lateral spine DXA, patients, on average, are 60 years of age when they are classified as osteoporotic by WHO criteria (Faulkner *et al.,* 1999). This discrepancy is due to: (1) inconsistent young normal reference databases among the devices, and (2) differences in the biology of bone, cortical vs cancellous, with varying rates of bone loss from these sites after the menopause.

Data suggest that if the BMD is measured on a patient at the spine and the hip using the same DXA machine, the *T*-score derived from different young normal populations may be very different (Table I) (Ahmed *et al.,* 1997; Simmons *et al.,* 1997). These data included over 2000 postmenopausal Caucasian women who were scanned at the PA spine and femoral neck using the same central DXA machine. Their *T* scores were calculated using a young normal population obtained at the study site and a young normal population provided by the manufacturer. Table I demonstrates that, for example, when the *T* score was calculated from the study site young normal database vs the manufacturer's young normal database at the hip, the percentage classified as osteoporotic by WHO criteria (in parentheses) increased from 3 to 23%. This difference cannot be due to bone biology or different devices since the same skeletal site was measured on the same DXA machine. These differences, therefore, are due to the differences in the two young normal reference populations from which the *T* scores were derived. If one examines the equation for the determination of the *T* score, it is clear that if the mean BMD or the SD of the mean of the young normal reference population are not identical, then the derived T-score will be different:

$$T\text{ score} = \frac{\text{patient's BMD} - \text{mean BMD of the young normal reference population}}{\text{SD of the young normal reference population}}.$$

The only database consistently being used by manufacturers is the National Health and Nutrition Examination III (NHANES III) database, which includes both young normal and age-matched BMD data from males and females of various ethnic backgrounds (Looker *et al.,* 1995). The FDA Regulatory Device Division strongly urged the manufacturers to incorporate the NHANES III hip database into their software, when it was demonstrated that the *T* score at the hip could differ by as much as 1.0 SD in the same patient as a function of which database was used (Faulkner *et al.,* 1996). The use of the NHANES III database uniformly across all machines has removed this source of *T*-score discrepancy. For the most part, manufacturers have incorporated this consistent database, yet, some software may not include NHANES III, or may allow the operator to include/exclude it. Each operator and analysis physician need to know which normal database is being used, and the implications if different databases are used when *T* scores from different central DXA machines are compared.

Additional data suggest that the *T*-score discrepancy may be reduced by using a common young normal database, even when patients are measured using different technologies at different skeletal sites (Greenspan *et al.,* 1995). The authors measured the BMD of elderly subjects using a variety of heel ultrasound devices, heel DXA and hip DXA. The subjects' *T* scores were calculated using a small ($n = 55$) yet consistent young normal database. Despite using different technologies and measuring different skeletal sites, the *T* scores were similar. These results, which have been confirmed by other

Table I Classification, Using the WHO Criteria, of a Group of Patients Using *T* Scores Calculated from a Group of Study Young Normals vs the Manufacturer's Young Normals

	Spine no. (%)		Femur no. (%)	
WHO criteria	Study young norm	Manufacturer young norm	Study young norm	Manufacturer young norm
$T > -1.0$	1320 (64)	990 (48)	1385 (67)	625 (30)
$-2.5 < T < -1.0$	625 (30)	759 (37)	626 (30)	970 (47)
$T < -2.5$	123 (6)	319 (15)	57 (3)	473 (23)
Total	2068	2068	2068	2068

Note. Modified from Ahmed AIH *et al.* (1997).

studies, suggest that despite differences in bone structure or varying rates of bone loss, diagnostic discordance can be minimized by using a consistent young normal database.

On May 17, 1999, the FDA Regulatory Device Panel met to consider ways of mitigating the *T*-score discrepancies, which were becoming more apparent as more devices were being used for testing in the United States. One solution offered was to abolish the *T* score and move to absolute BMD determinations and absolute fracture risk estimation. This approach has merit since low BMD is a very important risk factor for the estimation of fracture risk, and all devices (central and peripheral) are nearly equal in their ability to predict fracture risk at skeletal sites other than those measured (i.e., heel or wrist or finger measurements can be used predict hip or spine fracture) in the elderly population (Marshall *et al.,* 1996). Finally, data suggest that when other risk factors are removed, thereby isolating BMD, the absolute vertebral or hip fracture risk is identical for men and women at the same BMD level (Ross *et al.,* 1993; De Laet *et al.,* 1998). One of the limitations of linking absolute BMD to absolute fracture risk is that the absolute BMD also differs between manufacturers even at the same skeletal site. Though a standardized BMD (sBMD) has been established for both the spine and hip DXA on all three central DXA manufacturers' equipment (Steiger, 1995; Hanson, 1997), the relationship between sBMD and fracture risk is unknown. Hence, the currently defined relationships between absolute BMD and fracture risk are manufacturer-specific. The standardized database project described below will establish an sBMD for all currently available central and peripheral devices that is related to vertebral and hip fracture risk.

The International Osteoporosis Foundation (IOF) has recommended that *T* scores for the diagnosis of osteoporosis be calculated only for the hip and that all other measurements be used for risk prediction (Kanis and Gluer, 2000) as a means of dealing with the *T*-score discrepancy. Such a proposal has merit and may work well in those countries that have either no reimbursement schedules for densitometry or a payment system that is not linked to diagnostic, ICD-9 codes (International Classification of Diseases). However, in the United States reimbursement is linked to a diagnosis which requires clear, definable criteria. Hence, the recommendation of the International Society of Clinical Densitometry and the National Osteoporosis Foundation (NOF) to retain the *T* score for diagnostic purposes was accepted by the FDA Regulatory Device Panel.

The short-term solution for alleviating *T*-score discrepancies is to establish a "*T*-score equivalence" among the various devices. The *T*-score equivalence will be calculated primarily from data available from the Study of Osteoporotic Fractures (SOF) (Black *et al.,* 1992). Since the prevalence of osteoporosis varies as a function of the device used, the site measured, and differences in young normal databases, fracture risk would be used as the basis for calculations of equivalency. Using 5-year absolute risk figures for hip fracture in the elderly population (average age 70 years), this analysis established the feasibility of identifying a *T* score as equivalent to a given fracture risk for each device. Table II shows the various *T*-score cutoff points, derived from different young normal databases using various devices, which yield a 6% risk of hip fracture in the 70-year-old population. It is apparent that in this age group a *T* score of -2.0 from a heel ultrasound measurement is equivalent to a *T* score of -2.5 at the femoral neck for determining hip fracture risk over the next 5 years. Hence, the *T*-score equivalence for ultrasonometry of the heel is -2.0.

The concept of *T*-score equivalence offers a temporary solution to discrepancies in *T*-scores. A physician, using a particular BMD device, would know the *T*-score cutoff derived from that device which predicts the same hip fracture

Table II *T* Score Cutoff Points for Various Bone Density Devices Based on Equal Hip Fracture Risk (6%)

		BMD	
Site/technology	Hip fx risk (5 yr) (%)	Value	*T* score
Femoral neck/DXA	6	0.58[a]	−2.5
Spine/DXA	6	0.63	−3.8
Calcaneus/ultrasound	6	46.8	−2.0

[a]Reference point, 0.58 g/cm^2 is $T = -2.5$ at the femoral neck by Hologic DXA.

risk as a *T* score of −2.5 derived from DXA at the *femoral neck*. The *T*-score equivalence analysis also has problems, which include:

1. SOF data were not determined concurrently on all devices in a prospective fashion, since different devices were introduced into the trial at different times;
2. fractures, other than the hip, have not yet been assessed and the data may be inadequate;
3. some manufacturers do not have adequate hip fracture data;
4. the data available do not include the early postmenopausal population.

The long-term solution to the *T*-score discrepancy is either to abolish the *T* score and move to a standardized absolute BMD cutoff level linked to absolute fracture risk and/or to reduce the *T*-score differences by developing consistent young normal and elderly reference databases which would be incorporated into all bone mass measurement devices. This data would be collected at the same time point from all skeletal sites on all measurements devices.

The development of a consistent BMD database, would require at least 2 years to complete. This database would require 2000 young normal women of various ethnicities and 3500 older women, both with and without prevalent vertebral fractures who would have BMD measurements performed on all available FDA-approved devices. In addition, a case-controlled study of 100 women with hip fractures would be conducted. This study would define how much of the *T*-score discrepancy is due to site-specific differences in rates of bone loss, machine variance, and inconsistent young normal reference populations. In addition, by completing the fracture cohort with all devices, analysis could establish an sBMD for all devices linked to fracture risk. This information would make it possible to decrease the discordance in *T* scores and thereby improve the diagnostic capability of the various bone mass measurement devices. It would also define an sBMD, which is a single number for all devices, linked to vertebral and hip fracture risk.

Prevalence versus Risk in Reporting Bone Mineral Density Device Results

The second controversial issue in bone densitometry is the use of prevalence versus risk for reporting BMD results. Prevalence is used for diagnosis and risk is used for fracture prediction. The prevalence of osteoporosis by WHO criteria is dependent upon:

1. the young normal database used;
2. the cutoff point chosen to define osteoporosis (i.e., −2.0 SD vs −2.5 SD);
3. the skeletal site measured;
4. the accuracy of the measurement; and
5. the gender and ethnic group used to define prevalence.

The cutoff point chosen determines the prevalence, which is the percentage of a population that is diagnosed with osteoporosis. In 1992, the WHO strongly considered using a *T*-score of −2.0 as the cutoff point. Estimates of the number of women with osteoporosis using this cutoff were undertaken (Melton *et al.,* 1992). In 1994 the WHO decided that a *T* score of −2.5 would be the cutoff point for the diagnosis of osteoporosis in the postmenopausal population. Therefore, a revised calculation of the number of women with osteoporosis was necessary (Melton, 1995). In the postmenopausal population, measurements of the same skeletal site using the same machine and a consistent young normal reference database for the calculation of the *T* score, will determine the prevalence of osteoporosis defined by a given cutoff point. The prevalence would increase if the cutoff point were changed to −2.0 rather than −2.5.

The WHO selected −2.5 as the cutoff point for the diagnosis of osteoporosis based on two observations. First, the percentage of postmenopausal Caucasian women whose *T* scores were −2.5 or less at the hip (16%), was approximately the same as the lifetime hip fracture rate in this population (16%). Second, approximately 40% of the postmenopausal women had *T* scores at or below −2.5 at any of the three measured skeletal sites (hip, spine, and wrist), approximating the lifetime *osteoporotic fracture* risk at these same skeletal sites. Hence, the prevalence of osteoporosis using −2.5 as the cutoff was consistent with estimated fracture risk data. The WHO criterion was an important milestone because it provided, for the first time, a BMD level that allowed clinicians to make a diagnosis of osteoporosis before the first fracture occurred. The use of BMD criteria to make a diagnosis before fracture has occurred is analogous to the diagnosis of hypertension before a stroke occurs. This BMD cutoff was especially important since the risk for a second osteoporotic fracture is much greater after the first fracture has occurred (Ross *et al.,* 1998; Nevitt *et al.,* 1999). Prevention of the first fracture is possible only if the population with low bone mass who are at risk can be identified.

Differences in Ethnic- and Gender-Specific Databases among Bone Density Devices

The next step was to determine cutoff points for the male population and different ethnic groups. The prevalence of osteoporosis in the NHANES III study, using *T*-scores and the WHO criteria, was different if males were compared to a female young normal reference database. Prevalence also differed if males or females of one ethnic group were compared to males or females of a different ethnic group reference database (Looker *et al.,* 1995). Likewise, the impact of gender-specific databases on the prevalence of osteoporosis using the WHO criteria was documented in a separate study (Table III) (Melton *et al.,* 1998). *T* scores calculated for elderly men from a female young normal database are different from *T* scores calculated from a male young normal database. Therefore, it is recommended, at the current time, that the *T*-score

Table III Prevalence of Osteoporosis in Men Male vs Female Reference Database

Femoral neck		Spine, femoral neck, radius	
Male reference	Female reference	Male reference	Female reference
6%	4%	19%	3%

Note. NHANES III data (Looker, *et al.,* 1997); Rochester, Minnesota, data (Melton *et al.,* 1998).

used for the diagnosis of osteoporosis be calculated using a gender-specific young normal database. Hologic, Lunar, and Norland provide gender- and ethnic-specific young normal databases for the calculation of *T*-scores. Since the *T*-score calculation is based on the mean and the standard deviation of the reference population, small differences in these values influence a patient's *T*-score value. Therefore, *T*-score discrepancies are compounded when males are compared to female young normal populations. Therefore, *T*-score discrepancies for males can be reduced by the use of gender-specific young normal databases.

The prevalence of osteoporosis in various ethnic populations is being examined in the NORA (National Osteoporosis Risk Assessment) study. This longitudinal BMD and risk-factor project examines the prevalence of osteoporosis, based on the WHO criteria, in a group of more than 200,000 postmenopausal women of various ethnicities, more than 50 years old in the United States (Siris *et al.,* 1998). *T* scores for the central DXA and peripheral measurements were calculated using a Caucasian young normal database. As expected, African-American women had a higher mean BMD and therefore a higher mean *T* score than Caucasians, Asian-Americans, non-Black Hispanic-Americans, and Native Americans. The rate of bone loss was identical in all the ethnic groups measured. By age 70, the prevalence differences among ethnic groups was very small. Therefore, NORA data suggests that the rate of bone loss in postmenopausal women of various ethnic groups in the United States is similar, and by the age of 70, the prevalence of osteoporosis may be similar in all groups. This appears to be true for U.S. ethnic populations that are three to five generations from their origin, whose gene pools have mixed, and whose diets are all similar. The prevalence of osteoporosis may have been different if the *T* scores had been calculated using ethnic-specific young normal databases. This analysis is in progress. The NORA data, to date, calculating *T* scores from Caucasian young normal women, suggest that this may be an acceptable method of assessing the prevalence of osteoporosis in elderly (over 70 years), using peripheral devices, in U.S. ethnic groups.

In contrast to the prevalence issue, there is little data regarding the effects of gender or ethnicity on the determination of fracture risk using either *T* scores or BMD. The absolute BMD values of the spine by DXA or the heel SXA have shown similar absolute vertebral fracture risk and non-

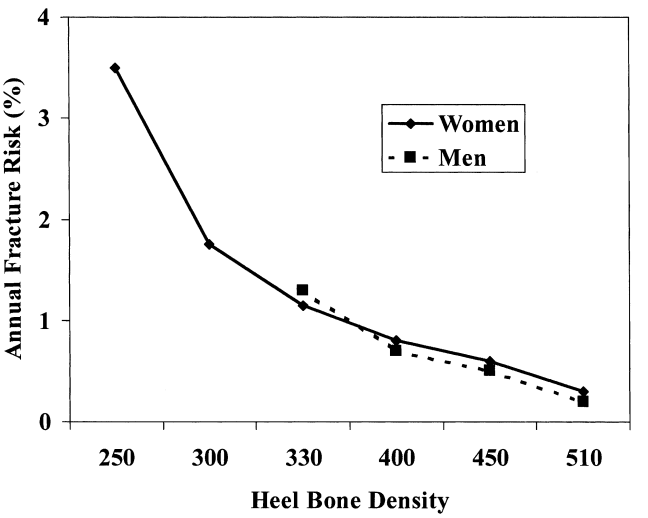

Figure 2 The relationship between heel bone density and annual fracture risk (%) in Caucasian and Japanese-American males and females adjusted for other risk factors.

vertebral, nonhip fracture risk between Caucasian and Japanese-American males and females (adjusted for other risk factors) in a prospective fracture trial (Fig. 2) (Ross *et al.,* 1995). Also, recent data have shown that the 1-year absolute hip fracture risk is identical in elderly age-matched Caucasian Dutch men and women at comparable absolute femoral neck BMD measurements (Fig. 3) (De Laet *et al.,* 1998). Finally, a 10-year prospective hip fracture study in Swedish men and women, using *T* scores calculated from the NHANES III Caucasian female young normal database rather than absolute BMD, showed similar 10-year absolute hip fracture risk in men and women at similar *T* scores (Table IV) (Kanis and Gluer, 2000). In a cross-sectional study, historical fracture risk, relative risk per SD (RR/SD) derived from elderly Caucasian men and women, was calculated using either a male or female young normal database. The RR for fracture per SD reduction in BMD was higher in women than in men (2.0/SD vs 1.4/SD), but the RR for fracture per SD reduction was not different whenever the RR per SD was calculated from the male young normal or female young normal reference population database (Table V) (Melton *et al.,* 1998). Other data suggest that men may fracture at a higher BMD or higher *T* score, calculated from a male young normal reference database, than women (Orwoll, 2000). Though the 5- to 10-year prospective fracture data suggest that men may fracture at similar BMD or *T* score as women, it is well known that lifetime fracture risk (especially hip) is lower in men than in women. The lower lifetime risk of hip fracture in men may be due to fewer falls, greater muscle mass or greater bone size in men (Seeman, 1999; Seeman *et al.,* 2001).

Data on the effects of ethnicity on the determination of fracture risk is scant. Historically calculated fracture data from NORA suggests that the RR for global fracture per SD reduction using *T* scores calculated from a female Caucasian young normal database, is similar in elderly

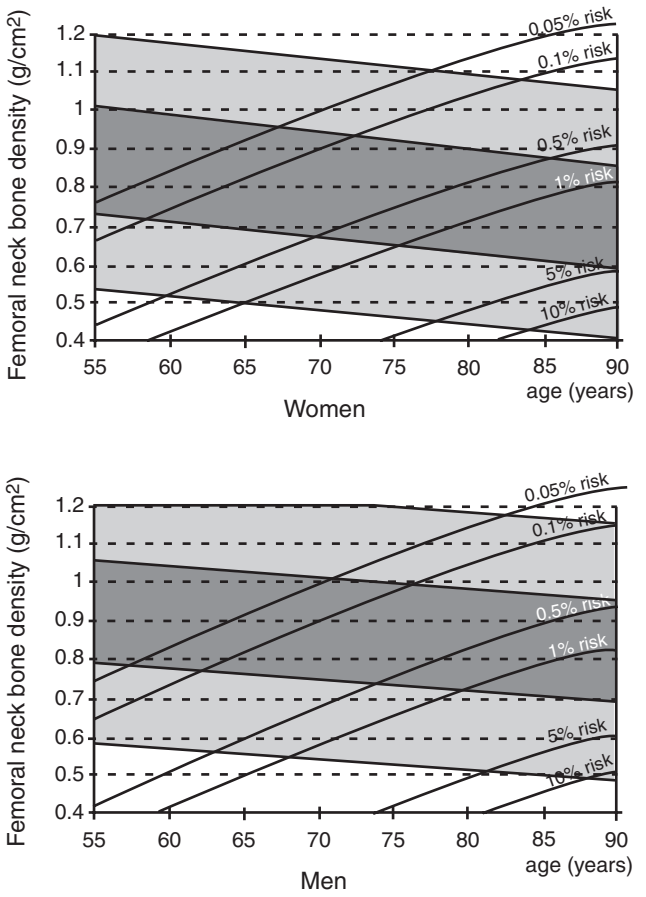

Figure 3 One-year hip fracture risk by age and BMD in women and men. The dark gray area indicates average BMD ± 1 SD, the light gray area indicates average BMD ± 2.5 SD. Reproduced from De Laet *et al.* (1998).

Caucasian, Hispanic, Native American, and Asian women and only slightly less in African-American women (Fig. 4).

Data from China, where the gene pool probably includes no Caucasian genes and the population has not been exposed to U.S. culture and nutrition, suggest that vertebral fracture risk calculated from absolute BMD may be similar to that of American Caucasians. However, hip fracture risk in China, calculated from either absolute BMD or *T* scores related to a Chinese-specific young normal database, is lower than in U.S. Caucasians (Ling *et al.,* 2000), even though the Chinese absolute hip BMD is lower than the absolute BMD of the U.S. Caucasians. The reason for these lower fracture rates in the Chinese is not clear, but shorter hip axis length, lower bone turnover rate, and fewer falls may be contributing factors independent of absolute BMD. Some of these fracture incidence differences are mitigated if the BMD is adjusted for the smaller body mass index (BMI) and smaller bone width observed in the Chinese. At comparable BMI, the absolute BMD to hip fracture risk is comparable to the U.S. Caucasian population.

Therefore, the data suggest that gender and ethnic-specific databases may be needed to determine prevalence, since differences in the mean BMD and SD for the young normal males and different ethnic groups influence the number of patients diagnosed with osteoporosis by the WHO criteria. Absolute 1- to 5-year fracture risk linked to absolute BMD may be similar regardless of gender, although the influence of bone size may alter this risk. Fracture risk data calculated from gender-specific *T* scores is not clear, although the two studies mentioned suggest that risk may be similar between men and women, even when the *T* scores are calculated from a female young normal database. Ethnic-specific global fracture risk (RR/SD) reduction may be similar in women when calculated from a female Caucasian young normal database, at least in the ethnic groups which make up the U.S. population. Ethnic-specific databases or absolute BMD linked to absolute fracture risk may be needed for risk assessments from continent to continent, especially in populations where there is little Caucasian influence on the gene pool. Recent Australian data suggest that hip fracture risk for Caucasian-Australian women is similar when the *T* scores are calculated from the U.S. Caucasian female NHANES III database or from a young normal Australian database.

Absolute risk calculated from absolute BMD may be the best approach for risk assessment regardless of race or gender, yet an sBMD calculated for all central and peripheral devices linked to fracture risk still needs to be completed since different devices give different BMD values. Presently, the sBMD available for central DXA spine and hip measurements is not linked to fracture risk. For now, the best suggestion is to use *T* scores calculated from a Caucasian female young normal database for the diagnosis of osteoporosis in

Table IV Ten-Year Absolute Hip Fracture Risk from Sweden

Males: *T* score −2.5	Females: *T* score −2.5
Age: 75 = 24%	Age: 75 = 24%
Age: 85 = 28%	Age: 85 = 28%
From NHANES III	From NHANES III
Female young normal database	Female young normal database

Note. From Kanis *et al.* (2000).

Table V Odds Ratios for Osteoporotic Fracture per 1.0 SD Decrease in BMD for Elderly Men and Women Using Different Young Normal Databases

Study group and criterion	Femoral neck
20- to 29-year-old male database	
Fx women (BMD)	2.25 (1.44, 3.51)
Fx men (BMD)	1.10 (0.82, 1.48)
20- to 29-year-old female database	
Fx women (BMD)	2.43 (1.49, 3.95)
Fx men (BMD)	1.11 (0.80, 1.54)

Note. Modified from Melton *et al.* (1998).

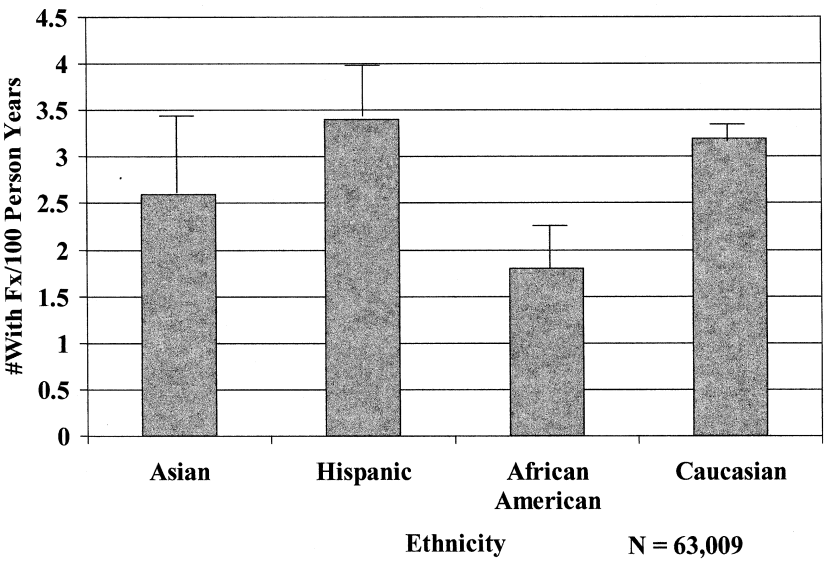

Figure 4 Fracture rates (per 100 person years) by ethnicity.

elderly Caucasian women. For the determination of prevalence, the *T* score calculated from ethnic-specific, young normal databases should be used. Peripheral BMD devices may or may not have gender or ethnic specific young normal databases, which may limit this approach with peripheral technologies, although the NORA data suggest that the Caucasian young normal database may suffice for the assessment of prevalence in U.S. ethnic groups. The diagnosis of osteoporosis in men should be made using *T* scores calculated from a male young normal database. Until more gender- and ethnic-specific fracture data is available using both areal and volumetric BMD measurements, absolute fracture risk (1–10 year) should be calculated using gender-specific absolute BMD for Caucasian men and women. Male and female Caucasian data for hip fracture and global fracture risk has suggested that risk may be the same using *T* scores derived from a young normal female database, although there is no consensus on this issue at this time.

Finally, patients who have already sustained fragility fractures can be diagnosed with established osteoporosis regardless of their absolute BMD or *T* score, assuming secondary causes of fracture, such as Paget's disease or malignancy, are excluded. The diagnosis of osteoporosis was based on the presence of a fragility fracture long before the WHO BMD criteria were established.

Value and Limitations of Serial Bone Mineral Density Measurements

The final controversy in bone density pertains to the use of serial BMD to monitor the effects of aging or disease states, or to monitor the effects of therapeutic agents on BMD.

The best skeletal site for detecting change of BMD, for most disease states that affect bone or pharmacological interventions, is the spine by DXA (Miller *et al.,* 1999; Miller, and Bonnick, 1999). This is due, in part, to the presence of abundant cancellous bone in the spine, a tissue-type which has a much greater surface area and higher metabolic activity than cortical bone. Quantitative computerized tomography (QCT) of the spine measures a larger volume of cancellous bone at the axial skeleton than does DXA, because it is able to isolate cancellous bone. Therefore, larger increases in BMD are observed with therapy. Moreover, QCT measures volumetric density (g/cm3), a parameter believed to be more accurate than the two-dimensional density measured by DXA. However, QCT's ability to monitor longitudinal changes is limited by its relatively poor precision compared to spine DXA. The precision error may be comparable to spine DXA in QCT centers that calibrate their equipment prior to each axial bone density assessment. This process is often unrealistic when the scanner is used for multiple types of scans (chest, head, etc.) rather than being dedicated to bone scans (Grampf *et al.,* 1997).

Other skeletal sites are less useful for monitoring disease states or pharmaceutical effects, with the exception of the distal forearm (1/3 site) which may be profoundly effected by excess parathyroid hormone activity. Patients whose forearm *T* score by DXA is less than that observed at the spine or hip should be assessed for parathyroid abnormalities (Dempster *et al.,* 1999). The hip shows significant serial change in response to pharmacological therapies, although the rate of change per year is less than the rate of change at the spine. The total hip is the best region of interest (ROI) of the hip for monitoring serial change since it is larger than the ROI for the femoral neck. The larger the ROI, the better the precision. However, the femoral neck is the best ROI of the hip for the diagnosis of osteoporosis and the calculation of prevalence, since the total hip BMD may be artifactually elevated in elderly patients by sclerosis below the lesser trochanter, much as osteophytes may artifactually

elevate the PA spine by DXA. None of the peripheral BMD measurements, including ultrasound, have shown significant serial changes in BMD in response to treatment. This is not due to the precision of the technology, which is as good as central devices. It is unclear why the peripheral techniques are not able to show changes in response to therapy. It may be related to the surface area, differences in blood flow, or differences in the bone marrow environment of the peripheral bone measured.

There are three common mistakes made in calculations of serial BMD changes:

1. using BMD values from two different manufacturer's equipment rather than calculating sBMD for both, and then determining serial changes;
2. failure to incorporate the *in vivo* precision error (CV%), determined for each specific device, into the calculation; and
3. using *T* scores rather than absolute BMD for the calculation of serial changes.

As previously discussed, the absolute BMD may vary among manufacturers' equipment, even if the same skeletal site is measured, due to differences in ROI and machine calibration. These differences might be misinterpreted as changes in BMD due to the biology of a disease or the response to a therapeutic intervention. It would be ideal if patients could be monitored on the same DXA machine, or, at the very least, equipment from the same manufacturer, every year. However, this is unrealistic since patients change health insurance, physicians, or move to different locations. In response to this problem, sBMD was developed to equalize the absolute BMD values among the three central DXA manufacturers for the PA spine and the total hip (Hanson *et al.,* 1997; Steiger, 1995). This was achieved by measuring 100 postmenopausal women on each central device and then performing a linear regression to derive an equation to equate the absolute BMD measurements. sBMD provides some degree of equality when it is necessary to compare serial BMD measurements from different manufacturers' central DXA instruments. Most manufacturer software now calculates sBMD as well as their own proprietary absolute BMD. However, sBMD can be calculated using the appropriate equations (Hanson *et al.,* 1997; Steiger, 1995). Even though sBMD provides comparability of measurements from two different devices, there is an additional effect on the precision error. Precision error may be increased as much as 3–5% above the *in vivo* precision error determined for each facility when two machines from different manufacturers are used. Thus, the least significant change (LSC), which is based on the difference of the two sBMD numbers, the precision error of the facility and the precision error between machines, may be increased. The clinician must make the final decision if a particular serial change is significant and thereby warrants a change in patient therapy.

It is incumbent upon each densitometry facility to determine its *in vivo* precision error at all skeletal sites, in addition to the daily *in vitro* phantom calibration that is a routine part of the quality control (QC). Daily phantom scanning for QC detects "drift" of the densitometer, but does not substitute for the additional determination of *in vivo* precision error (CV%) (Miller and Bonnick, 1999). The *in vivo* precision error is affected by changes in patient positioning and absolute BMD, which explains why *in vivo* precision error is higher than *in vitro* precision error. In addition, as Fig. 5 shows, the precision error (CV%) increases with lower BMD values. This is due to the poorer ability of the DXA system to distinguish between soft tissue and bone when there is less bone present. Hence, each densitometry facility must perform *in vivo* QC studies in patients with normal BMD as well as in a group of patients with low BMD to accurately assess their precision error. Precision studies should be

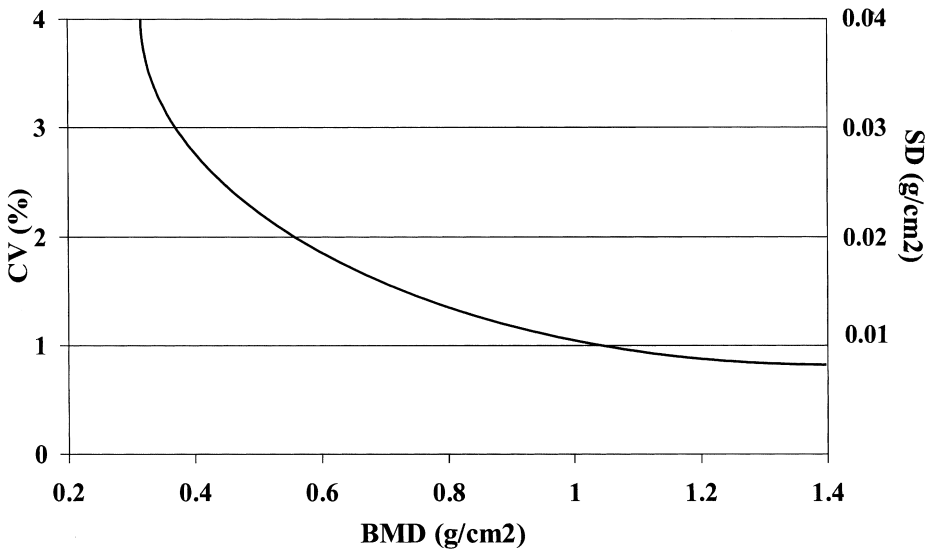

Figure 5 Precision expressed as %CV or SD across a range of bone mineral density. Reproduced from K. Faulkner, personal communication.

repeated whenever a technologist changes, machine location changes, or major software changes are made (Bonnick, 1998).

The least significant change (LSC) for two serial measurements is a function of the precision error. At the 95% confidence level, the difference between two measurements must be at least 2.77 times the precision error to be significant and not simply a measurement error. At the 80% confidence level, which is often acceptable in clinical measurements, the LSC between two BMD measurements must be at least 1.8 times the precision error. Many errors in patient management are made because the densitometry facility does not know their precision error. For example, a patient receiving a bisphosphonate has an increase in BMD of 4% at the PA spine and a decrease of 5% at the total hip and is taken off the bisphosphonate because it is assumed that there has been no response at the hip. In fact, in most instances, these changes at the hip are within the precision error of a hip measurement. The total hip precision error may be 2–3%, especially in patients with a low BMD (<0.60 g/cm^2), which requires a change of 6–9% to be significant at the 95% confidence level. Therefore, the patient had no significant change at the hip, which, if appreciated, may have changed the physician's decision to stop the bisphosphonate therapy.

The calculation of percentage change requires the following steps:

1. 1st BMD − 2nd BMD/1st BMD × 100 = % change
2. 2.77 (for 95% confidence) × precision error = LSC.

If step 1 is greater than step 2, then a LSC has occurred at the 95% confidence level. If step 2 is greater than step 1, then a change which is within the error of the measurement has occurred.

Changes in *T* score values should never be used to monitor patients. *T* scores are dependent on the mean and SD of a young normal reference population. As previously stated, the young normal reference population differs between manufacturers and, in some cases, between models of the same manufacturers' equipment. Over time, manufacturers upgrade their software, which may include changes in the mean and SD of the reference population used to calculate the *T* score.

Recently, the concept of "regression to the mean" has been applied to serial BMD measurements for monitoring the response to pharmacological therapy. In a recent publication, BMD data from 1 and 2 years of treatment with alendronate (5 mg/day) and raloxifene (60 mg/day) were analyzed using the premise of regression to the mean (Cummings *et al.,* 2000). This analysis showed that patients who demonstrate a fall or rise in BMD after the first year of treatment often demonstrate a change in the opposite direction during the second year of treatment. In other words, their BMD changes approach the mean of the group. The paper also stated that patients whose BMD decreased during the first year should not necessarily be taken off treatment, since most will show an increase during the second year. Regression to the mean is a useful concept when applied to groups of patients, however, it cannot be applied to therapeutic decisions for individual patients. This type of analysis is valid only in groups of patients. In addition, regression to the mean occurs in both treated and untreated groups, so in order to determine if the groups are different, regression to the mean in the untreated group must also be assessed (Bonnick, 2000). This has not been presented in the current publication. Finally, if one calculates the LSC range at the 95% confidence level for the total hip BMD in the referenced regression to the mean analysis, nearly all the datapoints fall within this range. The BMD changes at the PA spine were significant in many of the patients in this article (~4.5%), so the medications effectively increased BMD in most patients. Additionally, those patients who had a decline in BMD that was greater than the LSC are nonresponders. Clinic patients are more likely to demonstrate poor adherence, poor absorption, or secondary medical conditions mitigating their response to therapy than their clinical trial counterparts, due to careful selection criteria for participation in a clinical trial (Dowd *et al.,* 2000). Therefore, regression to the mean does not discount the value of serial BMD, but instead enforces the need to perform *in vivo* QC to determine LSC in BMD.

Finally, the relationship between pharmacologically induced increases in BMD and fracture reduction related to improvement in bone strength is presently under investigation. Animal models and *in situ* human studies have shown that 80% of the variance in bone strength is related to bone mineral content (BMC). A linear relationship exists between BMC and bone strength. The issue of fracture reduction has been raised due to observations made comparing changes in BMD versus placebo for calcitonin, alendronate, raloxifene, and risedronate. There are substantial differences in the BMD increases observed but overlap in the 95% confidence limits for relative incident vertebral fracture risk reduction (Faulkner, 2000). This might appear to suggest that changes in BMD are of little importance as an index of changes in bone strength. However, it should be noted that these comparisons are not from head-to-head trials. These different clinical trials recruit from different populations at different times, in contrast to a single head-to-head trial in which patient randomization reduces selection bias and ensures that placebo and treated groups are recruited from the same patient population. Therefore, the conclusions drawn from comparison of BMD changes from separate trial are often incorrect. In contrast, the highest order of evidence-based medicine is a meta-analysis which includes a sensitivity analysis. The data from such analyses suggests that the greater the increase in BMD over the treatment period, the greater the reduction in incident vertebral fractures (Fig. 6) (Wasnich and Miller, 2000). The incident vertebral fracture analysis indicates that there are factors, in addition to increased BMC, which contribute to the increased bone strength produced by the antiresorptive agents. These other factors improve bone strength but cannot presently be measured clinically. Bone architecture, bone size and/or width, and periosteal bone accretion may be altered by antiresorptive agents resulting in increased bone strength which is independent of increased BMC. The mechanism(s)

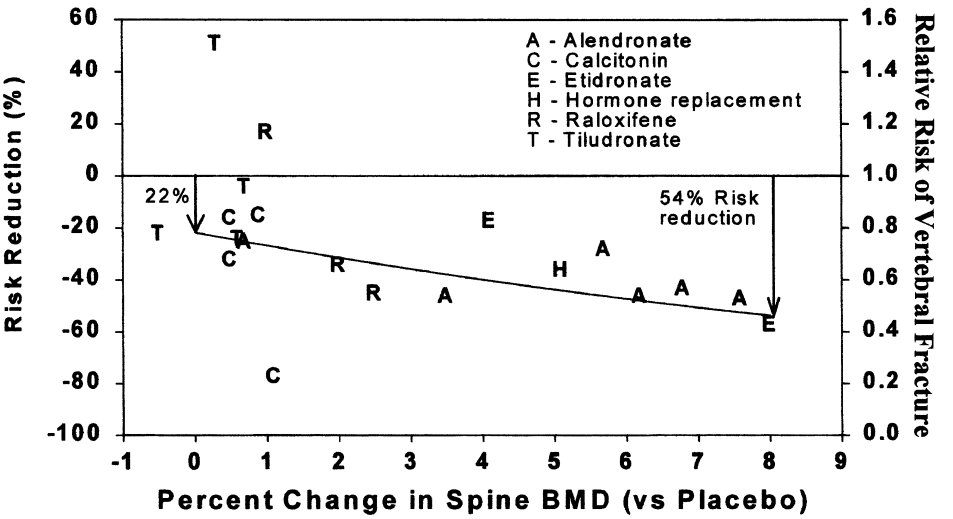

Figure 6 Relative risk of new vertebral fracture vs change in spine BMD (vs placebo) for randomized controlled trials of antiresorptive agents. The solid line represents the Poisson regression results. Reproduced from Wasnich *et al.* (2000).

whereby the various antiresorptive agents increase bone strength are under investigation. It is probable that each antiresorptive agent acts differently to increase bone strength. It is also possible that areal BMD, measured by DXA, underestimates the BMC added by antiresorptive agents, due to the method by which areal BMD is calculated (BMD = BMC/area). In elderly humans, bone size increases with increasing age and it may increase with subperiosteal bone formation induced by various agents. As the bone area increases and the BMC increases, the true increase in BMC may be underestimated by areal BMD measurements. Support for this hypothesis is observations that BMC, assessed by volumetric BMD (QCT), increases more than areal BMC, assessed by DXA with antiresorptive therapy.

In summary, serial BMD measurements should be carefully interpreted to maximize their usefulness. Physicians should never assume that a decline in BMD is not significant as long as the decline exceeds the LSC. It is essential for physicians to monitor the often asymptomatic condition of osteoporosis with BMD measurements. The serial determinations provide feedback and reassurance to ensure patient compliance with treatment regimens. Careful quality control, correct interpretation of serial changes, and proper patient assessment ensures that clinical medicine is not reduced to guess-work.

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CHAPTER 93

Macro- and Microimaging of Bone Architecture

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Introduction

Bone is a matrix of mineral and organic materials with a certain architecture and texture. The biomechanical competence of bone depends on two variables: its geometric properties and its material properties (Jiang *et al.,* 1999a). In terms of fracture risk, decreasing bone mineral leads to an exponential increase in fracture risk (Geusens, 1992). Yet, other studies have found that bone strength is only partially explained by bone mineral and that bone architecture has great influence on bone strength and the risk of fracture. Bone mineral values in normal and osteoporotic people overlap widely, and the expected correlation between the severity of osteoporosis and the degree of loss in bone mineral has not always been found (Podenphant *et al.,* 1987). The influence of factors other than bone mineral is thought to explain to some extent the overlap in bone mineral measurements between patients with and without osteoporotic fractures. Just as with bridges and buildings, the biomechanical competence or fracture resistance of bone depends not only on the amount of material but also on its spatial distribution, its structure, and its architecture.

Several noninvasive/nondestructive techniques have been developed for measuring bone structure and applied to the study and treatment of osteoporosis. These techniques allow early detection and diagnosis, monitoring of progression and response to therapy, improved estimates of bone strength, and improved prediction of fracture risk (Hassager and Christiansen, 1993; Genant *et al.,* 1996a, 2000). Conventional radiography, microradiography, and radiogrammetry have been used for many years to detect and quantify bone changes. Considerable progress has been made in advanced imaging techniques for noninvasive and/or nondestructive assessment of 2D and 3D bone structure. High-resolution computed tomography (hrCT) and magnetic resonance imaging (MRI) microscopy can be used to examine both cortical and trabecular bone *in vivo.* Much progress has been made in developing micro-CT (μCT) for noninvasive and/or nondestructive assessment of 3D trabecular structure and connectivity. The availability of 3D measuring techniques and 3D image processing methods allows direct quantification of unbiased morphometric parameters, such as direct volume and surface determination (Guilak, 1994), model independent assessment of thickness (Hildebrand *et al.* 1997b), and 3D connectivity estimation (Odgard and Gundersen, 1993).

Assessment of 3D trabecular structural characteristics may further improve our ability to understand the pathophysiology and progression of osteoporosis and other bone disorders. 3D structure is typically inferred indirectly from histomorphometry and stereology on a limited number of 2D sections based on a parallel plate model (Parfitt *et al.,* 1983a,b). With age and disease trabecular plates are perforated and connecting rods are dissolved, with a continuous shift from one structural type to the other. The traditional histomorphometric measurements based on a fixed model type will lead to questionable results (Hildebrand *et al.,* 1999). The introduction of 3D measuring techniques in bone research makes it possible to capture the true trabecular architecture without assumptions of the structure type.

Radiography

Conventional Radiography

Conventional radiography is a widely available, noninvasive means of visualizing bone structure. It also provides qualitative information on bone density. The term osteopenia, "poverty of bone," is used to describe visually decreased bone density—or radiolucency—on radiographs. This decrease in density is a result of a decrease in both mineralized bone volume and total calcium. The amount of calcium per unit of mineralized bone volume remains constant at about 35% (Albright *et al.,* 1941) in osteoporosis and osteopenia, resulting in decreased absorption of the X-ray beam and changes in the bone structure on radiographs. Alone or in conjunction with other advanced imaging techniques, conventional radiography is still widely used to detect osteoporotic fractures and to differentiate various disorders associated with osteoporosis and osteomalacia (Jergas *et al.,* 1999).

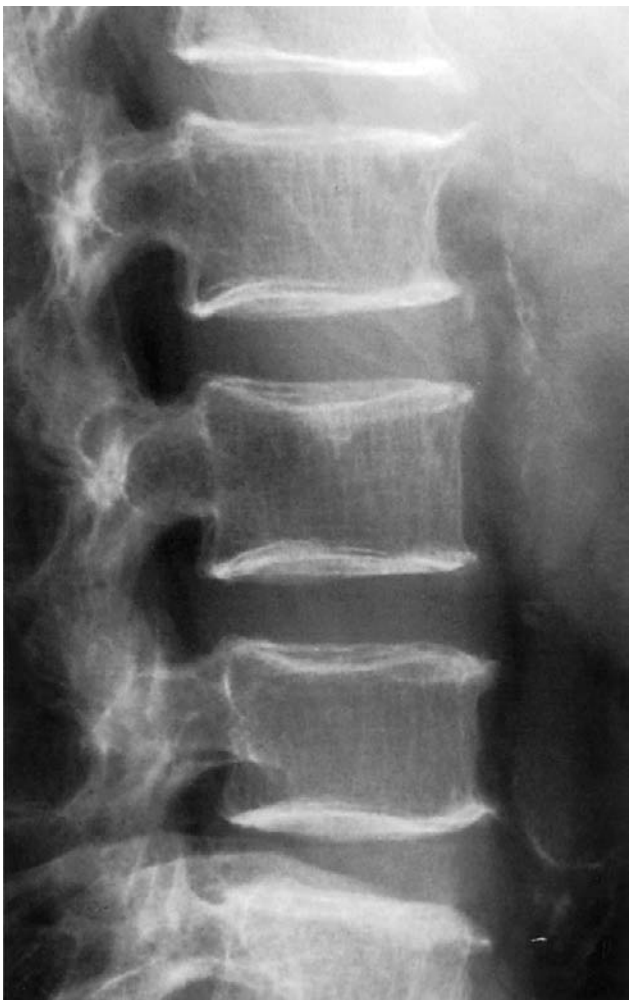

Figure 1 General decreased visual bone density results in increased radiolucency of the vertebrae with relative accentuation of the cortical rim, or "picture framing." There is also increased vertical striation due to the proportionally greater loss of horizontal trabeculae and compensatory hypertrophy of the vertical trabeculae.

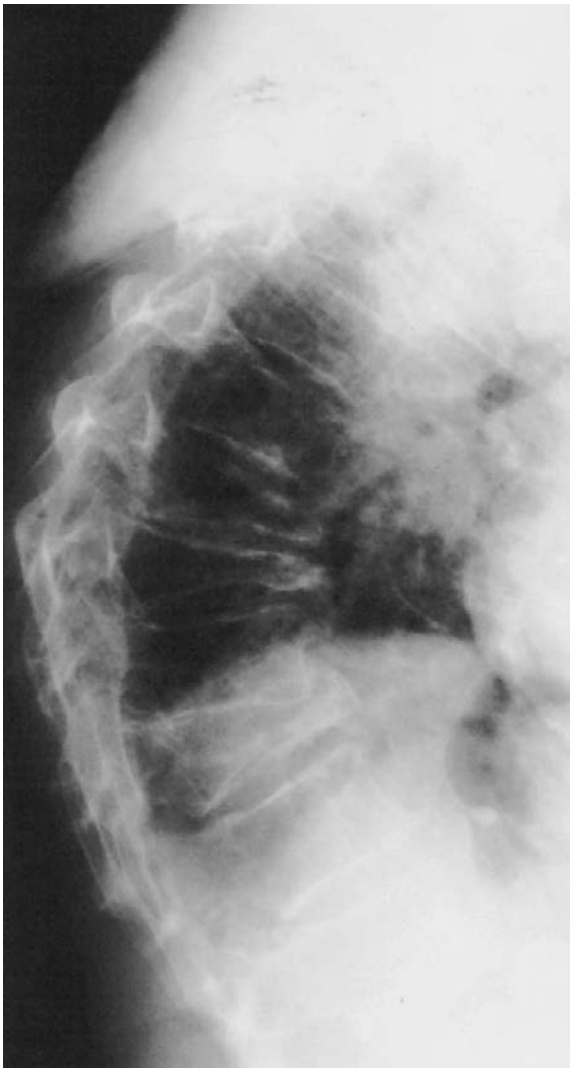

Figure 2 Multiple fractures in the thoracic spine in advanced involutional osteoporosis, including end plate, wedge, and compression fractures.

Changes in the axial skeleton and the ends of the long tubular bones are most prominent, since these sites have a relatively greater proportion of trabecular bone which has more surface and responds more quickly than cortical bone. As bone mineral is lost, non-weight-bearing connecting trabeculae are resorbed first, while the remaining weight-bearing trabeculae are more widely separated. Some may undergo compensatory thickening in the direction of mechanical stress. Trabecular anisotropy increases, resulting in distinct patterns on radiographs, such as the organized sequential trabecular changes along the compressive and tensile stress in the proximal femur, and an appearance of vertical striation in the lumbar spine in early stages of osteopenia caused by rarefaction of the horizontal trabeculae and a relative reinforcement of the vertical trabeculae (Fig. 1).

On radiographs vertebra may appear as a "picture frame" or as an empty box because of accentuation of the

cortical margins surrounding the lucent trabecular center. Increased bioconcavity of the vertebral endplates due to protrusion of the intervertebral disk into the weakened vertebral body can be seen in osteopenic vertebrae. It has been estimated that as much as 20–40% of bone mass must be lost before a decrease in bone visual density can be seen in lateral spine radiographs (Lachmann *et al.,* 1936).

Vertebral fracture, the hallmark of osteoporosis, has a wide range of morphologic appearances from increased concavity of the end plates, anterior wedging, to a complete destruction of the vertebral anatomy in vertebral crush fractures (Figs. 2–4). Fractures can be graded by radiologists or experienced clinicians and quantified with morphometric methods to reduce the subjectivity inherent in grading (Genant *et al.,* 1996b; Wu *et al.,* 2000; Jiang *et al.,* 1997c). The Genant scoring method (Genant *et al.,* 1993) grades 0 for normal, 1 for mildly deformed (20–25% reduction in anterior, middle, and/or posterior height, and 10–20% reduction in the projected area of the vertebral body), 2 for moderately deformed (25–40% reduction in height, and 20–40% in area), and 3 for severely deformed (40% or greater reduction in height and in area) (Fig. 5). This method offers high reproducibility inter- and intrareader and inter- and intratechnique for multiple experienced bone radiologists who graded both conventional radiographs and laser-digitized images at a 200- μm pixel size in a picture archiving and communication systems (Wu *et al.,* 2000; Jiang *et al.,* 1997c). It has been widely used to evaluate most important end points in epidemiological studies and in various clinical studies investigating the efficacy of osteoporotic drugs (Ettinger *et al.,* 1999; Harris *et al.,* 1999; Chesnut *et al.,* 1999).

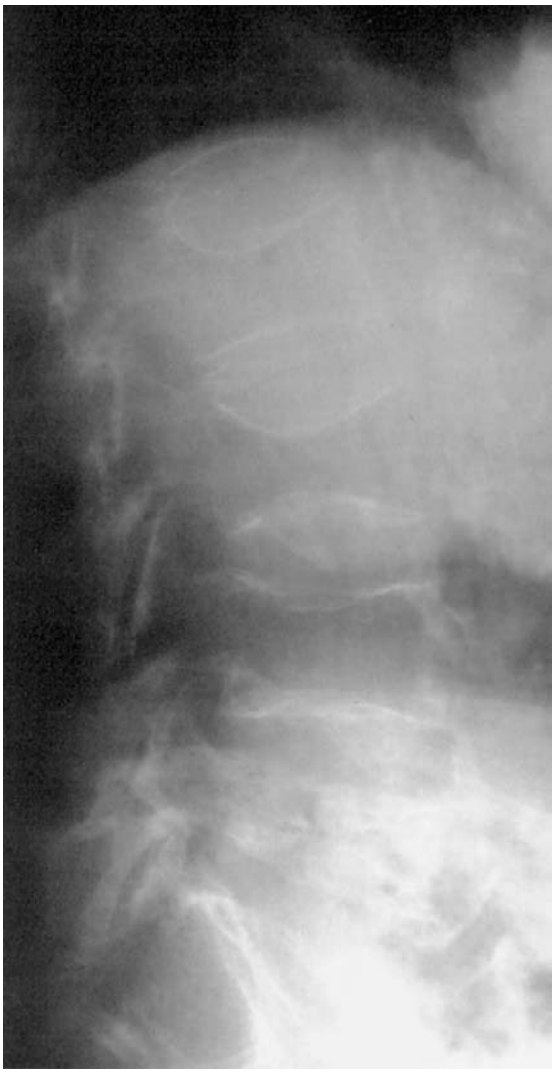

Figure 3 Severe osteoporosis. The vertebrae can hardly be distinguished on the lateral lumbar spine radiograph. There are multiple endplate and compression fractures.

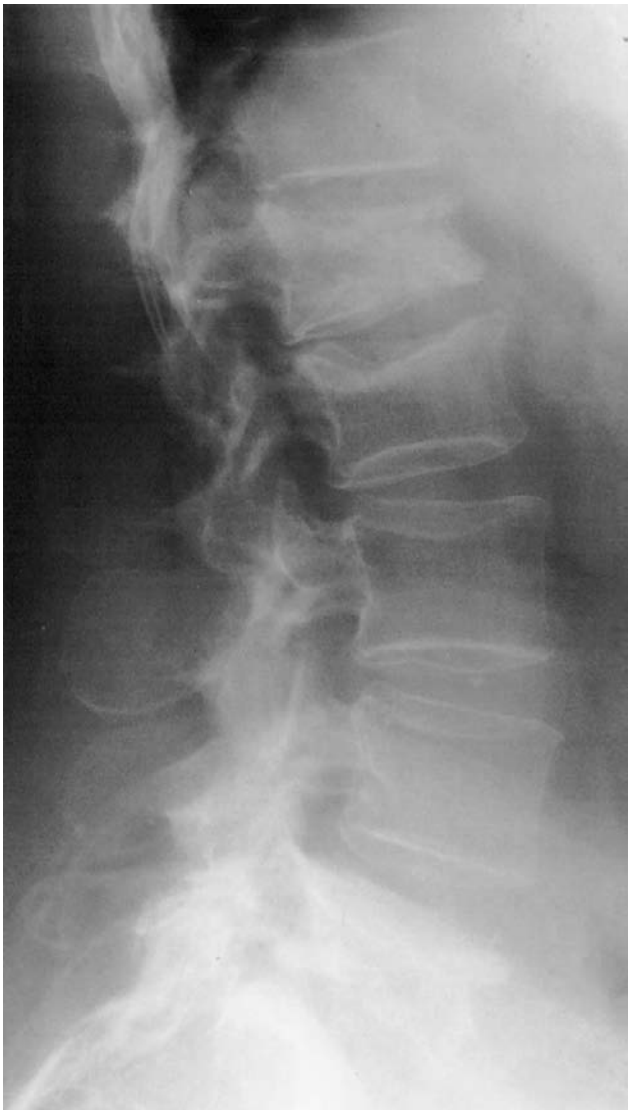

Figure 4 Lateral radiograph of the lumbar spine of steroid-induced osteoporosis demonstrates increased radiolucency, moderate wedge deformity of the first lumbar vertebra, and a mild biconcave deformity of the second lumbar vertebra.

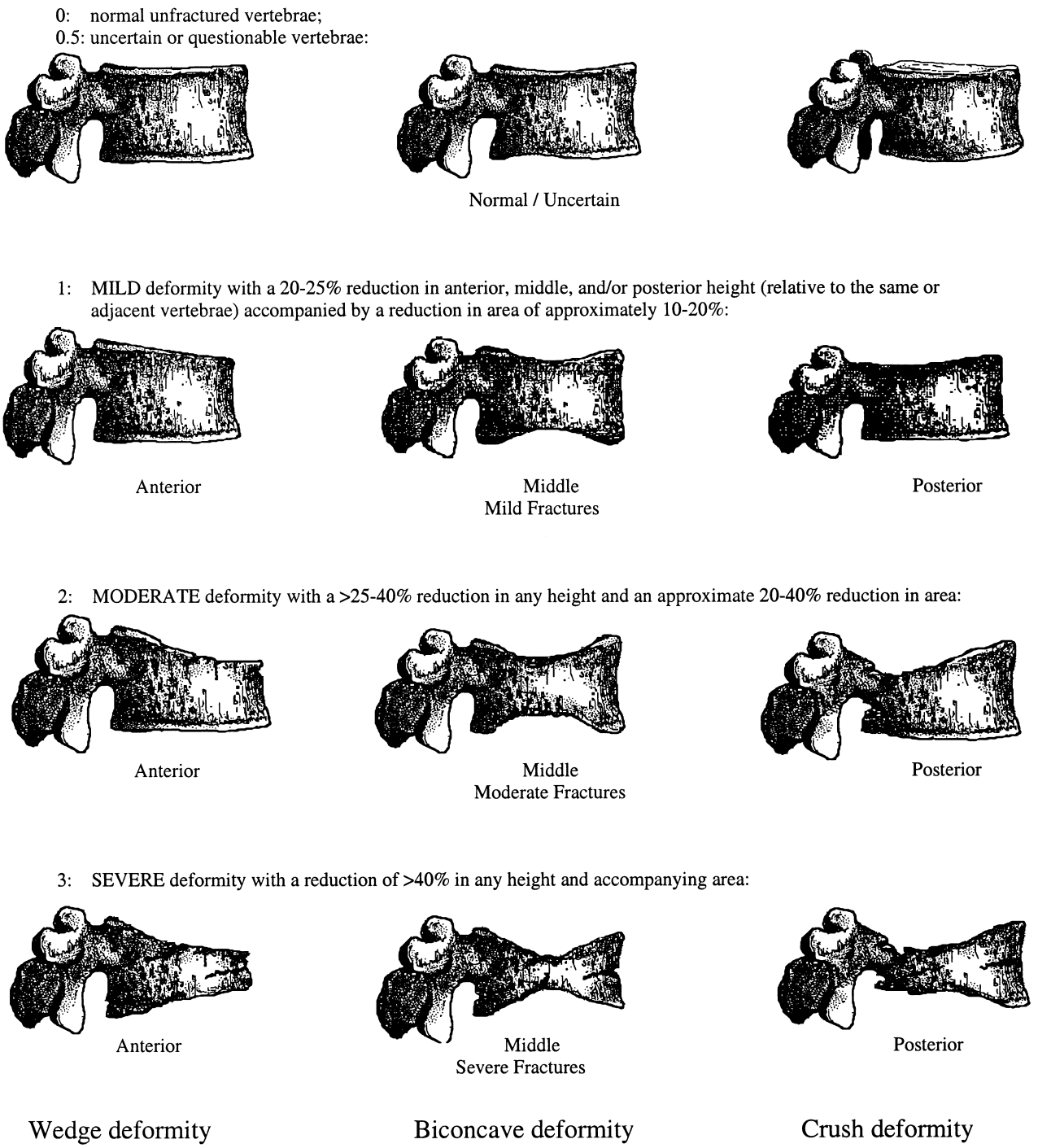

Figure 5 The Genant grading method for a semiquantitative evaluation of osteoporotic vertebral fracture grades fracture severity from normal (grade 0) to severe deformity (grade 3). The scoring scheme illustrates reductions of the anterior height that correspond to the grade of deformity. Reductions of the middle or posterior heights or combinations thereof can be evaluated accordingly.

Of most concern in osteoporosis are fractures of the hips, vertebral bodies, and wrists. Changes in the bone cortex or spongiosa may make such injuries more likely to occur. The shape, size, and structure of bone as well as its mass affect its resistance to these loads. Fractures of the vertebral bodies are a result of predominantly axial compressive loads. The decreased load-bearing capacity of each vertebra is related to the material properties of the bone and changes in the vertebral trabeculae from postmenopausal and age-related bone loss. Absorption and removal of the horizontal trabeculae or lateral support cross-ties change the architectural arrangement and result in a decrease in load-bearing capacity

(Mosekilde *et al.,* 1985). The vertical trabecular bone, therefore, behaves as a column and is prone to critical buckling loads (Townsend, 1975). A 50% decrease in cross-sectional area as a result of absorption of the horizontal trabeculae is associated with a 75% decrease in the load-bearing capacity of the vertebral body (Einhorn, 1992).

In addition to osteoporotic vertebral fracture, loss of horizontal trabeculae increases the likelihood of intertrochanteric hip fractures. Fractures of the wrist and hip usually begin in the metaphyseal–epiphyseal regions of the bone, which depend heavily on the trabeculae to support loads (Brown and Ferguson, 1978; McBroom *et al.,* 1985). Osteoporotic fractures usually do not occur in the mid-shaft of the femur or radius (Smith and Smith 1976), which is mainly cortical bone. Since the diameter of the bone expands with age with an increase in geometrical properties such as cross-sectional moments of inertia, particularly in men, there is more resistance to bending and torsional loads. However, the femoral neck, though also mainly of cortical bone, is not protected in the same way, since the periosteum is arguably absent from the part of the femoral neck within the hip joint for periosteal bone apposition (Pankovich, 1975; Phemister, 1939; Einhorn, 1992). Endosteal bone resorption during the aging process results in cortical bone thinning but is not associated with compensation of periosteal bone apposition to increase geometry such as cross-sectional diameter and moments of inertia. Consequently, the femoral neck with its unique anatomy becomes prone to fracture under deforming forces. A recent study in healthy men and men with hip or spine fractures using DXA shows that advancing age is associated with increased femoral neck width in healthy men, suggesting some capacity for intracapsular periosteal apposition. Bone fragility leading to hip or spine fractures in men may be the result of fracture site-specific deficits in bone size and volumetric BMD that have their origins in growth, aging, or both (Seeman *et al.,* 2001).

The trabecular patterns in the proximal femur have been scored to grade VII (Singh *et al.,* 1972) for individuals with dense trabeculae, even in the Ward's triangle enclosed by compressive and tensile trabeculae, and from grade VI as normal for all compressive and tensile trabeculae visible to grade I for marked reductions in the principal compressive trabeculae (Fig. 6) (Singh *et al.,* 1972). The Singh index has been shown to give different interobserver variation and different results regarding its relationship with bone mass and vertebral fracture (Cooper *et al.,* 1986; Singh *et al.,* 1972, 1970; Dequeker *et al.,* 1974).

In postmenopausal osteoporosis and other high-turnover metabolic bone disorders such as hyperparathyroidism (Fig. 7), renal osteodystrophy, and hypervitaminosis D (Fig. 8, see also color plate). (Jiang *et al.,* 1991), other structural changes in the cortical bone can be observed, such as longitudinal striation and tunneling in the cortical bone,

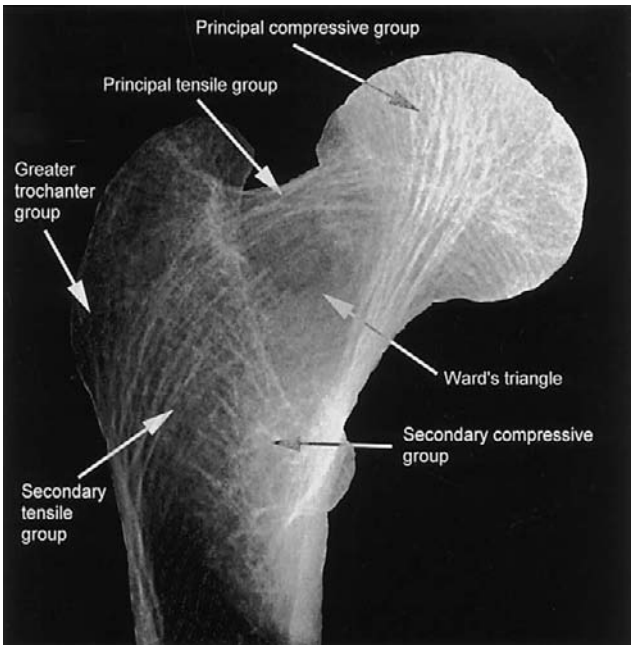

Figure 6 Schematic representation of the five normal groups of trabeculae in the Singh index which is based on the assumption that the trabeculae in the proximal femur disappear in a certain sequence. Ward's triangle is an area enclosed by the principal and secondary compressive as well as principle tensile groups.

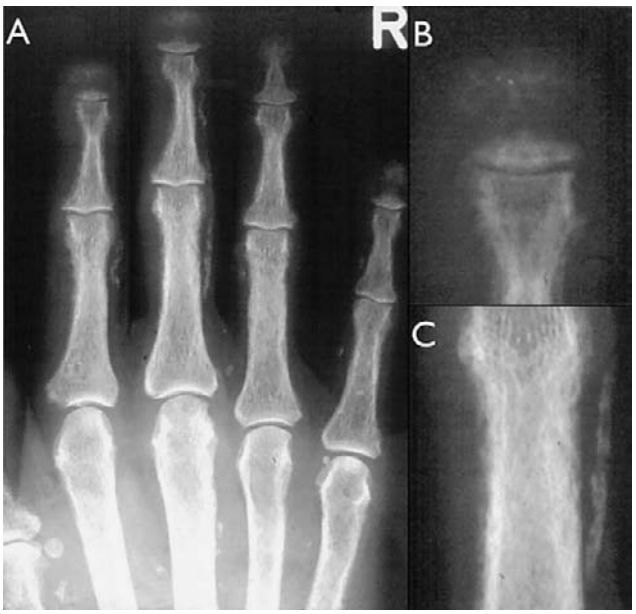

Figure 7 Patterns of bone resorption: subperiosteal bone resorption with scalloping and speculation of the outer cortex, acro-osteolyses, and soft tissue calcifications characterizes primary hyperparathyroidism; endosteal resorption and tunneling is prominent in senile osteoporosis; intracortical porosity and trabecular resorption are features of postmenopausal osteoporosis.

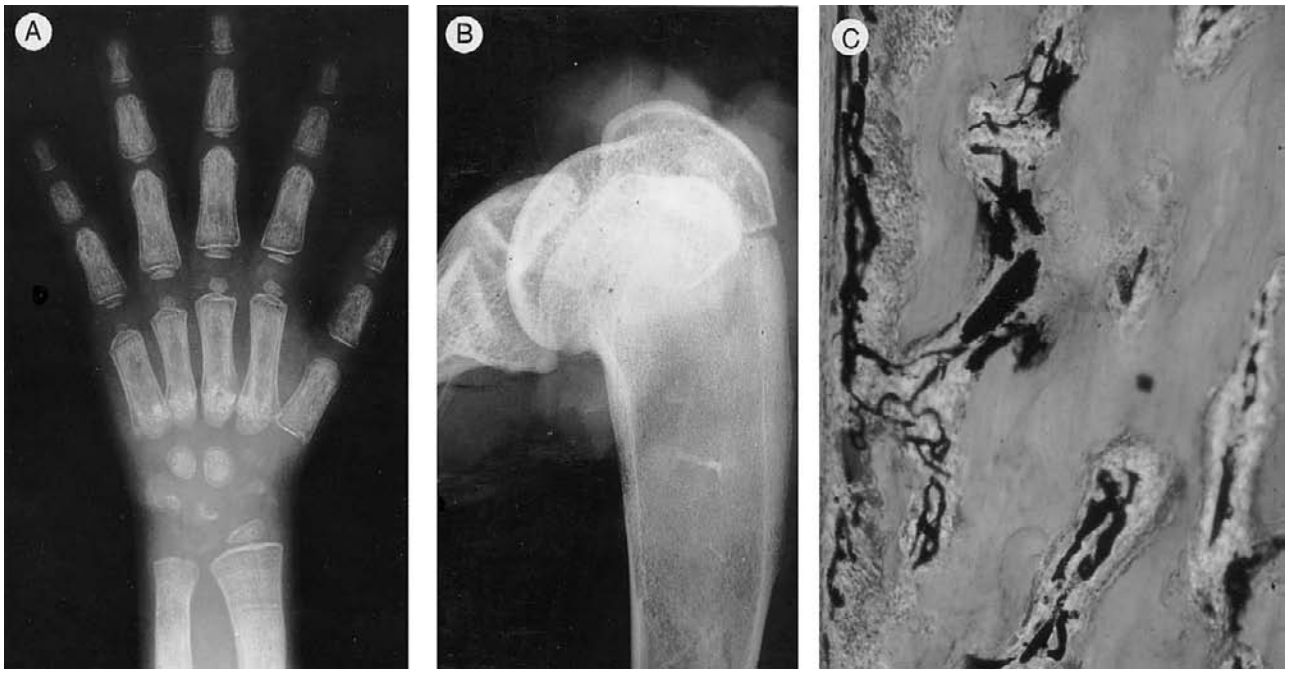

Figure 8 Remarkable increase in intracortical remodeling and aggressive resorption results in "trabeculization" in the metacarpi and phalanges in a child with hypervitaminosis D. There is metastatic calcification in the soft tissues in the wrist (A). Contact radiograph of a rabbit humerus with hypervitaminosis D (B) shows longitudinal striation and tunneling in the cortical bone because of increased intracortical remodeling and resorption, which represent on corresponding histology (C) increased cortical bone porosity, enlargement of the Haversian canals and Volkmann canals, and proliferation of microvasculature in the canals as a result of increased bone resorption by cutting cones (microvascular injection with Chinese ink and HE staining). (See also color plate.)

intracortical porosity, endosteal scalloping, subperiosteal resorption or irregular definition of the outer bone surface, and cortical thinning (Genant, 1983). The increased cortical bone remodeling and aggressive intracortical bone resorption leads to cortical bone "trabeculization."

Radiogrammetry

Radiogrammetry can be performed to measure cortical thickness, vertebral deformity, and bone size. It is simple, inexpensive, and reproducible. Radiogrammetry offers information on bone mass, although long before fractures are manifested radiologically, the patient will have already lost 30–50% of his or her bone mass over the previous 10–20 years (Dequeker, 1976). This technique does not measure cortical porosity or trabecular bone status.

Because of increased endocortical resorption and decreased bone formation with aging and senile osteoporosis, cortical thinning and widening of the medullary space is commonly seen (Fig. 9) and can be quantified using radiogrammetry applied to the metacarpal bones (Dequeker, 1976; Jiang, 1995) or in preclinical studies (Jiang *et al.,* 1996a). Annual decreases in combined cortical width of 0.4% before menopause and of 1.3% after menopause have been reported (Falch and Sandvik, 1990), which is comparable to the 1.45%

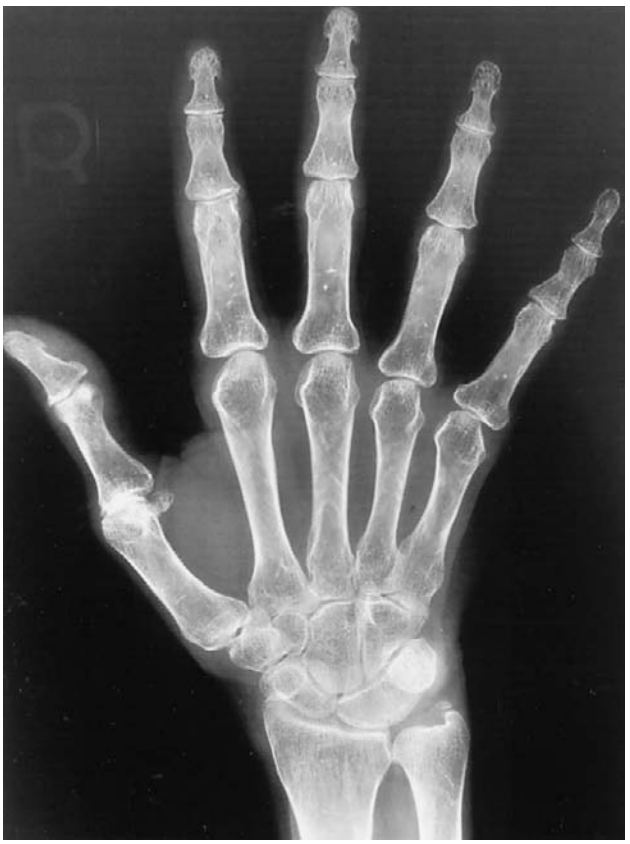

Figure 9 Conspicuous general cortical thinning in senile osteoporosis primarily as the result of endosteal bone resorption, decreased visual bone density, and loss in trabecular network.

in untreated women after oophorectomy reported by Genant *et al.* (1982).

The measurements of geometric properties of the proximal femur, including cortical thickness of the femoral shaft and the femoral neck, width of the trochanteric region, and number of tensile trabeculae, have been reported to be associated with the risk of hip fractures (Gluer *et al.,* 1994; Faulkner *et al.,* 1993). DXA can also provide lumbar spine morphometry and femoral neck geometry (Yoshikawa *et al.,* 1994; Seeman *et al.,* 2001) but yields little information on trabecular structure and intracortical changes because of limited resolution. Hip axis length determined from conventional radiographs or DXA can predict hip fracture independently of densitometry, as a longer hip axis length is associated with an increase in femoral neck and trochanteric fracture risk (Faulkner *et al.,* 1993; Gluer *et al.,* 1994). Instead of varying with age after midadolescence, the hip axis length seems to be mainly influenced by genetic factors (Flicker *et al.,* 1996), which supports other reports that differences in the hip axis length may partially explain racial differences in hip fracture (Cummings *et al.,* 1994). Hip axis length adds information to BMD and appears to be useful for cross-sectional studies of risk factors for osteoporosis, but has little value in longitudinal studies.

High-resolution radiographic techniques using high-detail films and small spot X-ray tubes, like the ones used in mammography, are important for depicting changes in bone, since the detection of osteopenia by conventional radiography can be inaccurate and insensitive (Genant *et al.,* 1975). It is influenced by many factors such as soft-tissue thickness, radiographic exposure, the film used, and film processing.

Morphometric radiogrammetry measurements of the long tubular bones on conventional radiographs include bone length (L), the outer diameter or periosteal width (D), and inner diameter or medullary space (d) of the cortical bone at 50% of the length. The cortical thickness can be obtained by simple subtraction ($D - d$). Since the long tubular bones are approximately circular at midshaft and the medullary cavity is nearly centered in the tubular bone cylinder at that point, $D^2 - d^2$ can be regarded as an index of the cortical cross-sectional area after omitting the constant $\pi/4$; $(D - d)/D$ the ratio of cortical thickness to the midshaft width; and $(D^2 - d^2)/D^2$ the ratio of cortical cross-sectional area to the total midshaft cross-sectional area. The reproducibility as coefficient of variation (CV) of six determinations with repositioning of the radiographs at each measurement is *L,* 0.2%; D, 1.2%, *d,* 2.1%; DDM, 1.5%; DPM, 1.8% for conventional radiographs of the radius of baboons and *L,* 0.07%; *D,* 0.65%; *d,* 1% for microradiographs ($\times$100, with focal spot 5 μm) of rat femurs. High correlations are found in both baboons and rats between midshaft periosteal diameter (D) and torsional strength and stiffness ($r = 0.7$); between cortical area index ($D^2 - d^2$) and stiffness ($r = 0.6$); and among D, $D^2 - d^2$, and dual X-ray absorptiometry (DXA) measurements ($0.7 \leq r \leq 0.9$), indicating that radiogrammetric measurements may be useful for estimating bone mineral and strength in preclinical studies (Jiang 1995).

Microradiography

With the expansion of contact radiography, microfocus radiography, and specimen slab radiography, more details of bone structure have been clearly demonstrated (Genant *et al.,* 1973, 1975; Jiang *et al.,* 1990, 1991, 1995; Wevers *et al.,* 1993).

Because trabecular structure can be clearly displayed on radiographs, especially on high-resolution radiographs or microradiographs, sophisticated image processing techniques have been designed to segment and extract trabecular morphometric parameters, such as trabecular bone volume, trabecular thickness, number, separation, and other connectivity information. Fractal analysis and fast Fourier transforms have also been applied to the evaluation of trabecular structure (Benhamou *et al.,* 1994; Ouyang *et al.,* 1998; Jiang *et al.,* 1996b). For structural analysis, gray-scale images with a range of values must be segmented into binary images with only two values, white and black, representing bone and no bone.

Quantitative microradiography using an aluminum calibration step wedge, similar to quantitative backscattered electron imaging and small-angle X-ray scattering imaging, can measure the focal degree of mineralization and secondary mineral apposition. Mineralization of the trabecular bone consists of primary and secondary mineral apposition. Primary mineral apposition is mineralization of osteoid, which can be detected using traditional histomorphometry that measures the amount of bone matrix or bone mass. Secondary mineral apposition is a slow and gradual maturation of the mineral component, including an increase in the amount of crystals and/or an augmentation of crystal size toward their maximum dimensions. Increased bone turnover, as in postmenopausal osteoporosis, results in hypomineralization, because resorption begins prematurely, before mineralization is complete. The least mineralized bone is highly radiotranslucent and is the most recently formed, or has just achieved its primary mineralization, and represents about 75% of the complete mineralization. It contrasts with the adjacent fully mineralized interstitial bone, with low radiotranslucency. Oral administration of alendronate for 2 or 3 years in osteoporotic women increases BMD, but does not increase trabecular bone volume in the iliac crest bone biopsy, which can be explained by an increase in secondary mineralization as a result of reduced bone turnover (Meunier and Boivin, 1997).

Microfocus radiography and specimen slab radiography have been employed in preclinical studies (Jee *et al.,* 1990; Miller and Jee, 1979) and in toxicological and safety studies, e.g., to investigate effects of on/off treatments of high doses of a bisphosphonate, an inhibitor of osteoclastic activities and bone resorption, on bone modeling and bone remodeling in growing nonhuman primates (Fig. 10) and rodents (Fig. 11). Radiological changes are very closely

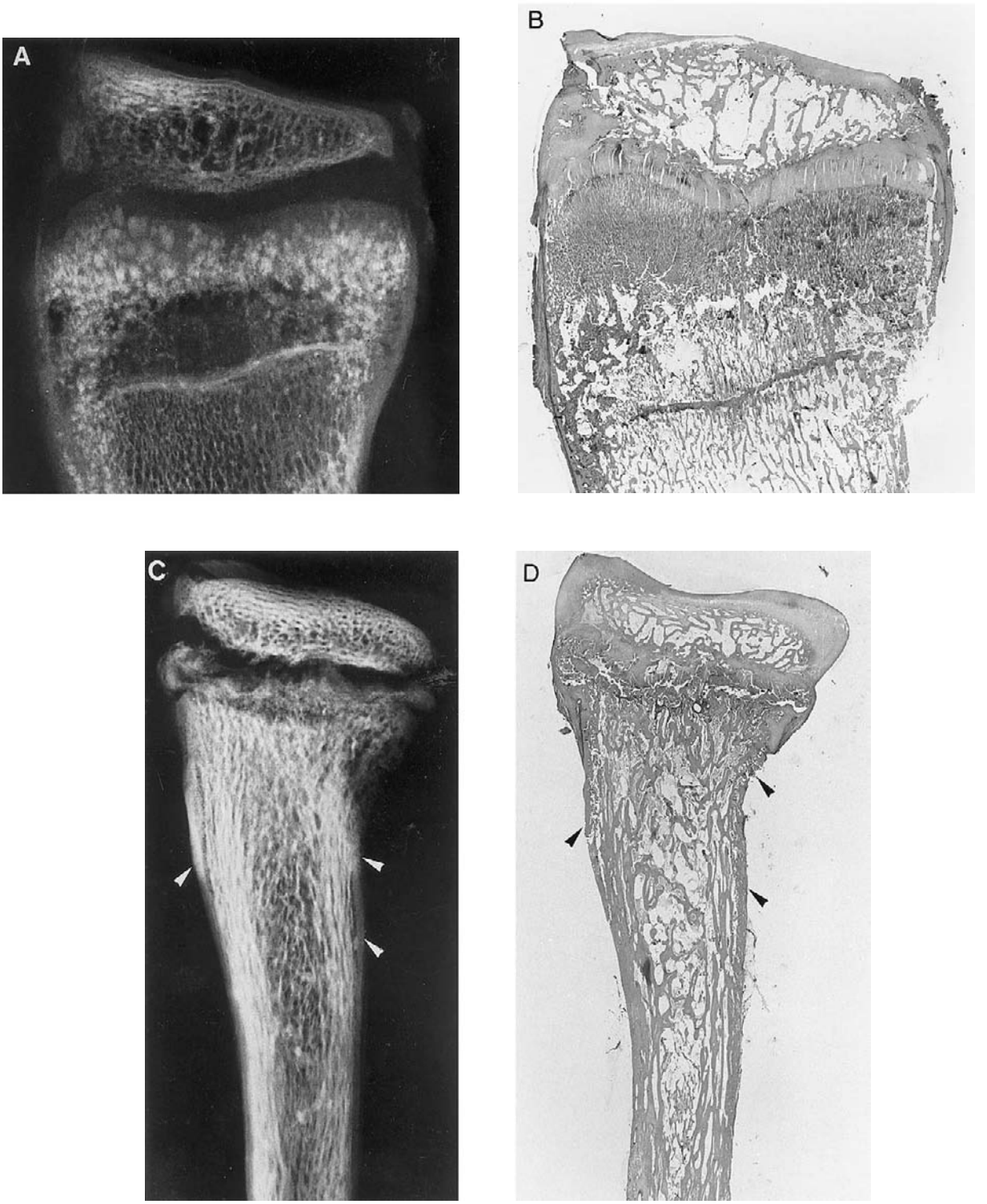

Figure 10 A baboon treated with a high dose of a bisphosphonate. Slab contact radiograph (A) of the distal end of the radius reveals an increase in width of the growth cartilage. The scattered dense amorphous dots on the radiograph represent localized areas of persisting calcified cartilage cores correlative to histological macrosection (B, HE staining). The sharp arrest line, also representing persisting calcified cartilage cores, is the transition between the induced changes and the original normal bone. The periosteal reaction represents woven bone formation. Slab contact radiograph (C) of the proximal end of a baboon treated with a higher dose of a bisphosphonate shows irregularity of the growth plate with dense dots, increased density in the spongiosa, intracortical lucent lines, and periosteal reaction (arrowhead), which correspond in the histologic macrosection (D) to cartilage calcification in the growth plate, increased number of trabeculae in the metaphysis, enlargement of the Haversian canals in the cortical bone, and periosteal woven bone formation (arrowhead).

correlated with corresponding histopathological findings (Jiang, 1995). The radiologic manifestations in baboons are very similar to changes in humans treated with pamidronate (Liens *et al.,* 1994).

CT

There are currently major research initiatives in the areas of high-resolution and microcomputed tomography (hrCT/μCT) for assessment of bone structure. The spatial resolution of clinical CT scanners is inadequate for highly accurate analysis of discrete trabecular morphological parameters and cortical measurements, and newer CT developments address these issues with different approaches: new image acquisition and analysis procedures using state-of-the-art clinical CT scanners; new ultra-high-resolution scanners for *in vivo* investigations of the peripheral skeleton; and new μCT scanners for *in vitro* three-dimensional structural analysis of small bone samples or for preclinical studies. The discrete elements in digital images are called pixels for 2D and voxels for 3D. From a physical perspective, spatial resolution is typically given in line pairs per millimeter or as a 5 or 10% value of the modulation transfer function, requiring measuring the modulation transfer function or the point spread function of the imaging system. For simplicity, pixel or voxel dimensions are used which may result in a "pseudo" resolution that typically overestimates the physically "correct" resolution by a factor of 2–4. Therefore, to image 50-μm-thick structures the "correct" resolution must be at least 50 μm, requiring pixel/voxel size of 25 μm or smaller according to Nyquist's theorem (Engelke *et al.,* 1998a).

High-Resolution CT

High-resolution (hrCT) thin-slice CT, adapted from existing body CT scanners for high-resolution measurements, can be used to assess trabecular and cortical bone structure and cross-sectional geometry of virtually any bone in the body. Volumetric bone mass or density in trabecular and cortical bone can be determined separately using QCT (Genant *et al.,* 1996a). HrCT, using standard body CT scanners, typically has pixel sixes of 300–500 μm and slice thickness of 1–1.5 mm, and can depict the trabecular network of the spine and hip, but requires a higher radiation exposure than standard QCT. As the trabecular thickness is about 100–150 μm and trabecular separation about 500–1000 μm, the depiction of individual trabecula is limited by the spatial resolution and signal to noise ratio as a result of the low radiation dose involved. It is still possible to extract some measures of apparent trabecular structure, though it may not accurately determine trabecular structure. The image processing procedures and threshold used to segment bone structure (Fig. 12) can also influence trabecular structure measurement. Using a textural analysis in osteopenic women with DXA T scores of less than -2.5, with or without vertebral fractures, structural parameters allowed discrimination of fractured from nonfractured women. (Wang *et al.,* 1996)

In CT images of lumbar vertebrae with 1.5-mm slice thickness, trabecular structural analysis can provide differential information, *in vivo,* about the state of trabecular structure, particularly in osteopenic subjects (Chevalier *et al.,* 1992; Gordon *et al.,* 1998). Chevalier *et al.* (1992) used hrCT to measure a feature called the trabecular fragmentation index (length of the trabecular network divided by the number of discontinuities) to separate osteoporotic subjects from normal subjects. In another study using CT images of the vertebral body with 2-mm slice thickness, trabecular separation significantly increased with age in women 50 years of age or older, while women with vertebral fractures had significantly lower BMD and significantly higher trabecular separation (Ito *et al.,* 1995). Gordon *et al.* (1998) reported on an hrCT technique that extracted a texture parameter reflecting trabecular hole area, analogous to star volume (Croucher *et al.,* 1996), which appeared to enhance vertebral fracture discrimination relative to BMD. In other reports, no significant difference was found in pQCT trabecular BMD at the distal radius *in vivo* from premenopausal and postmenopausal women, although the number of trabecular perforations was significantly higher in the postmenopausal women. This suggests that loss of connectivity occurred before a significant reduction of BMD and that trabecular bone connectivity is more sensitive than BMD to the early changes of postmenopausal osteoporosis (Takagi *et al.,* 1995).

Trabecular bone structural parameters derived from *in vitro* CT images correlated moderately with BMD, with r^2 values ranging from 0.37 to 0.64 (Jiang *et al.,* 1998). In CT images of the lumbar vertebrae using 1.5 mm slice thickness, the correlation between trabecular structural parameters and BMD was 0.60 (Chevalier *et al.,* 1992). The same observation has been reported in a study of MRI trabecular structure and CT BMD (Majumdar *et al.,* 1997). This moderate correlation indicates that trabecular structural analysis in CT or MR images — in addition to bone mineral measurement — may provide new information about the state of trabecular bone.

Combining volumetric BMD with trabecular structural parameters provides better prediction of bone biomechanical properties than either parameter alone (Jiang *et al.,* 1998; Mundinger *et al.,* 1993). Osteoporotic fractures are most likely to occur at sites with a considerable amount of trabecular bone such as the distal radius, the vertebral body, and the proximal femur, so the development of diagnostic procedures for detecting individuals at fracture risk has focused on the trabecular compartment at these sites. Low BMD at these sites — hip, spine, and forearm — is significantly associated with the risk of osteoporotic fractures (Hui *et al.,* 1990; Melton *et al.,* 1993; Cummings *et al.,* 1990; Seeley *et al.,* 1991) However, there is often considerable overlap in BMD values between fracture and nonfracture subjects (Cann *et al.,* 1985; Cummings *et al.,* 1993). The overall risk of fracture is determined by the mechanical strength of the bone and other factors such as falls. BMD alone only partially explains the mechanical competence of

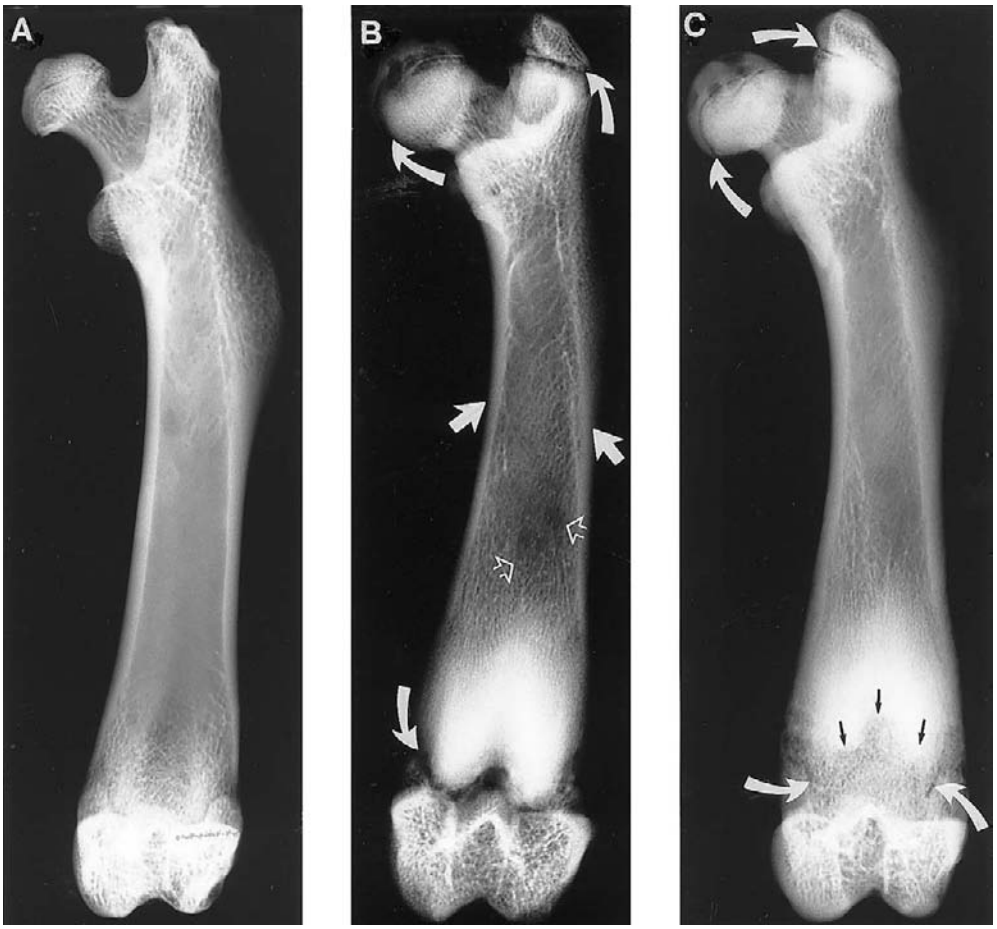

Figure 11 Microradiographs of rat femur specimens were taken using a microfocus X-ray system. Compared with a control (A), treatment with a high dose of bisphosphonate for 3 months (B) resulted in an enlarged club-shaped deformity with increased density of the metaphyses and thickening of the growth plates (curved arrows) at both distal and proximal ends, linear intracortical lucencies (arrows) in the diaphysis and femoral neck, and patches of relatively decreased density (open arrows) in middiaphysis. Three months after withdrawal from treatment, the bone in the distal metaphysis normalized so that the originally increased density area progressively moved toward the diaphysis, causing alternate bands of increased and decreased (arrows) density, while the cortical bone and growth plates (curved arrows) became normalized (C). Corresponding μMRI of the same specimens imaged in saggital plane, using a spin echo sequence, in-plane resolution of 78 μm and slice thickness of 150 μm on a Varian Unity 400 NMR instrument at 9.4 T, show normal cortical bone, growth plate, and trabecular network in the metaphysis and epiphysis of a control rat (D), increased bone mass in the metaphysis and epiphysis and increased thickening of the growth plate in the treated rat (E), and normalization of the thickness of the growth plate and trabecular network in the metaphysis and the movement of the increased bone mass toward the diaphysis after withdrawal from treatment (F).

cancellous bone; and trabecular architecture is an important additional factor in assessing bone strength (Vesterby *et al.,* 1991; Dalstra *et al.,* 1993; Chevalier *et al.,* 1992; Wallach *et al.,* 1992).

Analyzing bone strength biomechanically and correlating it to BMD shows correlation coefficients (r) between bone strength and BMD ranging from 0.7 to 0.95 (Linde *et al.,* 1991; Biggemann *et al.,* 1988; Martens *et al.,* 1983; Lang *et al.,* 1988; Lotz *et al.,* 1990; Esses *et al.,* 1989; Jiang *et al.,* 1998). Trabecular structural parameters from *in vitro* CT images of the human vertebral body are significantly correlated with elastic modulus, with r^2 ranging from 0.58 to 0.72 (Jiang *et al.,* 1998). The maximum compressive strength and the shear strength of human L2 vertebral bodies show significant correlations with trabecular architectural parameters determined by hrCT or histomorphometry, with correlation coefficients ranging from 0.88 to 0.55 (Cendre *et al.,* 1999). Consideration of architectural properties by noninvasive imaging techniques seems to increase the predictability of the *in vitro* fracture load beyond BMD measurement alone (Faulkner *et al.,* 1993; Augat *et al.,* 1996).

Because of the resolution and slice thickness used in clinical CT, only the thick plates of the trabeculae are depicted and the dominant structures result in a textural appearance, while the thinner trabecular structure is affected by partial

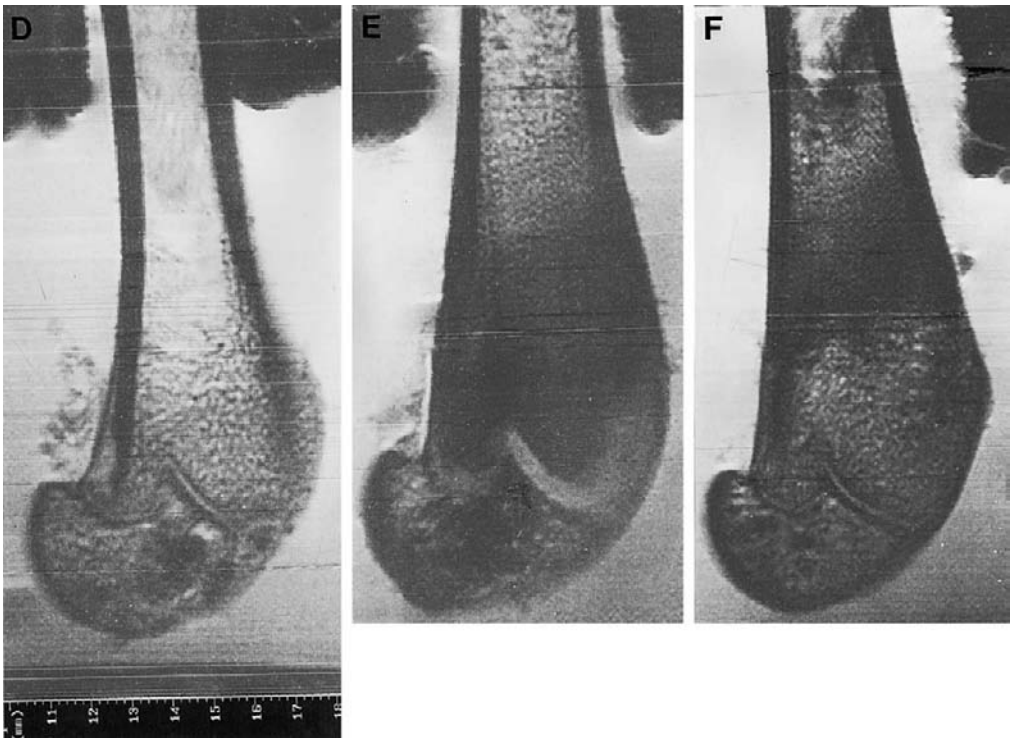

Figure 11 *(Continued)*

volume effects. The segmentation algorithm is important to accurately and reproducibly classify pixels from images in a fast, objective, non-user-specific manner. Segmentation is usually threshold- or edge-based. The threshold depends on the absolute gray value of the pixel, i.e., bone and marrow densities. Global thresholds apply the same threshold to the whole image which works well in high-resolution and high-contrast images, while local thresholds adapt this value to a neighborhood of a selectable size. Edge-based methods apply the first- or second-order derivative and therefore detect changes in gray values (Engelke *et al.,* 1998a). Although some decisions have to be made by the operator, such as placement of the region of interest (ROI), the important advance is that the algorithm is implemented in a uniform manner across all images. An *in vitro* study showed that precision error as coefficient variation (CV) and standardized CV of trabecular structural parameters was $<5\%$ (Jiang *et al.,* 1998). In a validation study, hrCT of a human L2 vertebral body with 150 μm spatial resolution and 330 μm thickness and with edge enhancement to segment bone structure, showed significant correlations with histomorphometry with 7 μm slice thickness, with correlation coefficients ranging from 0.87 to 0.63. Because hrCT has lower spatial resolution and thicker slices, bone volume fraction (bone volume/ total volume, BV/TV) and trabecular thickness determined by hrCT were overestimated by a factor 3.5 and 2.5, respectively, as compared with histomorphometry, while trabecular separatioin was underestimated by a factor of 1.6 (Cendre *et al.,* 1999).

Volumetric CT

Standard CT 2D acquisition is useful for assessing vertebral trabecular bone. Limitations of the single slice standard CT 2D acquisition include a high degree of operator dependence, the need for careful slice positioning, ROI selectioin and placement, and the lack of a technique to assess the proximal femur. Because of the availability of helical CT systems and powerful and relatively inexpensive computer workstations to handle large volumes of image data, new developments in true volumetric acquisition can address some of the limitation of standard 2D CT by providing rapid and highly precise volumetric measurements. The 3D volumetric approaches are optimal for highly complex structures such as the proximal femur and to improve spinal measurements (Fig. 13, see also color plate).

The volumetric acquisition encompasses the entire object of interest either with stacked-slice or spiral CT scans, and uses anatomic landmarks to automatically define coordinate systems for reformatting CT data into anatomically relevant projections. For example, using the vertebral endplates this technique can automatically determine 3D orientation of the vertebral body and does not need an operator for careful slice positioning (Heitz and Kalender, 1994). Measurement accuracy can be improved in patients with scoliotic or lordotic curvature.

With appropriate image processing techniques to reproducibly delineate volumes of interest, spiral CT systems can determine bone mineral content (BMC) or bone mineral

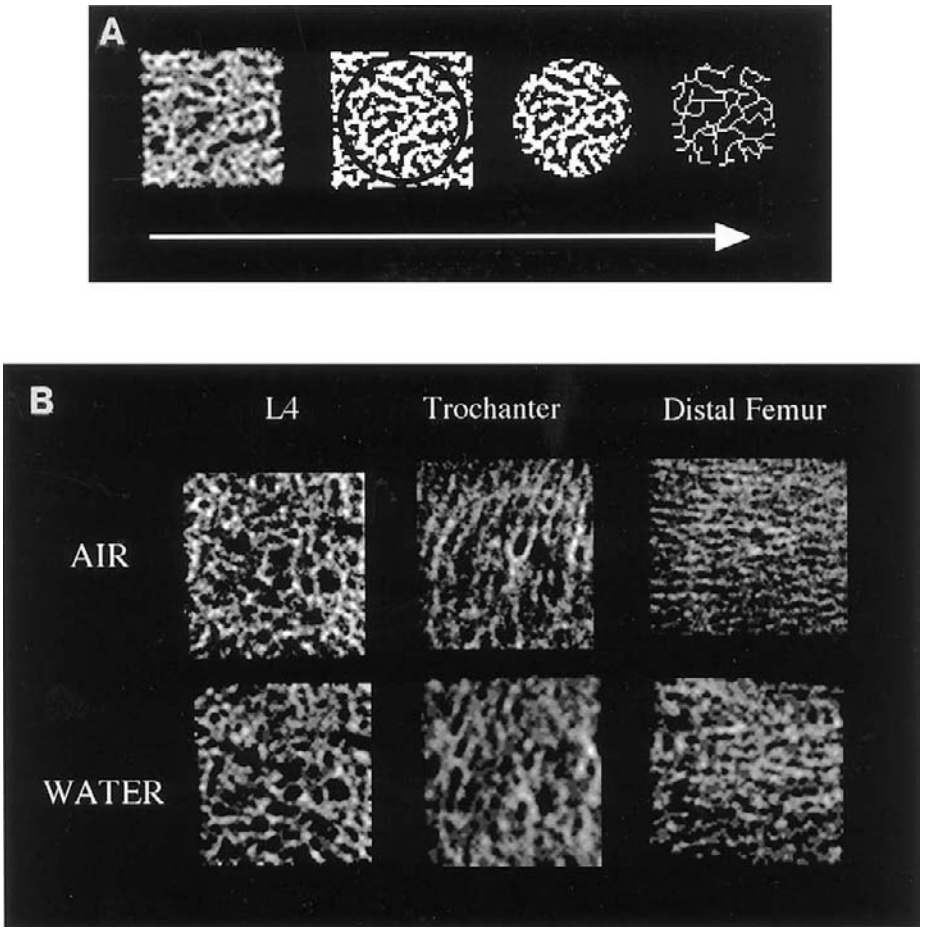

Figure 12 Schematic representation of image processing procedures of trabecular bone structure analysis using AVS, advanced visual system, on a Sun workstation. The analysis procedures are from left to right. This first image on the left is a pQCT image of a bone cube from a vertebral body scanned in water. A threshold is applied to segment the trabecular bone. A region of interest is selected from the binary images to calculate apparent trabecular bone volume, thickness, and separation. Finally, the image is skeletonized to analyze apparent trabecular structure and connectivity (A). Sample images of cubic human specimens from different anatomic locations scanned in air or in water using pQCT (B).

density (BMD) in the trabecular or cortical components separately and of the entire bone or subregion, i.e., the vertebral body, femoral neck, intertrochanteric subregions, etc. In addition, volumetric acquisition can provide highly accurate true volumetric rendering and can calculate important geometrical and other biomechanical measurements, such as cross-sectional area, cross-sectional moments of inertia, and finite element analyses (Faulkner *et al.,* 1990; Heitz and Kalender, 1994; Keyak *et al.,* 1998; Lang *et al.,* 1997; Lotz *et al.,* 1990; McBroom *et al.,* 1985). Such an approach is particularly useful with the proximal femur because of its complex anatomy, dramatic 3D variations in BMD, and biological relevance to osteoporosis, arthritis, and other disorders. Using volumetric acquisition and finite analysis modeling (Lotz *et al.,* 1990; Keyak *et al.,* 1998) can improve estimations of proximal femoral strength over global projectional densitometry. Finite element analysis of bone has shown that bone structure itself rather than density values averaged over a larger volume can be used as a starting point (van Rietbergen *et al.,* 1995), Potentially the interaction of the cortex with the trabecular structure, the transmission of forces inside the bone and the contribution of the cortex to bone strength can be investigated in

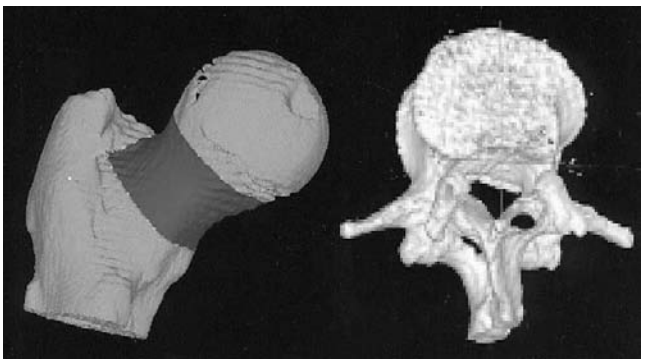

Figure 13 Three-dimensional representation of the lumbar spine and the proximal femur from 3D or volumetric CT acquisition. The lumbar spine and the proximal femur were encompassed with 3-mm contiguous slices, and segmentation was obtained by mapping the bone surface using a contour-tracking algorithm. (See also color plate.)

greater detail. *In vitro* studies using subregions of the hip and volumetric acquisition can enhance prediction of biomechanical strength (Lang *et al.,* 1997; Heitz and Kalender, 1994). It will be of interest to explore the application of these approaches to longitudinal studies, to monitoring response in drug therapies, and to diagnostic measurements.

Ultra-High-Resolution Peripheral CT

Improving spatial resolution by decreasing pixel size and slice thickness improves the accuracy of structural assessment. Several investigators have developed and evaluated special-purpose ultra-high-resolution CT scanners for peripheral skeletal measurements *in vivo* (Müller *et al.,* 1996c). They have achieved resolutions of 0.17 mm isotropic for trabecular structure in the distal radius and obtained morphological data that were comparable to direct histomorphological measurements of bone. The results of the morphometric analysis from the three-dimensional stack of multiple thin CT slices at a resolution of 0.25 mm support the assumption that cancellous bone structures based on noninvasive high-resolution CT measurements are representative for 2D trabecular microstructures assessed from histologic bone sections (Müller *et al.,* 1996a). The images can be used for quantitative trabecular structure analysis and also for a separate assessment of cortical and trabecular BMD. Radiation exposure and acquisition time are the most limiting factors for the *in vivo* system. High quantum detection efficiency is important to reduce radiation exposure. Powerful X-ray tubes and fast readout electronics are essential for minimizing scanning time to avoid motion artifacts (Kapadia *et al.,* 2001).

These state-of-the-art scanners approach the limits of spatial resolution achievable *in vivo* with acceptable radiation exposure (Engelke *et al.,* 1993). The radiation dose can limit spatial resolution and the signal to noise ratio that can be optimized for X-ray-based techniques, as theoretical calculations show that a spatial resolution of 100 μm approaches the limit for human *in vivo* examination (Graeff and Engelke, 1991). An increase in image resolution by a factor of 2 without an increase in noise will result in an increase of dose by a factor of 8, should slice thickness remain unchanged, while dose will be increased by a factor of 16 should slice thickness be further decreased by a factor of 2 (Graeff and Engelke, 1991; Engelke *et al.,* 1991, 1998a), as the applied surface dose (D) is affected by the resolution (w) and slice thickness (h): $D \sim 1/w^3h$ (Graeff and Engelke, 1991; Engelke and Kalender, 1998a). Contrast, noise, and resolution are all important factors for successful detection of trabecular structure. An increase in noise limits the detectibility of finer details of trabeculae. Usually the slice thickness for standard CT is larger than the in-plane pixel size, leading to lower spatial resolution but higher contrast than isotropic resolution, because the pixels representing trabeculae are filled with a larger amount of bone and a smaller amount of marrow as a result of partial volume effect, and because an increase in slice thickness can reduce the noise by a factor of 5 (Engelke and Kalender, 1998a).

Other CT

Peripheral quantitative CT (pQCT) has been explored in clinical and preclinical studies, *in vitro* and *in vivo,* and in the assessment of BMD and cortical bone geometry (Augat *et al.,* 1996; Grampp *et al.,* 1995, 1996; Butz *et al.,* 1994, Neu *et al.,* 2001). In preclinical studies, or *ex vivo* specimen studies, it might be convenient to examine the bones after dissection and exposure to air by densitometry, as pQCT can comparably and reproducibly quantify both volumetric trabecular BMD in either water or in air (Jiang *et al.,* 1998).

pQCT has been used in the study of osteoporotic animal models, such as mice (Turner *et al.,* 2001) but mostly in rats, with osteopenia induced by ovariectomy (Sato *et al.,* 1995, Cointry *et al.,* 1995), orchiectomy (Rosen *et al.,* 1995), immobilization (Ma *et al.,* 1995), and corticosteroid administration (Ferretti 1995). It has also been employed to evaluate effects of pharmaceutical intervention in osteopenic models using antiresorptive (Cointry *et al.,* 1995) or anabolic agents (Ma *et al.,* 1995). pQCT can determine cortical volumetric BMD and geometry (Ma *et al.,* 1995) which significantly contribute to the estimation of cortical bone biomechanical strength (Ferretti *et al.,* 1995, Cointry *et al.,* 1995) and trabecular volumetric BMD (Sato *et al.,* 1995; Rosen *et al.,* 1995; Ma *et al.,* 1995). Longitudinal analysis of the proximal tibia *in vivo* showed a significant reduction of 17% in BMD 31 days after ovariectomy (Sato *et al.,* 1995). It has been reported that pQCT volumetric BMD is more sensitive to bone loss in the rat than DXA (Sato *et al.,* 1995). pQCT was used in a study that found that a low dose of a Chinese herbal preparation containing epimedium leptorhizum was effective in preventing osteoporosis development in weight-bearing proximal femur and tibia but not in the lumbar spine in ovariectomized rats (Qin *et al.,* 2001). pQCT and μCT examinations showed that the distribution of the vertebral mineral into cortical and trabecular compartment is regulated genetically in 12BXH recombinant inbred mice (Turner *et al.,* 2001). It is difficult to determine the trabecular structure in preclinical studies because of limited spatial resolution and increased slice thickness in the pQCT images, though apparent trabecular structural parameters can be derived from pQCT images of human cubic bone specimens using special imaging processing techniques (Jiang *et al.,* 1998).

The scatter or diffraction patterns depend on the molecular structure of scattering tissues and can be used to identify the materials present. Coherent-scatter CT, based on the low-angle X-ray scatter properties of tissues, generates images of scatter intensity from which the scatter patterns corresponding to each pixel are determined. Material-specific tomographic images of mineral, collagen, fat, and water distributions can be obtained, and the gray level in the images provides the volumetric density (g/cm^3) of each material independently. It could be useful for imaging extremities in patients with bone mineralization disorders such as osteomalacia or hypermineralization after certain pharmaceutical intervention, using a radiation exposure about twice that of standard CT (Cunningham and Batchelar, 2001).

μCT

The μCT system was first introduced by Feldkamp and Goldstein (Feldkamp *et al.,* 1989; Kuhn *et al.,* 1990), who used a microfocus X-ray tube as a source, an image intensifier as a 2D detector, and a cone-beam reconstruction to create a 3D object. It was originally designed to detect small structural defects in ceramic materials. Instead of rotating the X-ray source and detectors during data collection as in clinical CT, the specimen is rotated at various angles. X-rays are partially attenuated as the specimen rotates in equal steps in a full circle about a single axis. At each rotational position, the surviving X-ray photons are detected by a planar 2D array. A 3D-reconstruction array is created directly in place of a series of 2D slices. Morphological measures such as the Euler characteristic to quantify connectivity, a measure of the maximum number of branches which can be removed before the structure is divided into multiple pieces, can be determined in the μCT data set without prior skeletonization (Odgaard and Gundersen, 1993; Feldkamp *et al.,* 1989). The connectivity, *c,* of a two-component system such as bone and marrow can be derived directly from the Euler characteristic, *e,* by $c = 1-e$ (Odgaard and Gundersen, 1993), if all the trabeculae and bone marrow cavities are connected without isolated marrow cavities inside the bone. Usually it is normalized by examined tissue volume and reported as connectivity density in $1/mm^3$. Discrepant results of the connectivity measurements, ranging from highly linear to nonlinear relationships with bone volume, have been reported (Odgaard and Gundersen, 1993; Feldkamp *et al.,* 1989; Kinney *et al.,* 1995; Geldstein *et al.,* 1993), which might be due to different specimen and sample sizes studied. Goulet (Goulet *et al.,* 1994) utilized images of bone cubes and related these image-based parameters to Young's modulus, a measure of elasticity of bone. Based on data sets from Feldkamp's μCT, Engelke (Engelke *et al.,* 1996) developed a 3D digital model of trabecular bone that could be used to compare two- and three dimensional structural analysis methods and to investigate the effect of spatial resolution and image processing techniques on the extraction of structural parameters.

The 3D cone-beam μCT imaged the trabecular bone architecture in small samples of human tibias and vertebrae, *ex vivo,* with a spatial resolution of 60 μm (Feldkamp *et al.,* 1989). A resolution of 60 μm, although acceptable for characterizing the connectivity of human trabeculae, may be insufficient for studies in small animals like the rat, where the trabecular widths average about 50 μm and trabecular separations average 150 μm or less (Kinney *et al.,* 1995). Furthermore, Smith and Silver (Smith and Silver, 1994) have reported that 3D images from cone beam scanners are inevitably distorted away from the central slice because the single-orbit cone beam geometry does not provide a complete data set. These distortions and associated loss of spatial resolution have been particularly evident in samples containing plate-like structures, even when the cone beam angle is less than 6.5° (Smith and Silver, 1994). A recent study with direct comparison of fan beam (obtained on the central plane) and cone bone (obtained from a divergent section near the periphery of the volume) techniques over a full cone angle of 9°, using a 100-μm microfocus X-ray tube and isotropic 33-μm voxels, showed that the bone volume fraction based on a gray-scale threshold in the excised lumbar vertebrae from normal adult rats was not adversely affected by cone-beam acquisition geometry for cone angles typically used in μCT (Holdsworth *et al.,* 2000, 2001).

The method was further enhanced by resorting to synchrotron radiation with a spatial resolution of 2 μm (Bonse *et al.,* 1994), or with applications to live rats (Kinney *et al.,* 1995). The X-ray intensity of synchrotron radiation is higher in magnitude than X-ray tubes. When scanning time is important, and for resolution less than 1–5 μm, synchrotron radiation is a better choice than X-ray tubes. Adaptation of the X-ray energy to the sample can be optimized by using monochromatic radiation because of the continuous X-ray spectrum of synchrotron radiation, which can minimize radiation exposure for examining small animals *in vivo.* Synchrotron μCT has spatial resolutions of 2 μm because of high brightness and natural collimation of radiation sources (Bonse *et al.,* 1994). It uses parallel beam-imaging geometry, and avoids the distortions and loss of resolution inherent in cone beam methods and can make distortionless images of human trabecular bone using a CT at a synchrotron electron storage ring (Bonse *et al.,* 1994). Recently, μCT using high-intensity and tight collimation synchrotron radiation that achieves spatial resolution of 1–2 μm has provided the capability to assess additional features such as resorption cavities (Peyrin *et al.,* 1998).

Synchrotron μCT at 23 μm/voxel in the proximal tibial metaphysis of live rats (Lane *et al.,* 1995, 1998, 1999) shows that trabecular connectivity decreased 27% by days 5 and 8 postovariectomy and continued to decrease up to day 50 after ovariectomy. The trabecular BV/TV decreased 25% by 8 days after ovariectomy, and it continued to decrease through day 50. These changes were more rapid than biochemical markers. Estrogen replacement therapy initiated at 5–13 days after ovariectomy can restore BV/TV—but not connectivity—to baseline levels by allowing bone formation to continue in previously activated bone remodeling units while suppressing the production of new remodeling units. Intermittent human parathyroid hormone (1–34) treatment in osteopenic ovariectomized rats increased trabecular BV/TV to control levels or higher by thickening existing trabeculae. Human parathyroid hormone (1–34) did not reestablish connectivity when therapy was started after 50% of the trabecular connectivity was lost. At 120 days after ovariectomy, there was a small but significant decrease in trabecular bone volume, and a significant decrease in trabecular plate thickness. The decrease in trabecular thickness was associated with an increase in connectivity, in contrast to the proximal tibia where connectivity always declines (Kinney *et al.,* 2000).

In addition, synchrotron radiation X-ray microtomography (μT) using new X-ray optic components, has been designed to assess the ultrastructure of individual trabeculae

with a resolution of 1 μm, and to describe microscopic variations in mineral loading within the bone material of an individual trabecular rod. Artifacts from X-ray refraction and diffraction require methods different from those used for other μCT techniques. Delicate and minimal individual trabecular specimen handling and no microtome cutting preserve the specimen geometry and internal microfractures. The histological features of the mineral ultrastructure can be evaluated using volumetric viewing. The volume, shape and orientation of osteocyte lacunae and major cannaliculae can be observed. Quantitative measures of trabecular ultrastructure are now being considered including BMU (basic multicellular unit of bone remodeling with activation → resorption → formation sequence) volume, lamellar thickness, and density gradients (Flynn *et al.,* 2001).

The hardware for synchrotron radiation μCT, however, is not readily accessible. Electron storage rings are stationary and cannot be operated in a small laboratory and only a few synchrotron radiation centers are available worldwide. Rüegsegger *et al.* (1996) developed a μCT device dedicated to the study of bone specimens, without synchrotron radiation. It has been used extensively in laboratory investigations. Image processing algorithms, free from the model assumptions used in 2D histomorphometry, have been developed to segment and directly quantify 3D trabecular bone structure (Hildebrand *et al.,* 1999, 1997ab). Trabecular thickness is determined by filling maximal spheres in the structure with the distance transformation and then calculating the average thickness of all bone voxels. Trabecular separation is calculated with the same procedure, but the voxels representing nonbone parts are filled with maximal spheres. Separation is the thickness of the marrow cavities. Trabecular number is taken as the inverse of the mean distance between the mid-axes of the observed structure. The mid-axes of the structure are assessed from the binary 3D image using the 3D distance transformation and extracting the center points of nonredundant spheres which fill the structure completely. Then the mean distance between the mid-axes is determined similarly to the separation calculation; i.e., the separation between the mid-axes is assessed.

The deterioration of trabecular bone structure is characterized by a change from plate elements to rod elements. Consequently the terms "rod-like" and "plate-like" are frequently used for a subjective classification of cancellous bone. A new morphometric parameter called the structure model index (SMI) makes it possible to quantify the characteristic form of a 3D structure in terms of the amount of plates and rods composing the structure. The SMI is calculated by 3D image analysis based on a differential analysis of the triangulated bone surface. The SMI value is 0 and 3 for an ideal plate structure and a rod structure, respectively, independent of the physical dimensions. For a structure with both plates and rods of equal thickness the value lies between 0 and 3, depending on the volume ratio of rods and plates. The geometrical degree of anisotropy (DA) is defined as the ratio between the maximal and the minimal radius of the mean intercept length (MIL) ellipsoid. The MIL distribution is calculated by superimposing parallel test lines in different directions on the 3D image. The directional MIL is the total length of the test lines in one direction divided by the number of intersections with the bone marrow interface of the test lines in the same direction. The MIL ellipsoid is calculated by fitting the directional MIL to a directed ellipsoid using a least square fit. Also, the relationship of these parameters to *in vitro* measures of strength and their application to microfinite element modeling has been shown (Müller *et al.,* 1996b).

The early uses of 3D μCT focused on the technical and methodological aspects of the systems, but recent developments emphasize the practical aspects of microtomographic imaging. One recent study compared iliac crest bone samples from premenopausal women with iliac crest bone samples taken from the same women after menopause (Fig. 14, see also color plate). The samples were examined using μCT with an isotropic resolution of 20 μm^3. The postmenopause samples showed significant changes in 3D trabecular structural parameters. The annual rate of change from plate-like structure to rod-like structure was 12%, which may have important consequences for bone biomechanical properties.

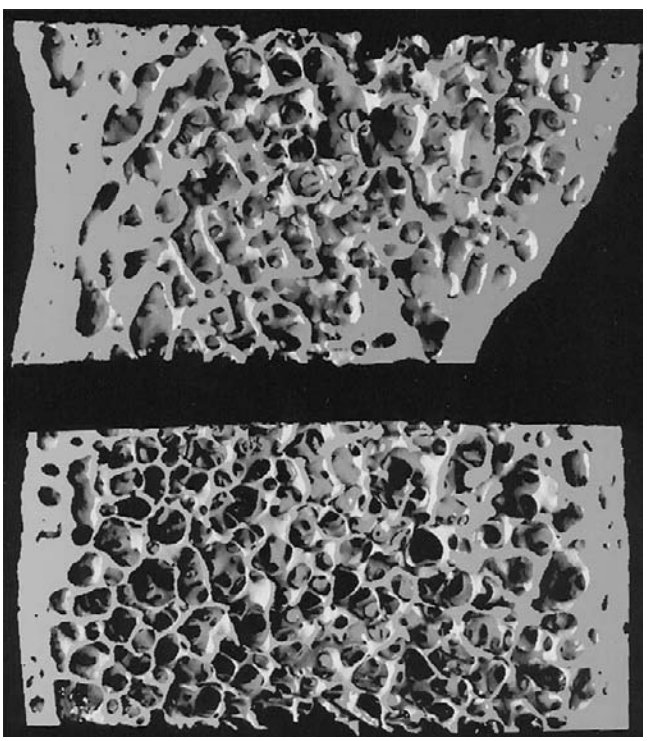

Figure 14 These two images are 3D μCT reconstruction of two paired iliac crest bone biopsy samples from one woman. The first biopsy was taken when she was healthy, premenopausal, and 53 years old. The second biopsy was from the opposite side, 5 years later, after menopause. The samples were examined using μCT with an isotropic resolution of 20 μm^3. The cortical bone of the iliac crest on the sides and trabecular bone between the cortex can be appreciated in both images. The trabecular structure is mainly plate-like and quite anisotropic in general before menopause (A). The changes in 3D trabecular structure after menopause (B) are dramatic: 3D trabecular bone volume has decreased; trabecular separation has increased; the trabeculae have become thinner and more isotropic, and there has been a shift from plate-like to rod-like structure. (See also color plate.)

Trabecular bone became more isotropic after menopause, with an annual decrease rate in DA of 0.7%. Interestingly, annual change in trabecular thickness (−3.5%) is greater than in trabecular number (−1.6%) and separation (+2%) (Jiang *et al.,* 2000a). These findings are quite different from those of traditional 2D histomorphometry. In histomorphometry, it is still a matter of debate whether trabeculae thin or simply disappear after menopause and with aging. It is possible that the loss of entire elements because of trabecular perforation forms the main mechanism of structural changes in osteoporosis. The remaining trabeculae are more widely separated and some may undergo compensatory thickening. Trabecular anisotropy will increase. In another study of paired biopsies taken before and after treatment with human parathyroid hormone, 3D μCT showed increased 3D connectivity density and confirmed the preservation of 2D histomorphometric cancellous bone volume and trabecular number and thickness. Cortical width from 2D histomorphometry increased in osteoporotic men treated with parathyroid hormone PTH and in osteoporotic women treated with parathyroid hormone (PTH) and estrogen (Dempster *et al.,* 2000).

μCT has been used to measure trabecular bone structure in rats. Most studies have focused on the trabecular bone in the proximal tibial metaphysis, but the trabecular bone in rat vertebrae is of interest because of its similarity to the human fracture site and because biomechanical testing is practical (Jiang *et al.,* 1997b). μCT with an isotropic resolution of 11 μm^3 has been used to examine the 3D trabecular bone structure of the vertebral body (Fig. 15, see also color plate) in ovariectomized rats treated with estrogen replacement therapy (Jiang *et al.,* 1999b). μCT 3D determined trabecular parameters show greater percentage changes than those observed with DXA, and they show better correlation with biomechanical properties. Combining trabecular bone volume with trabecular structural parameters provides better prediction of biomechanical properties than either alone. A study of the anabolic effects of low-dose (5 ppm in drinking water) long-term (9 months) sodium fluoride (NaF) treatment in intact and ovariectomized rats shows that NaF treatment increases trabecular bone volume, possibly by increasing trabecular thickness through increasing bone formation on existing trabeculae, rather than by increasing trabecular number. NaF in sham-operated rats increases trabecular structural parameters and bone mineral, but decreases compressive stress in the vertebral body. NaF partially protects against ovariectomy-induced changes in bone mineral and structure but this protection does not translate into a corresponding protection of bone biomechanical properties (Zhao *et al.,* 2000a,b).

The wide availability of genetically altered mice has increased the usefulness of the murine model for investigating osteoporosis and other skeletal disorders. Independent genetic regulation of 3D vertebral trabecular microstructure in 12BXH recombinant inbred mice as measured by μCT contributed information regarding the variation in biomechanical properties among the strains (Turner *et al.,* 2001). Mice homozygous for a null mutation of the PTH-related peptide (PTHrP) gene die at birth with severe skeletal deformities. Heterozygotes survive and by 3 months of age develop osteopenia characterized by decreased trabecular bone volume and increased bone marrow adiposity. PTHrP wild type and heterozygous-null mice were ovariectomized at 4 months of age and sacrificed at 5 weeks. 3D μCT was use to examine the trabecular structure of the mice, with an isotropic resolution of 9 μm^3. Bone specimens from mice heterozygous for the PTHrP null allele demonstrate significant changes as compared to wild-type litter mates in most parameters examined. However, measurements of trabecular number and trabecular thickness were not significantly different between the two groups. These findings support the notion that PTHrP haploinsufficiency leads to abnormal bone formation in the adult mouse skeleton (He *et al.,* 2000).

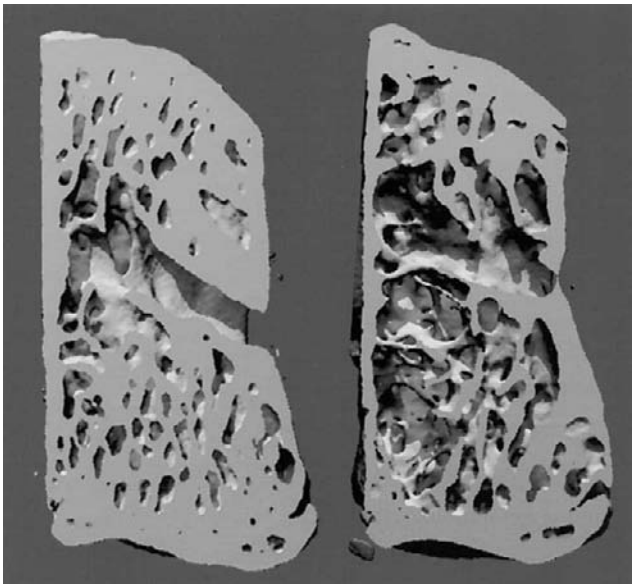

Figure 15 μCT 3D images of rat vertebral body with isotropic resolution of 11 μm^3. Compared with age-matched sham-operated control (left), ovariectomy results in a remarkable decrease in the trabecular bone volume, thickness, number, and a conspicuous increase in trabecular separation. The different trabecular patterns are noticeable, i.e., plate-like trabeculae in the control and rode-like trabeculae in the ovariectomized rat. The cortical thickness is also decreased after ovariectomy. (See also color plate.)

Ovariectomy induces short-term high-turnover accelerated deterioration of 3D trabecular structure in mice (Jiang *et al.,* 2000c). In a study using 3-month-old Swiss Webster mice 5 and 13 weeks after ovariectomy, 3D μCT trabecular structure was measured in the secondary spongiosa of the distal femur with an isotropic resolution of 9 μm^3. The trabeculae become more rod-like and more isotropic, thinner, and more widely separated after ovariectomy (Fig. 16, see also color plate) (Zhao *et al.,* 2000c). HRT prevented ovariectomy-induced bone loss. Percentage changes in pQCT volumetric BMD were similar to the changes measured by μCT but less pronounced (Jiang *et al.,* 2001).

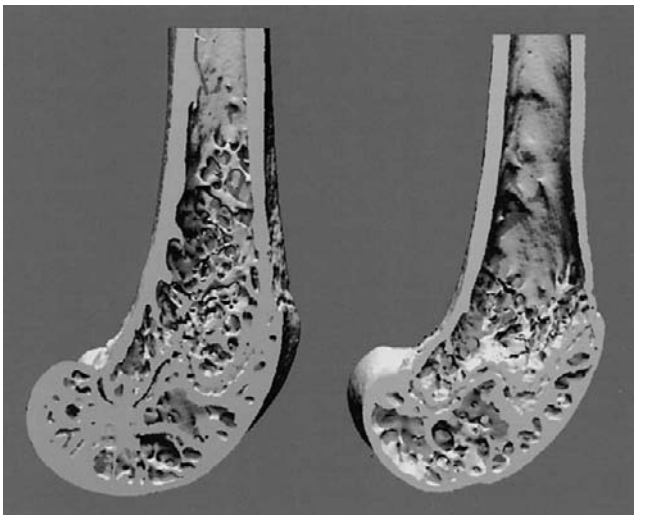

Figure 16 μCT 3D images of trabecular and cortical bone structure of mice with isotropic resolution of 9 μm^3. Compared with age-matched sham-operated control (left), ovariectomy results in a dramatic loss trabecular bone volume and other trabecular bone microstructure (right). (See also color plate.)

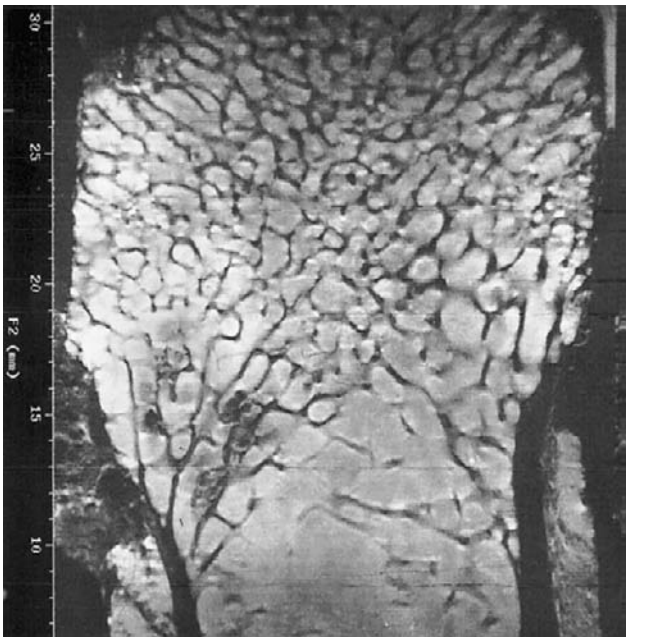

Figure 17 Fine trabecular network on μMRI of the femoral neck specimen of an ewe imaged in coronal plane with a spin echo sequence, in-plane resolution of 78 μm and slice thickness of 150 μm on a Varian Unity 400 NMR instrument at 9.4 T.

μCT has other research applications, for example quantifying osteogenesis in mouse Ilizarov leg lengthening procedures and quantifying osteoconduction in a rat cranial defect model (Jiang *et al.,* 2000b,d). In a model of osteoarthritis in dogs, μCT with a nominal resolution of 66 μm was used to examine the subchondral bone changes. 3D μCT and 2D histomorphometry showed a decrease in bone volume fraction and an increase in bone surface fraction in the femur and patella of dogs 3 and 6 months after an anterior cruciate ligament transection. Histomorphometry also shows an increase in bone formation rate. μCT images of the femur showed multiple pits on the bone surface of femoral condyles and femoral trochlear ridges, while the gross inspection of the cartilage showed only a discoloration in the corresponding areas, indicating that the subchondral bone erosions preceded articular cartilage damage in the development of osteoarthritis (Han *et al.,* 2001).

MRI Microscopy

High-resolution MR and μMR, collectively called MR microscopy, have received considerable attention both as research tools and as potential clinical tools for assessment of osteoporosis. As a noninvasive, nonionizing radiation technique, MR can provide 3D images in arbitrary orientations and can clearly depict trabecular bone. MRI is a complex technology based on the application of high magnetic fields, transmission of radiofrequency waves, and detection of radiofrequency signals from excited hydrogen protons. MRI can delineate bone so well (Figs. 11 and 17) because bone mineral lacks free protons and generates no MR signal while adjacent soft tissue and marrow contain abundant free protons and give a strong signal. The great difference in the magnetic properties of trabecular bone and bone marrow produces distortions of the magnetic lines of force which make the local magnetic field within the tissue inhomogeneous and alters the relaxation properties of tissue, e.g., the apparent transverse relaxation time T_2^* in gradient-echo images. This shortening of relaxation time is greater in normal dense trabecular networks than in rarefied osteoporotic trabeculae. T_2^* is directly related to the density of the surrounding trabecular network and its spatial geometry. There is a highly significant correlation between the relaxation rate $1/T_2^*$ by MR and BMD determined by QCT. Measurement of changes in the apparent transverse relaxation time $T2^*$ shortening can discriminate healthy normal dense trabecular networks from rarefied osteoporotic trabeculae (Genant *et al.,* 1996a; Grampp *et al.,* 1996, Chung *et al.,* 1990). $T2^*$ maps of the proximal femur could be used to differentiate between postmenopausal women with and those without osteoporotic hip fractures (Link *et al.,* 1998a). In addition to the indirect measures of the relaxation time, high-resolution images have been obtained to assess bone structure.

Currently, assessment of trabecular structure using MR microscopy is restricted to the peripheral skeleton unlike CT which can be applied to the spine and peripheral bones. The parameters derived from the low-resolution images account for 91% of the variation in Young's modulus, suggesting that noninvasive assessment of the mechanical competence of trabecular bone in osteoporotic patients may be feasible (Hwang *et al.,* 1997). High-field MR spectrometers with imaging possibilities or small-bore MR imaging scanners can be used to obtain ultrahigh spatial resolution. Using a rapid

spin-echo technique and three-dimensional imaging at 9.4 T, an isotropic resolution with 78 μm of human and bovine bone cubes has been reported (Chung *et al.,* 1995a,b; Hwang *et al.,* 1997). Good correlations between MR-derived parameters and standard histomorphometric measures were found. Antich *et al.* (1994) found changes in accordance with histomorphometry measures. Comparison of MR microscopy and the μCT system developed by Feldkamp (Hipp *et al.,* 1996; Kuhn *et al.,* 1990; Simmons and Hipp, 1997) showed that precision for trabecular bone volume is better with μCT (3% vs 6%) but trabecular number is better with MR microscopy (1% vs 2.5%).

In MR the appearance of the image is affected by many factors beyond spatial resolution, including the field strength—since signal is related to the field strength, the specific pulse sequence used, the echo time, and the signal-to-noise achieved. Acquisition, analysis, and interpretation of MR images are more complicated than for X-ray-based images of CT. Both spin-echo (Jara *et al.,* 1993) and gradient-echo sequences (Majumdar *et al.,* 1995) can be used to generate high-resolution MRI or bone structure. Gradient-echo techniques require less sampling time and may be better suited to *in vivo* clinical examinations. A 180° radio frequency (RF) pulse is applied to obtain an echo signal for generating an image in spin-echo sequences, while a reversal of the magnetic field gradient is used for such an echo in gradient-echo sequences. The apparent trabecular size depicted in both spin-echo and gradient-echo images may differ from their true dimensions (Jara and Wehrli, 1994). The overestimation of trabecular dimension is more pronounced in gradient-echo images, especially when TE, echo time, is increased. An increase in TR, the repetition time, leads to an increase in signal to noise ratio, but also an increase in the total scan time. The higher the bandwidth or the total time in which the MR signal is sampled, the shorter the achievable echo time, and the lower the signal to noise ratio. All these parameters need to be carefully weighed and adjusted as they interact in multiple ways and affect resolution and quality of the image (Majumdar *et al.,* 1995).

Segmentation to derive binary images for structural measurement can be challenging, since adaptive threshold- and edge-based methods tend to amplify susceptibility differences in the bone marrow, resulting in misclassification of marrow as bone. Global thresholds based on the histogram of the gray-value distribution (Majumdar *et al.,* 1995), or local thresholds using internal calibration based on fat, air, tendon, and cortical bone (Ouyang *et al.,* 1997) have been used (Fig. 18). The local threshold results in precision of 3–5% for trabecular histomorphometric parameters of the calcaneus, and can discriminate trabecular thickness and separation between pre- and postmenopausal healthy women (Ouyang *et al.,* 1997). Hipp and colleagues (1996) examined bovine cubes in a small bore microimaging spectrometer at 60 μm^3 resolution and found 3D results heavily dependent on the threshold and image processing algorithm. Majumdar *et al.* (1996) examined human cadaver specimens using a standard clinical MR scanner at 1.5 T and a spatial resolution of 117 × 117 × 300 μm and compared these images with μCT images and with serial grindings to determine the impact of in-plane resolution and slice thickness on both 2D and 3D structural and textural parameters. Considerable resolution dependence was observed for traditional stereological parameters, some of which could be modulated by appropriate thresholds and image processing techniques. As results can be affected by operator-adjustable parameters in the threshold-based methods, it is recommended that the same segmentation scheme be used for all patients or specimens in a specific project.

Examination of the distal radius using MRI with a spatial resolution of 156 μm^2, a slice thickness of 0.7 mm, and a modified gradient echo sequence at a 1.5 T clinical imager (Majumdar *et al.,* 1994) clearly depicted the loss of the integrity of the trabecular network with the development of osteoporosis (Fig. 19). Similar images of the calcaneus of normal subjects showed that the orientation of the trabeculae is significantly different in various anatomic regions. Ellipses, representing the mean intercept length, showed a preferred orientation and hence mapped the anisotropy of trabecular structure. Resolutions of 78–150 μm and slice thicknesses of 300 μm have been achieved in the phalanges, a convenient anatomic site that is particularly suitable for obtaining high signal-to-noise and high spatial resolution images *in vivo,* using clinical imagers at 1.5 T with a special RF coil (Jara *et al.,* 1993; Stampa *et al.,* 1998), and quantitative 3D parameters based on an algorithm and model for defining trabecular rods and plates have been derived (Stampa *et al.,* 1998). It has been reported that using trabecular structure or textural parameters from *in vivo* MR images of the radius (Fig. 19) or calcaneus can discriminate spine and/or hip fracture (Link *et al.,* 1998b; Majumdar *et al.,* 1997; Wehrli *et al.,* 1998).

Although high-resolution MR has been used successfully for *in vitro* quantitative evaluation of human trabecular bone, application of this technology to small animal bones is more demanding, as resolution requirements are more stringent because of the considerably smaller trabecular size. The need for higher resolution, dictated by the thinner trabeculae, entails a significant penalty for signal to noise ratio and acquisition time. Recently, the ability of MRI to assess osteoporosis in animal models has been explored in depth. One study demonstrated that using a small high-efficiency coil in a high-field imager, MRI can provide resolutions sufficient to discriminate individual trabeculae. MRI of trabecular structure in the distal radius shows trabecular bone loss after ovariectomy. μMRI of a rat tail showed 3D cortical bone, trabecular network, and other soft tissue. μMRI shows increased bone mass in the distal femoral metaphysis of rats treated with a bisphosphonate, increased cartilage thickness in the growth plate, and corresponding recovery changes after withdrawal of treatment. The trabecular structure in the femoral neck of a ewe can be clearly shown on MRI (Jiang, 1995). In this study, with appropriate choices, it was possible to image trabecular bone in rats *in vivo* and *in vitro.* Segmenting trabecular

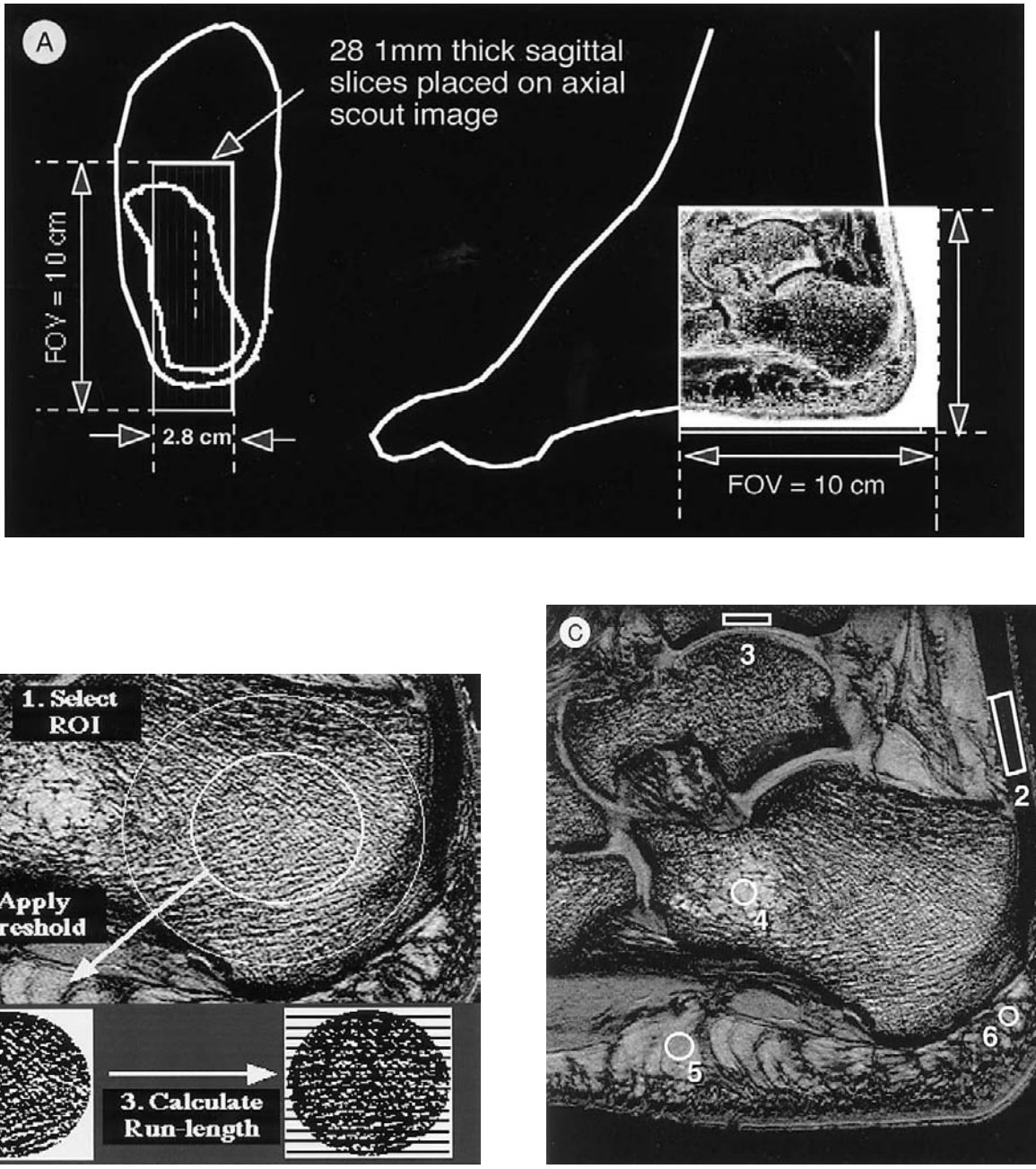

Figure 18 Procedures of trabecular bone structure acquisition and analysis using high resolution MR imaging. (A) Schematic representation of MR imaging acquisition of the calcaneus on a 1.5-T GE Signa imager using a modified 3D GRE pulse sequence (TE/TR/α = 11 msec/35 msec/30°), with in-plane pixel resolution of 195 × 260 μm^2, encompassing the entire central portion of the calcaneus. (B) Illustration of the MR image analysis procedure: (1) selection of the analysis ROI; (2) gray-level thresholding of the ROI to obtain a binary image of bone/marrow; (3) calculation of trabecular structure parameters using run-length analysis. (C) Positioning of ROIs used for internal calibration of the segmentation threshold. Six ROIs are defined in regions of the MR image representing: (1) air, (2) tendon, (3) cortical bone, (4) bone marrow, (5) and (6) subcutaneous fat.

bone from adjacent tissues has been a useful technique in the quantification of trabecular bone in MRI images. In a study of ovariectomy in rats, analysis of MRI demonstrated differences in rat trabecular bone that were not detected by DXA measurements (White *et al.,* 1997).

MRI showed ovariectomy induced losses in trabecular bone volume fraction and structure that ERT prevented (Kapadia *et al.,* 1998). There are excellent correlations between MRI with resolution up to 24 × 24 × 250 μm and histological assessment of intact rat tibiae and vertebrae (Kapadia *et al.,* 1993). It has been reported that rat tibiae were imaged at 9.4 T *in vitro* with isotropic resolution of 46 μm^3. It has also been shown that alendronate maintains trabecular bone volume and structure about midway between intact and ovariectomy, whereas prostaglandin E_2 returned them to intact levels (Takahashi *et al.,* 1999).

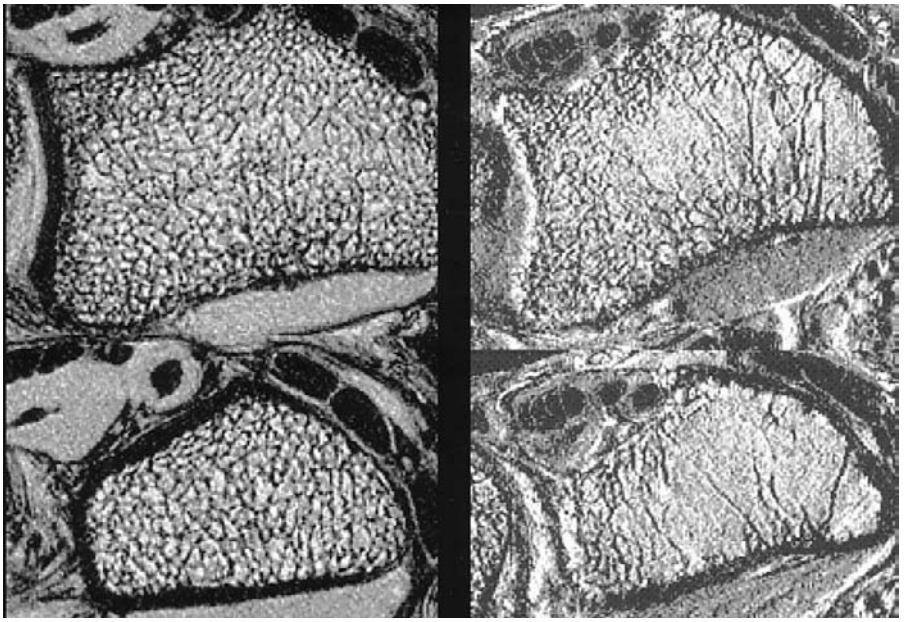

Figure 19 Trabecular network in the distal radius from a healthy, 35-year-old, prememopausal woman (left) and a 76-year-old, osteoporotic postmenopausal woman with vertebral fracture (right), imaged using an 1.5-T Signa SR/230 MR magnet (General Electric) with fast gradient echo imaging sequence at a bandwidth of $\pm$ 8 kHz, in-plane resolution of 156 μm, slice thickness of 700 μm, TE 7.8 msec, TR 31 msec, flip angle 30°. A remarkable decrease in the apparent trabecular bone volume and loss in trabecular connectivity in osteoporotic radius can be appreciated.

MRI shows 3D bone structure and some other tissues at the same time. In the rabbit knee, MRI shows trabecular structure and cartilage. In an osteoarthritis model induced by menisectomy (Jiang *et al.,* 1997a) or anterior cruciate ligament transection (Zhao *et al.,* 1999), MRI shows subchondral osteosclerosis, and decreased cartilage thickness. MRI also shows osteophytes in a rabbit osteoarthritis model. However, radiographs show only subchondral osteosclerosis, while osteophytes could not be found in a rabbit osteoarthritis model.

The usefulness of this powerful tool is balanced by disadvantages: it is expensive to obtain, operate, and maintain, and it is rarely available for routine nonclinical use. Dedicated extremity MR systems, which are low-field and less expensive, can quantify geometry of the cortical bone such as cross-sectional area and cross-sectional moments of inertia (CSMI) in the distal radius and the muscle mass in the forearms, which contribute significantly to the biomechanical strength of the radius (Jiang *et al.,* 1998). The mechanical behavior of a bone reflects the material property within the bones as well as the bone geometry. CSMI describes the geometric configuration or distribution of the bone mass around the central axis. The magnitude of the CSMI depends on both the cross-sectional area and the squared distance of each unit area from the sectional neutral axis that always intersects the centroid, or center of the area of the section. In torsion, deformation would be resisted more efficiently if bone were distributed further away from the neutral torsional axis. Ideally, in bending or torsion, bone should be distributed as far away from the neutral axis of the load as possible (Ruff and Hayes, 1983).

Summary

Many studies have shown that changes in bone quality and structural characteristics lead to changes in bone biomechanical competence or individual risk of fracture independently of BMD. New techniques and imaging modalities have been developed to quantify bone architecture in addition to bone density. Structural measurements using advanced imaging techniques contribute to our understanding of osteoporosis and other bone disorders, and provide insight into their pathomechanisms.

Though progress has been made in the noninvasive and/or nondestructive imaging bone of architecture, there are many issues that require further investigation: the balance between spatial resolution and sampling size; between signal-to-noise and radiation dose/acquisition time; between the complexity/expense of equipment and availability/accessibility of the methods; the potential biological differences between the peripheral appendicular skeleton and the central axial skeleton, and their clinical diagnostic applications that require high accuracy while application of monitoring diseases progress and treatment response that require high precision.

Although they are demanding in terms of equipment and technique, advanced imaging methods have many advantages.

They are unbiased, free from the model assumptions used in 2D histomorphometry. Since a true 3D assessment of the trabecular bone structure is possible, rod or plate model assumptions are no longer necessary. They are able to directly measure 3D structure and connectivity in arbitrary orientations in a highly automated, fast, objective, non-user-specific manner, with little sample preparation, allowing greater numbers of samples for unbiased comparisons between controls and subjects. They can have large sample sizes and therefore less sampling error. They are nondestructive, which allows multiple tests such as biomechanical testing and chemical analysis on the same sample, and noninvasive which permits longitudinal studies. They are noninvasive, allow repeated measurements over time in longitudinal studies, reduce the number of subjects in each study, and may provide a reasonable surrogate for static histomorphometric measurement and a complimentary technique to dynamic histomorphometry. The noninvasive imaging techniques have an advantage over histologic biopsy, in that they allow repeated follow-up measurements, at a number of different skeletal sites. These methods also have weaknesses: they require robust image processing algorithms to segment and quantify bone structure, and may have limitations in spatial resolution for certain structures. They cannot provide information on cellular activities and on dynamic mineralization processes. Rather than replacing bone histomorphometry, these imaging methods provide additional and valuable information and are a useful complement to traditional techniques in the evaluation of osteoporosis and other bone disorders.

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CHAPTER 94

Transilial Bone Biopsy

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Introduction

Bone biopsy has been widely used as a research tool in clinical studies and animal work for nearly three decades. Fluorochrome tissue-time markers (Frost, 1969) have enhanced the value of the biopsy by giving dynamic information about bone formation in addition to static histologic information. The fluorochrome double label marks the location of mineralization at two nearly instantaneous points in time. The mineral apposition rate (MAR), the key dynamic measurement that opens the door to measurement of bone formation rates, is determined from the two labels. Thus, histologic and dynamic information from bone biopsies can be expressed in numerical terms and evaluated statistically.

Bone biopsy has limited application in clinical diagnosis of common bone disorders such as postmenopausal osteoporosis. However, bone biopsy data are very valuable for studying the etiology and pathogenesis of bone disease, as well as the short- and long-term effects of treatments and interventions, particularly early in the study of new pharmacologic agents.

The transilial biopsy locus has become the predominant one used for histomorphometry in clinical studies. There are shortcomings, to be sure: the tissue sample is small; only bone from the iliac crest is sampled; the morphology of samples varies considerably from core to core within the target area (and even within a single core); analysis of cortical bone is limited; the laboratory procedure is tedious, difficult, and expensive; and few laboratories are available to process and analyze specimens. Patient fear and discomfort, along with perceived risk, have contributed to the reluctance of many investigators to include transilial bone biopsies in their research protocols. However, when properly carried out, the procedure is quite safe, with minimal patient discomfort and minimal risk of morbidity.

In this chapter, we describe the surgical procedure for transilial bone biopsy and the laboratory methods for processing and analyzing the specimens. We then present reference data from both healthy postmenopausal women and patients with postmenopausal osteoporosis. Other chapters in this volume describe the histomorphometric findings in other metabolic bone diseases.

The Procedure

Though few clinicians or clinical investigators will require detailed knowledge of specimen processing and histomorphometric analysis, many of them will be called upon to obtain transilial bone biopsies. We therefore describe the first author's biopsy procedure in some detail and limit our description of laboratory methods to an overview.

Tetracycline Labeling

The tetracycline class of drugs presents the only practical fluorochrome labeling agent for human studies (Frost, 1969). They are safe, available for human use, accumulate at bone-forming sites, and fluoresce brightly when activated with fluorescent light of 350 nm in an epifluorescence microscope system. Although these properties are common to all tetracyclines, tetracycline HCl (250 mg *tid* or *qid*) and demethylchlortetracycline (150 mg *bid* or *tid*) are most commonly used. The drug is deposited at sites of ongoing mineral precipitation. Intensity of the label is dependent on the plasma concentration of the drug, which in turn is dependent on dose. The double-labeling process involves dosing for several days, a longer period off drug, a second dosing period, and biopsy 5 to 14 days after the last dose. The schedule

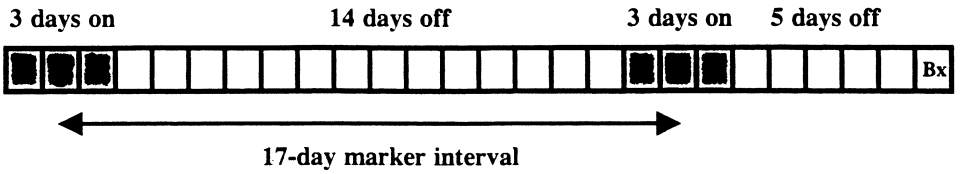

Figure 1 The 3–14–3:5 schedule for double-labeling of bone with oral tetracycline prior to biopsy (Bx). The marker interval is the number of days between the midpoints of the two dosing periods.

abbreviated as 3–14–3:5 (see Fig. 1) produces good results; it involves 3 days on, 14 days off, 3 days on, and 5 days off before biopsy. The number of days that span the midpoints of the first and second labeling periods is called the *marker interval*; this value is used to calculate the mineral apposition rate. As Fig. 1 indicates, the 3–14–3:5 schedule yields a marker interval of 17 days. While other schedules can be used, the marker interval *should not exceed 17 days* in order to minimize the label escape phenomenon (Schwartz and Recker, 1982).

Instructions to Patients

It is essential that patients follow the labeling regimen strictly, and the first and last of dose of each dosing period are the most crucial points of adherence to schedule. Calculations can be altered to accommodate a change in marker interval, *but only if the histomorphometry laboratory has been notified of changes in the dosing regimen.* To reinforce and clarify oral instructions, furnish each patient with a printout of instructions that include tetracycline labeling, preparation for the biopsy procedure, and postbiopsy precautions and wound care. Prior to the actual biopsy, the patient should avoid aspirin for at least 3 days and have nothing by mouth for 4 hr.

Instruments

Suitable specimens can be obtained by use of a trephine with inner diameter of no less than 7.5 mm, e.g., a Rochester bone biopsy trephine (Medical Innovations International, Inc., Rochester, MN). The needle should *always* be very sharp; it should be sharpened (and reconditioned, if necessary) after every three to five procedures. Manual operation of the needle results in better control, although some prefer using a power tool, such as a variable-speed cordless electric drill.

Obtaining the Biopsy

Schedule the transilial bone biopsy as an outpatient minor surgery. The patient wears a hospital gown and lies in the supine position on the surgical table. A folded towel or sheet under the hip will lift the pelvis about 1–2 inches on the side of the biopsy, to provide a better angle for the physician. Use fingertip and cuff *monitoring equipment.* The operator scrubs and uses a cap, mask, gown, and gloves.

Use the transilial approach exclusively. Locate the biopsy site about 2 cm posterior to the anterior–superior spine, which is about 2 cm inferior to the iliac crest. Give a sedative (Versed, 2.5 to 5 mg) through a forearm intravenous catheter. Prep and drape the site. Infiltrate the skin and subcutaneous tissues at the biopsy site with about 30 mL of local anesthetic. Infiltrate the inner wall of the ilium through the abdominal wall. Make a 2-cm skin incision and extend it to the periosteum with blunt dissection. After making the skin incision, use a dissecting scissors for all dissection. Minimal use of the scalpel reduces blood loss, eliminates the need for subcutaneous sutures, and reduces postoperative discomfort. Insert the trephine and remove a core of bone, including both cortices and the intervening trabecular bone. Pack the bony defect with 2 small strips of surgicel (2 by 0.25 inches), secure hemostasis with pressure, close the wound with 5-0 monofilament (3 to 5 stitches), and apply a pressure dressing.

The dressing is to be left in place and kept absolutely dry for 48 hr. The patient can then remove it and shower daily—but not bathe—until the suture is removed. Instruct the patient to keep physical activity to a minimum in the week following the procedure. The patient can expect localized aching for about 2 days, possible itchiness as the wound heals, and a small scar.

If a second biopsy is done, it should always be on the side opposite the first biopsy. Do not attempt more than two biopsies (one on each side) because of potentially confounding effects remaining from the previous biopsy.

Precautions

In order to obtain an intact biopsy, it is vital to use *gentle* pressure to advance the trephine. This is particularly the case in patients whose osteoporotic skeletons are very fragile. Make every effort to obtain an intact specimen, i.e., a core that contains both cortices and the intervening trabecular bone. Anything less is not usable. If the specimen is fractured during the procedure, one or two additional attempts can be made to get a good specimen through the same incision. However, make a new track with blunt dissection for each attempt to obtain an undamaged specimen.

The specimen is sometimes difficult to remove from the trephine; this problem results from wedging of the proximal cortex in the sleeve of the trephine. The specimen can usually be safely dislodged by use of a sharp blow to the handle of the trochar. Immediately transfer the specimen to a 20-mL screw-cap vial containing 70% ethanol.

Complications

PAIN

Pain during and after the biopsy procedure is the most feared complication. It is often reinforced by the stories of family or friends who have had a bone marrow *aspiration,* which typically involves sharp pain as the specimen is withdrawn. However, if generous amounts of local anesthetic are placed periosteally on both sides of the ilium, the bone biopsy procedure described here rarely evokes pain. (Injection of local anesthetic is, of course, associated with some discomfort.) Rarely, a patient will experience acute sharp pain during passage of the trephine through the marrow space, signaling the presence of a pain fiber within the marrow space. If this occurs, a small additional amount of intravenous analgesic can be injected through the indwelling catheter to aid in finishing the procedure. More commonly, patients describe passage of the trephine as feeling "like a cramp."

BLEEDING/HEMATOMA/OTHER

Bleeding during the procedure is minimal. Preoperative tests of the platelet and coagulation systems need not be carried out, unless there is some evidence of risk. Such risks exist in patients on hemodialysis, in those with liver disease, and among those with other conditions or medications that can compromise hemostasis. Prior surgery of any type accomplished without bleeding complications is evidence against an inherited clotting disorder. Aspirin interferes with platelet function (hence the caveat noted earlier). Local hematoma is uncommon, although bruising sometimes occurs around the wound. Occasionally there will be transient neuropathy due to entrapment of a local branch of the femoral nerve by hematoma, or perhaps from transection of a sensory nerve. The resulting paresthesia may take 2 or 3 weeks to resolve. Transient femoral nerve palsy has been reported, as well as postoperative trochanteric bursitis on the side of the biopsy. The first author has encountered two instances in which a sudden episode of severe pain occurred about 10 days after the biopsy in the general area of the biopsy site. These were due to fractures extending from the crest of the ilium to the biopsy site. Healing occurred with analgesics and restriction of activity. Rao surveyed a large number of physicians who performed transilial biopsies, asking them to enumerate the complications (Rao *et al.,* 1980). For 9131 biopsies, they reported 22 with hematomas, 17 with pain for more than 7 days, 11 with transient neuropathy, 6 with wound infection, 2 with fracture, and 1 with osteomyelitis for a total incidence of complications of 0.7%. There were no deaths and no cases of permanent disability among the group.

Specimen Processing

The following overview of laboratory methods associated with transilial bone biopsy includes a number of practical tips derived from the experience of our laboratory. Details of specimen processing can be obtained from the text edited by Recker (1983, though no longer in print) or through one of the laboratories that does this work.

Fixative

As noted earlier, our research unit uses 70% ethanol for fixing transilial bone biopsy specimens. In addition to providing adequate fixation, 70% ethanol is a suitable medium for long-term storage at room temperature, which is particularly important when specimens are shipped to a central biopsy-processing site. Fill specimen containers to capacity (eliminate air) in order to protect the specimen during handling. Biopsies to be stained for tartrate-resistant acid phosphatase require special handling (e.g., specimens in fixative kept on ice until processing). Our laboratory does not recommend use of phosphate-buffered formalin fixative, because it can dissolve bone mineral and distort tetracycline labels. It is possible to dissolve stain (Villanueva *et al.,* 1964) in the fixative so that block staining occurs during fixation. This is valuable for measuring static features at the same remodeling sites where the measurements of interlabel width are made.

Dehydrating, Defatting, and Embedding

Dehydrate and defat specimens slowly, carefully, and completely in order to achieve good quality embedding and avoid creating tissue artifacts. Pass specimens through graded alcohols and acetone, and de-gas to prevent the formation of bubbles during embedding. Embed samples in methyl methacrylate pretreated with appropriate plasticizing agents.

Sectioning and Staining

Trim and face the tissue blocks to provide a plane for sectioning that is parallel to the axis of the biopsy core. Section with a microtome equipped with a tungsten carbide blade. This is the most difficult part of the process, because the sections are very thin and fragile. The technician must grasp them and mount them on slides without creating excessive artifact. Make the first set of sections at 35–40% (about 2.5–3 mm) into the embedded specimen with a minimum side-to-side width of 5 mm. Collect subsequent sets at intervals of 250–300 μm further into the specimen. Obtain at least five 5- to 7-μm sections at each section depth for making Goldner's (Goldner, 1938) stained sections; obtain at least two 8- to 10-μm sections for making unstained slides; and obtain at least two 5- to 7-μm sections for making toluidine blue stained sections. Deplasticize slides with acetone before staining. Use unstained sections to quantitate tetracycline-labeled surfaces and to examine collagen architecture (i.e., lamellar or woven bone). Assess all other variables except for wall thickness with Goldner's stained sections. Use the toluidine blue-stained sections for wall thickness measurements. There are also other methods for measuring wall thickness (e.g., Matrajt and Hioco, 1966).

The Histology

Most of the measurements and calculations of bone histomorphometry reflect concepts about the organization and function of bone cells.

Intermediary Organization

Frost described several discrete bone cell functional activities (Frost, 1986) in what he called the intermediary organization (IO) of the skeleton. These are important in understanding the behavior of bone cells, and the reader is encouraged to consult reviews describing details (Frost, 1986). There are at least four levels of IO: growth, modeling, remodeling, and fracture repair. Growth is responsible for elongation of the skeleton; modeling is responsible for sculpting and shaping the skeleton during growth; remodeling is responsible for removal and replacement of bone tissue without change in volume or mass; and fracture repair is responsible for healing fractures. They all involve the same osteoclasts and osteoblasts, but the coordinated end result is much different in each case. This discussion concerns mainly remodeling, the IO most prominent in adult life.

Basic Multicellular Unit/Basic Structural Unit

Two terms used in histomorphometry need definition. First, the basic multicellular unit (BMU) (Frost, 1973) is the group of osteoclasts, osteoblasts, osteocytes, and lining cells that make up one remodeling unit. Second, the basic structural unit (BSU) is the unit of bone tissue formed by one BMU. It is also referred to as the osteon. These are brought into existence by the remodeling IO and are important because all adult metabolic bone disease occurs through derangement of the remodeling IO and the resulting derangement of BMUs and BSUs.

Osteoblasts and Bone Formation

Osteoblasts are bone-forming cells (Fig. 2). They produce the organic collagen matrix (and noncollagenous proteins) that undergoes mineralization to form both lamellar and woven bone. They originate from marrow stromal cell lineage (Owen, 1978) and appear at bone remodeling sites where osteoclasts previously resorbed bone. They also form bone in other IOs without prior osteoclast resorption. Prominent features are an eccentric nucleus, prominent Golgi apparatus, cell processes, gap junctions, endoplasmic reticulum, and collagen secretory granules. These plump cells appear in microscopic sections lined up at forming sites on the surface of unmineralized osteoid. As the apposition of matrix and its mineralization progresses, the cells become thinner. Some become incorporated into the bone matrix as osteocytes. Others disappear altogether or become thin lining cells on the finished bone surface. In addition to synthesis of osteoid and noncollagenous proteins, osteoblasts also participate in the mineralization process (Marotti *et al.,* 1972). Intensive study over the past decade has described many of the factors that influence osteoblast activity. However, a complete picture (including osteoblast recruitment, attraction to bone-forming sites, regulation of cell numbers, control of the bone formation rate and total amount of bone formed) remains to be pieced together.

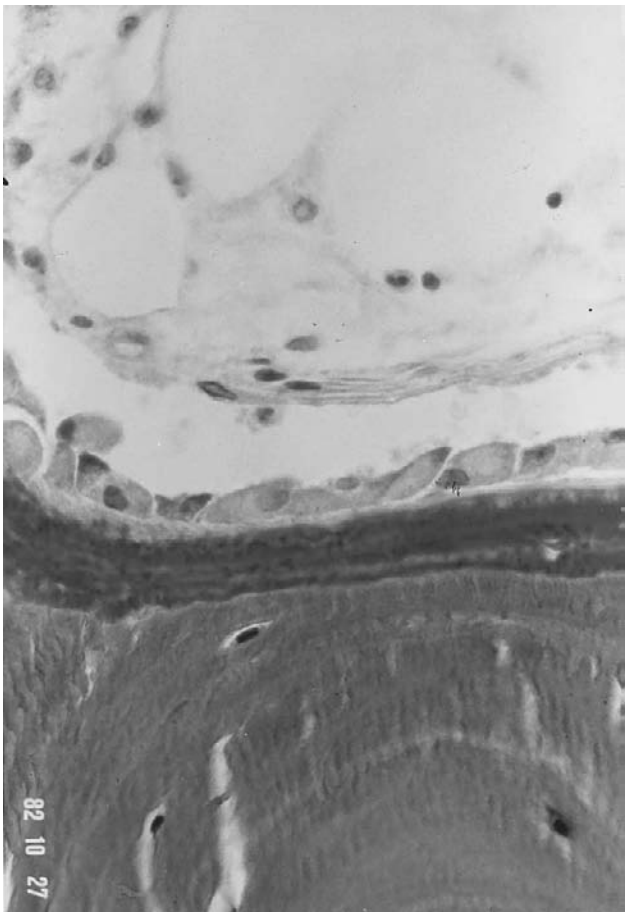

Figure 2 Osteoblasts lined in a palisade along an unmineralized osteoid seam. Goldner's stain; original magnification, ×150.

Osteoclasts and Bone Resorption

Osteoclasts (Fig. 3) are blood-borne multinucleated giant cells that originate from mononuclear hematopoietic stem cells (Jotereau and Le Douarin, 1978). They resorb both the mineral and the organic phases of bone. They contain 1 to 50 nuclei, and range from 20 to over 200 μm in diameter. In trabecular bone, they occupy shallow excavations on the surface, and in Haversian bone, they occupy the leading edge of "cutting cones." Light microscopic features include irregular cell shape, foamy, acidophilic cytoplasm, a striated perimeter zone of attachment to the bone, and positive staining for tartrate-resistant acid phosphatase. Electron microscopic features are numerous mitochondria, rough endoplasmic reticulum, multiple Golgi complexes, pairs of centrioles in a centrosome, vacuoles, and numerous granules.

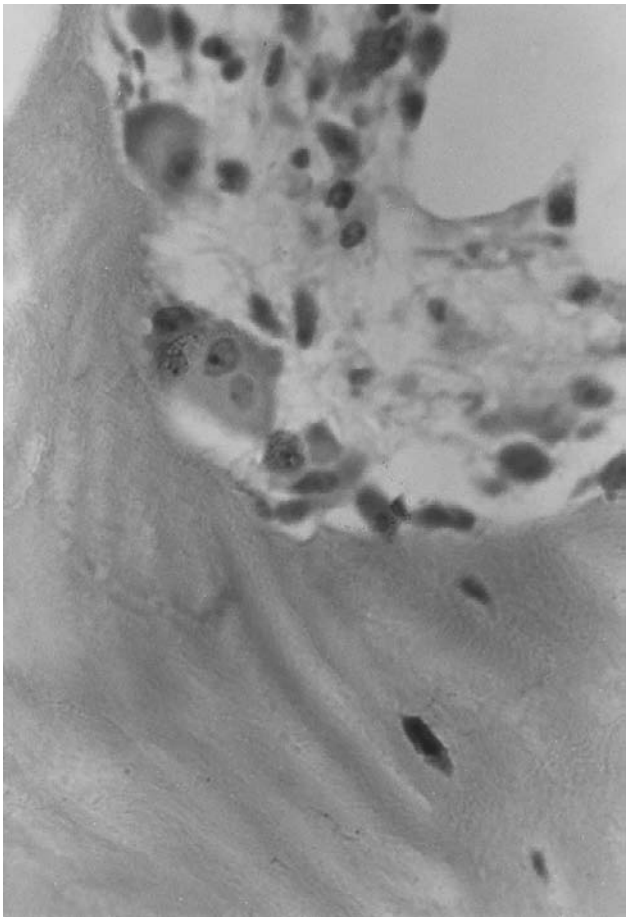

Figure 3 Osteoclast in a Howship's lacuna. Goldner's stain; original magnification, ×150. One multinucleated giant cell is seen, with other cells in the area.

A ruffled border, seen on electron and light microscopy, is located at the interface between resorbing bone surface and the cell surface. Osteoclasts secrete cysteine proteinases, collagenase, and acid phosphatase. Carbonic anhydrase is utilized for production of protons secreted at the ruffled border (Baron, 1985). The life span of osteoclast work at a trabecular resorption site is about 4 weeks on average (Recker *et al.,* 1988).

As noted above for osteoblasts, relatively recent research has described elements of the control mechanisms that influence osteoclast activity, but a fully integrated picture (including cell recruitment, attraction to sites to be resorbed, regulation of cell numbers, and control of the bone resorption rate) remains to be assembled.

Lining Cells

These are members of the osteoblast team (Fig. 4) that remain after bone formation has stopped (Parfitt, 1983). Morphologic features include location on trabecular surfaces; elongated, flattened, thin cytoplasm which cannot ordinarily be seen on light microscopy; and elongated, flattened, darkly stained nuclei. The function of lining cells remains uncertain, although they probably play a key role in the localization and initiation of remodeling (Mundy *et al.,* 1990) and in plasma calcium homeostasis (Parfitt, 1989).

Osteocytes

These, too (Fig. 5), are members of the osteoblast team that remain after bone formation has stopped (Parfitt, 1983), occupying small (10 μm) lacunae within the mineralized matrix of bone. The lacunae are interconnected by a fine network of canaliculi, measuring 1–2 μm in diameter, which contain cytoplasmic processes of osteocytes. Osteocytes can communicate with each other and pass oxygen, cell nutrients, and wastes to and from the canaliculi to the systemic circulation. The network of osteocytes may function to detect strain as mechanical force is applied to bone, and/or to convert changes in the strain environment into organized bone cell work. They may also be important in bone and plasma mineral homeostasis (El Haj *et al.,* 1990), although their function is not well understood.

Fluorochrome Labels

Tetracycline HCl labels are shown in Fig. 6. They were given on a 3–14–3:5 schedule and viewed in an epifluorescence microscope system. The distinction between the first and second labels is clear. The interlabel width measurements are made at several equidistant points at each formation site. The middle of each label is easier to locate than the leading or trailing edges; hence, the width measurements are made from the middle of the first label to the middle of the second label. Tetracycline HCl fluoresces a yellow color, and demethylchlortetracycline fluoresces a slightly orange color in systems activated with fluorescent light at a wavelength of 350 nm. This is helpful when two labelings are given at different points in time.

Regions Evaluated and Morphometric Methods

The area of an undecalcified section selected for histomorphometric analysis is permanently recorded by outline with an indelible mark on the slide. The analysis is restricted to the trabecular area. The distinction between endocortical and trabecular bone is made by pattern recognition by the histomorphometrist.

Few basic data are gathered at the microscope: volumes of bone, osteoid, and marrow; total trabecular perimeter; perimeters bearing the features of formation and resorption; thickness of osteoid and osteon walls; and interlabel width. The reader is encouraged to consult several texts and articles for detailed description of the numerous variables that can be measured or derived from microscopic sections, and for description of the methods of unbiased sampling of microscopic features (Recker, 1983). Several of the more frequently used variables and their abbreviations, as approved by the committee on nomenclature of the American Society

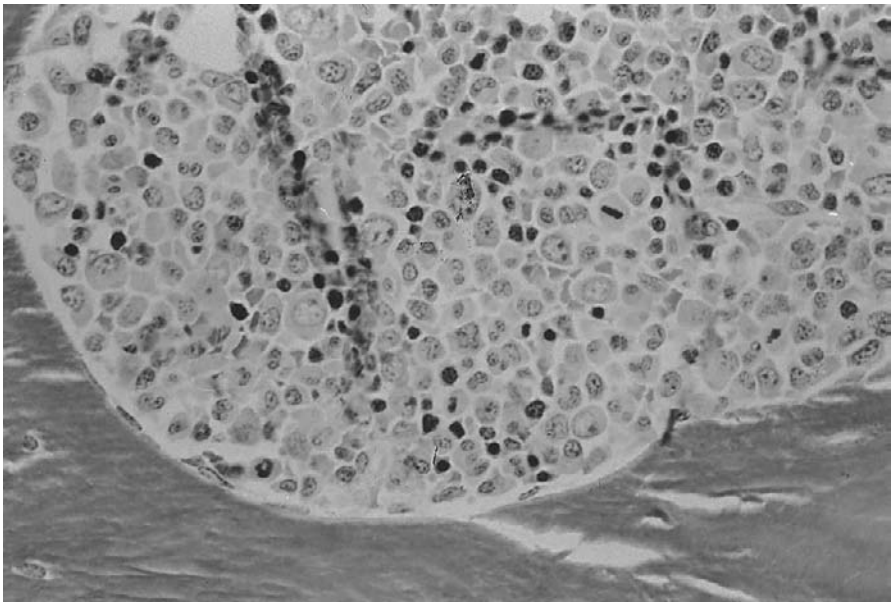

Figure 4 Resting surface covered with lining cells. Goldner's stain: original magnification, ×100. Flattened nuclei of lining cells can be seen; however, their cytoplasm is not visible.

Figure 5 Osteocytes within lacunae, with canaliculi radiating outward. Toluidine blue stain; original magnification, ×150.

Figure 6 Surface doubly labeled with tetracycline. Unstained; original magnification, ×100, epifluorescence system. Old buried label can be seen in the central part of the trabecula.

of Bone and Mineral Research (Parfitt *et al.,* 1987), are described in the following paragraphs.

Trabecular bone volume (BV/TV*)* is the ratio of the area of trabecular bone to the total area of marrow (including trabecular bone) expressed as a percentage. It is equivalent to the ratio of the volume of trabecular bone to volume of marrow. It is a "noisy" variable; however, it is important as part of the calculation of a number of other variables. *Osteoid surface* (OS/BS) is the percentage of trabecular perimeter that is occupied by unmineralized osteoid, with or without osteoblasts. *Osteoid thickness* (O.Th) is the average thickness of the osteoid covering trabecular surfaces. *Eroded surface* (ES/BS) is the percentage of trabecular surface that is occupied by Howship's lacunae, with and without osteoclasts. *Osteoblast surface* (Ob.S/BS) is the percentage of trabecular surface that is occupied by osteoblasts. *Osteoclast surface* (Oc.S/BS) is the percentage of trabecular surface that is occupied by osteoclasts.

Mineralizing surface (MS/BS) is the percentage of trabecular surface that is mineralizing and thus labeled. The definition of this surface must be specified; it may include both single and double label, double label only, or double label plus one-half the single label surface. The latter definition is the most accurate expression of mineralizing surface. MS/BS is used in the calculation of bone formation rates, bone formation periods, and the various expressions of mineralization lag time; thus, it is important to define it strictly.

Mineral apposition rate (MAR) is the rate of addition of new layers of mineral at trabecular surfaces. It is obtained by dividing the distance between labels by the time interval between labels. In a 3–14–3 schedule, the time interval is 17 days. The measurements are made from the middle of one label to the middle of the other, and corrected by $\pi/4$ for the randomness of the angle between the section plane and the plane of the trabecular surface. Some authors use an empirically determined refinement (1.199) of this factor, which was introduced because of the known departure from anisotropy of trabecular bone (Schwartz and Recker, 1981). All thickness measurements should be corrected by one of these factors in order to compare histomorphometric expressions of bone remodeling with other methods.

Wall thickness (W.Th) is the average distance between the resting trabecular surface (no osteoid or Howship's lacunae) and the cement lines. It is also corrected for section obliquity by one of the scaling factors. This measurement is used to calculate the formation periods.

Formation period (FP) is the average time required to complete a new trabecular BSU. It is calculated as the MAR divided by the W.Th. It can be used to calculate the resorption period (RP) by multiplying by the ratio of ES/BS to OS/BS.

Mineralization lag time (Mlt) is the lag between the time osteoid is formed and the mineral is added. It is calculated as O.Th/MAR. For a more accurate expression, it can be adjusted downward by the fraction of osteoid surface taking label (MS/OS). Thus, Mlt = O.Th/MAR*MS/OS. The Mlt can be used for detecting a mineralization defect, such as seen in frank vitamin D deficiency.

Bone formation rates (BFR/BV, BFR/BS): The two most useful expressions of bone formation rates are BFR/BV, the annual fractional volume of trabecular bone formed, and BFR/BS, the annual fractional volume of trabecular bone formed per unit trabecular surface area.

Trabecular connectivity: This concept expresses the extent of attachments between trabeculae. Loss of connectivity is a mechanism that might explain excessive (i.e., out of proportion to the deficit in bone mass) bone fragility. Parfitt (Parfitt *et al.,* 1983) described calculations for trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp). These calculations and measurements have become an integral part of the histomorphometric analysis of transilial bone biopsies. Other indices of trabecular connectivity have since been developed and gained a measure of acceptance, i.e., the ratio of nodes to free ends and related measures (Garrahan *et al.,* 1986), star volume (Vesterby *et al.,* 1989 and 1991), and trabecular bone pattern factor (TBPf) (Hahn *et al.,* 1992).

Still another approach involves analysis of superficially stained thick sections ("three-dimensional histomorphometry"), in which artifactual trabecular termini on the surface (created during sectioning) take up the stain, but *real* termini within the section do not. In a study of osteopenic women with and without vertebral fractures, this method disclosed a significant structural difference (Aaron *et al.,* 2000) but conventional histomorphometry did not (Hordon *et al.,* 2000).

Other additions to the list of histomorphometric variables are those associated with total reconstruction of the remodeling site (Agerbaek *et al.,* 1991). This allows calculation of bone balance at the individual BMU. The key maneuver permitting these measurements and calculations is estimating the position of the trabecular surface at the time osteoclasts began their resorption.

Reference Data

Table I contains selected histomorphometric data from a group of 90 untreated patients with postmenopausal osteoporosis compared to 34 healthy postmenopausal women (Kimmel *et al.,* 1990). The table is included here to characterize the findings in normals and in patients with osteoporosis, and to provide data for calculating statistical power and sample size. The patients were selected on the basis of at least one vertebral deformity coupled with low bone mass and no other cause of skeletal fragility. The normals were recruited as a convenience sample of ostensibly healthy postmenopausal women evenly distributed between ages 45 and 75. Careful clinical screening, including spine radiographs, was used to document absence of osteoporosis.

As Table I shows, the patients tended to be older, shorter, and lighter than the normals. It is somewhat surprising that

Table I Reference Data, Normals, and Osteoporosis Patients[a]

Variable			Osteoporosis		Normal		
Abbreviation	Units	Name	Mean ± SD	Median	Mean ± SD	Median	P<
N			90		34		
Age	Years		67.1 ± 7.2	67	60.0 ± 7.6	60	.001
Ht	m		1.56 ± 0.06	1.56	1.62 ± 0.05	1.60	.001
Wt	kg		58.3 ± 11.6	55.9	73.8 ± 12.2	70.2	.001
Static measures							
BV/TV	%	Bone volume	13.9 ± 4.5	13.42	21.2 ± 4.90	20.5	.001
W.Th	μm	Wall thickness	28.0 ± 4.44	28.2	32.1 ± 4.13	31.9	.001
Tb.Th	μm	Trabecular thickness	124 ± 27	119	138 ± 29	132	.02
Tb.N	mm^{-1}	Trabecular number	1.12 ± 0.24	1.09	1.55 ± 0.26	1.59	.001
Tb.Sp	μm	Trabecular separation	928 ± 218	894	654 ± 139	600	.001
O.Th	μm	Osteoid thickness	8.5 ± 2.1	8.2	9.6 ± 2.2	9.9	.02
OS/BS	%	Osteoid surface	16.0 ± 7.12	15.7	14.3 ± 6.3	12.5	NS
ES/BS	%	Eroded surface	4.8 ± 2.7	4.2	4.0 ± 2.0	3.0	NS
Oc.S/BS	%	Osteoclast surface	0.37 ± 0.50	0.13	0.71 ± 0.70	0.37	.005
Dynamic measures							
MS/BS	%	Mineralizing surface[a]	7.2 ± 4.5	6.6	7.0 ± 4.1	6.1	NS
MAR	μ/day	Mineral apposition rate	0.52 ± 0.08	0.49	0.55 ± 0.09	0.55	NS
BFR/BS	mm^2/mm/year	Bone formation rate[b]	0.0137 ± 0.0088	0.0116	0.0141 ± 0.0086	0.0114	NS
BFR/BV	mm^3/mm/year	Bone formation rate[c]	22.5 ± 14.4	19.6	21.1 ± 12.8	18.4	NS
Mlt	Days	Mineralization lag time	74.0 ± 47.1	52.29	87.5 ± 77.7	51.19	NS
FP	Years	Formation period	0.42 ± 0.30	0.31	0.48 ± 0.44	0.26	NS

[a]As double-label surface.
[b]Surface referent.
[c]Bone volume referent.

almost all the differences between the normals and osteoporotics are found in the structural variables and not the dynamic ones. This reflects the fact that the remodeling abnormalities in established osteoporosis cause loss of trabecular elements, rather than global thinning of trabeculae. However, wall thickness, a static structural variable that is unrelated to connectivity, is about 13 μm lower in the osteoporotic group. The greatest differences, however, are in trabecular number and trabecular separation (with median values for the osteoporotic group about one-third lower and one-half higher, respectively, than corresponding values for the normal group). Trabecular thickness is modestly affected (with a median value about 1/10th lower in the osteoporotic group). Together, these findings contribute importantly to our understanding of the pathogenesis of osteoporosis: the process leading to postmenopausal osteoporosis removes *entire trabecular elements.* Furthermore, loss of trabecular elements reduces trabecular connectivity, thereby producing bone fragility that is out of proportion to the reduction in mass.

Bone Biopsy in Patient Care

As noted earlier, most patients with osteoporosis can be managed without bone biopsy. However transilial bone biopsy is useful in the clinical setting when there is uncertainty about the nature of a patient's bone disease. A variety of rare metabolic bone diseases are diagnosed without bone biopsy, that is, by clinical, X-ray, and laboratory examinations. However, these diseases have important bone histomorphometric abnormalities, and a bone biopsy before and after starting treatment can be necessary to judge the adequacy of, or adherence to, a treatment intervention. Table II lists situations in which transilial bone biopsy *with tetracycline labeling* can aid in clinical decision making.

Table II Some Clinical Indications for Transilial Bone Biopsy

1. When there is excessive skeletal fragility in unusual circumstances (e.g., the patient younger than age 50)
2. When a mineralizing defect is suspected (e.g., due to occult osteomalacia or treatment with anticonvulsant drugs)
3. To evaluate adherence to treatment in a malabsorption syndrome (e.g., sprue)
4. To characterize the bone lesion in renal osteodystrophy
5. To diagnose and assess response to treatment in vitamin D-resistant osteomalacia and similar disorders
6. When a rare metabolic bone disease is suspected

Bone Biopsy in Clinical Research

Mechanisms, Pathogenesis, and Response to Treatment

The biopsy can give important clues to the pathogenesis of a metabolic bone disease, and the mechanism of a treatment intervention. Further, the information can be obtained rather quickly, at least quickly in the context of clinical bone research. For example, in the first year of a treatment that increases bone mass, the biopsy may be able to distinguish between a mechanism that reduces remodeling and one that is truly anabolic. The increase in bone mass might be similar in both cases. An agent that reduces the activation of new remodeling sites, while leaving the rest of the remodeling IO unaffected, will increase bone mass, sometimes to a striking degree (Heaney, 1994). This occurs by filling part or all of the remodeling space, with increases in bone mass that level off after about the first year or so. On the other hand, a truly *anabolic* agent would increase trabecular thickness, increase connectivity, and/or produce positive bone balance at the BMU. Such an anabolic effect might well continue for a longer period of time, result in a greater net increase in bone mass, and be more effective in preventing fragility fractures.

Prediction of Long-Term Safety

Prediction of long-term safety is perhaps the most valuable information that can be obtained from the bone biopsy in clinical trials. For example, an agent that stops the activation of remodeling completely would be worrisome for long-term safety in the treatment of osteoporosis. In the complete absence of remodeling, one would expect a reduction in the risk of fracture in the first few years of treatment. After several years, as unrepaired microdamage accumulates, the risk would actually increase even though bone mass remained stable. If transilial bone biopsy is part of the research protocol, this problem can be detected in time to adjust the dose or stop treatment.

The biopsy can also be used to identify abnormal bone formation, such as woven bone or osteitis fibrosa, before harm can occur. The histomorphometric evaluation of bone biopsies is particularly important in studying the effects of anabolic agents, where new bone must be normal in histologic appearance to be mechanically useful.

Practical Considerations, Training, Availability, etc.

Few physicians have been trained in the transilial biopsy technique, and few laboratories have the expertise and capacity to process and examine the number of specimens required for large clinical trials. However, not every patient in a large multicenter trial needs to undergo biopsy in order to obtain enough information to understand the mechanism of action or long-term safety of a new drug. Principal investigators or their physician associates can be trained in the biopsy procedure, and central laboratories can process and examine the specimens. Thus, the practical constraints of resources can be overcome to meet the needs of clinical treatment trials.

Timing of the Biopsy

A given patient can undergo no more than two biopsies, one on either side, without danger of confounding effects remaining from a previous biopsy. Thus, the research questions to be answered should be planned around a maximum of two biopsies, preferably one prior to an intervention, followed by another at least 1 year later. The first biopsy can be used to estimate the time required for one remodeling cycle. This is equivalent to the skeletal transient, the time required to reach a new steady state. A second biopsy can be staggered among study patients, at 1 year in some and at longer intervals in others. This requires sufficient numbers of subjects at each time point in order to detect treatment effects. Another strategy is to omit the baseline biopsy, and perform biopsies at time intervals during treatment, comparing controls with those treated. This approach generally requires a larger sample size.

Other strategies have been suggested that are not satisfactory. For example, one might consider multiple periodic labelings at time intervals over several years, followed by a single biopsy. The problem with this is that a profusion of labels appears in microscopic sections. It is impossible to match labels seen on the sections with the times they were given. Little or no useful dynamic information can be obtained under these circumstances.

Another strategy is to give continuous label. Any forming site that was active during administration of the label will contain a broad fluorescent label. The problem with this is that one cannot determine when formation stopped or started at any given forming site, and thus one cannot calculate MAR. Again, little or no useful dynamic information can be obtained under these circumstances.

Conclusion

While transilial bone biopsy has limited value in managing patients in clinical practice, there are a few instances where it can be helpful in making clinical decisions. On the other hand, the biopsy is indispensable in clinical bone research for studying the pathogenesis of bone diseases and the tissue-level mechanisms of treatment effects. It is particularly useful in evaluating and predicting long-term safety of treatment interventions. Finally, it is indispensable for evaluating whether new bone formed by a treatment intervention is histologically normal and likely to be mechanically sound.

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CHAPTER 95

Animal Models in Osteoporosis Research

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Introduction

The principal use for animal models of osteoporosis is to provide efficient experimental environments in which bone cells function within their intermediary organizations of modeling and remodeling. This chapter first summarizes important features of human osteoporoses detailed elsewhere in this volume. It then evaluates how a series of animal models provides *in vivo* skeletal behaviors that not only reflect tissue level organizations in humans, but also can be used to improve understanding of the pathogenesis, prevention, and treatment of osteoporosis.

Regulatory Recommendations for Animal Models of Osteoporosis

The most prevalent type of osteoporosis occurs in postmenopausal women. Because of the firm relationship of postmenopausal osteoporosis to estrogen deficiency, the United States Food and Drug Administration (USFDA) guidelines for preclinical testing of anti-osteoporosis agents recommend using animals either losing bone or that have already become osteopenic following ovariectomy (OVX) (USFDA, 1994; Thompson *et al.,* 1995). Before the existence of drugs proven to reduce osteoporotic fracture incidence in humans, the USFDA required data from one small and one large animal species. The guidelines suggest measuring endpoints used in human trials, such as: (a) bone density by densitometry, (b) biochemical markers of bone turnover, (c) bone strength by biomechanical testing (fracture surrogate), and (d) mineralization defect and turnover assessment by histomorphometry.

During the past decade, the de facto requirements for anti-osteoporosis drugs have evolved in several ways. First, because all current osteoporosis treatment drugs reduce fracture in humans, new anti-osteoporosis drugs need to prove anti-fracture efficacy in humans to compete in the marketplace. Experiments in large animals will likely not serve as a substitute for, or informative predecessor to, fracture data in humans. Second, existing guidelines are based principally on testing specific anti-resorptives that treat osteoporosis by slowing bone turnover and correcting the imbalance of bone resorption and formation (e.g., bisphosphonates, estrogen and selective estrogen receptor modulators [SERMs], and calcitonin). Current guidelines thus focus on detecting modest changes in bone mass over long periods of time. When agents that stimulate bone formation are considered, shorter experiments may suffice, due to more rapid changes in measurable endpoints. Improved serum, tissue, and urine bone turnover markers in animals may also shorten the required experimental durations.

A Perspective on *In Vivo* Experimentation

All animal models attempt to bridge the gap between molecular and cell level characterizations of diseases and agents and *in vivo* adult human trials that measure outcomes in afflicted adult humans. Past *in vivo* animal investigations of osteoporosis are a textbook example of how animal models are used to test agents and concepts that improve the understanding and control of human disease. Animal models are the lowest level at which the skeletal tissue level intermediary organizations of modeling and remodeling that govern the output of bone cells and the attendant skeletal

structural adaptations in adult humans can be examined (Frost, 1983).

The principal reason for the success of osteoporosis animal models is that the tissue level features of human osteoporosis are understood adequately to allow recognition of animals with skeletons that satisfactorily mimick those tissue level behaviors. Because of the variety of investigative techniques that can be applied and the extreme time-frame compression, animal studies are the most appropriate way to understand tissue-level mechanisms of drug actions. The combined results from recent Phase III and postapproval trials whose outcomes were accurately predicted by preclinical trials directly document the appropriateness of these animal models. The ongoing examination of human data continues to yield new understanding of human osteoporosis that will lead to further improvements in the animal models (Cummings *et al.,* 1995; Elffors *et al.,* 1994; Blunt *et al.,* 1994; Garnero *et al.,* 1996; Ross *et al.,* 1993).

The most certain way to learn the effects of a safe agent on the human skeleton is to study outcomes in an appropriately selected human population. However, when putative osteoporosis treatment agents need proof-of-concept or early phase efficacy testing prior to safety assessment, *in vivo* animal experimentation is the best choice. In addition, when questions about the effects of agents on turnover and cellular activity in humans in sites prone to osteoporotic fracture (e.g., vertebrae, hip, and wrist) arise, human experiments cannot generate suitable samples. Despite improvements in noninvasive imaging in humans (Link *et al.,* 1999), an appropriate *in vivo* study remains the best approach. The failure of early animal models to predict sodium fluoride effects in humans (Kleerekoper *et al.,* 1991; Riggs *et al.,* 1990, 1994) and failure of fracture reduction to follow bone mineral density (BMD) changes within early reports about etidronate (Chapuy and Meunier, 1995) led the USFDA to require that new classes of anti-osteoporosis agents demonstrate *both* BMD increases and anti-fracture efficacy in clinical trials. Now it is the marketplace that requires such performance.

Criteria for Animal Models in Osteoporosis

Full parallelism of *in vivo* animal models with human symptoms rarely exists. The criteria are flexible, creative, and open to revision as new evidence or needs appear. They place the highest value on animal models that match the tissue level behavior of human osteoporosis, allowing the detailed match of mechanisms to be provided by molecular- and/or enzyme-level characterization.

All bone researchers should participate in optimizing their end points, duration, and physiologic relevance. Clinical investigators can help refine *in vivo* animal models both by revealing new behaviors of the human osteoporotic skeleton, and ranking the importance of various tissue-level end points studied in human osteoporosis. *In vivo* animal investigators can help by developing end points that make the models more closely fit clinical needs. Skeletal research in the ovariectomized (OVX) rat is a critical achievement by bench scientists validating a highly relevant preclinical model (Wronski *et al.,* 1988a,b, 1989a) that led to efficacious treatments for human osteoporosis. That effort, completed in the late 1980s, was made possible by the dynamic characterization of human bone remodeling of the 1960s (Frost, 1969) and the bone mass and metabolic data collected about human osteoporosis during the 1970s (Recker *et al.,* 1978; Nordin *et al.,* 1978; Jensen *et al.,* 1982).

In vivo animal scientists have also done well with model refinement since that time. The introduction of standardized tests of bone strength as surrogates for osteoporotic fracture in the early 1990s is an example of targeted research that further refined the preclinical models (Mosekilde *et al.,* 1991; Toolan *et al.,* 1992; Kasra and Grynpas, 1994). New imaging techniques may further reduce the length of animal experiments by providing extremely precise measurement methods that focus on regions of bone showing the greatest post-OVX change (Kapadia *et al.,* 1998; Kinney *et al.,* 1995; Breen *et al.,* 1998). Relevant physiologic conditions studied in a complete manner should continue to be the hallmark of *in vivo* animal studies. For example, when the goal is to study an *adult* disease process, choosing a growing animal, simply to see quicker changes, makes little sense. Contributions from growth cartilage activity will certainly be absent from the adult human skeleton. Nerve resection experiments (Wakley *et al.,* 1988) best model the motionless, *denervated* limb, not the motionless limb. Combining extreme calcium deprivation with estrogen deficiency to accelerate (or even simply create) bone loss, produces a confused situation that bears limited resemblance to the clinical picture (Geusens *et al.,* 1991; Jerome *et al.,* 1995; Mosekilde *et al.,* 1993b). Though *in vivo* measurements of the animal skeleton are now routinely possible (Ammann *et al.,* 1992; Black *et al.,* 1989), the data often seem incomplete without quantitative histologic characterization of the tissue level events accompanying any bone mass and biochemical changes.

Summary

Using relevant, physiologic methods in an adult animal to produce a consistent, albeit incomplete set of symptoms, is the correct choice during early phases of osteoporosis model development. Additional experimental time, new end points, and more precise analysis tools are clearly preferred during model refinement to introducing irrelevant physiology. Models that give sporadic results across laboratories or require convoluted manipulations do not gain acceptance. Furthermore, today's understanding of animal models in osteoporosis research makes using an irrelevant animal model inexcusable and wasteful. Considering the general regulatory and practical requirement for human anti-fracture efficacy of new osteoporosis treatment agents, the need for studies of a relevant, costly model for terminal studies should be carefully considered. The continuous evaluation of animal models for relevance, need, and cost-effectiveness benefits all.

Postmenopausal Osteoporosis

In vivo animal models for the skeleton have been reviewed elsewhere (Rodgers *et al.,* 1993; Lerome, 1998; Bellino, 2000; Bonjour *et al.,* 1999; Charassieux, 1990; Grynpas *et al.,* 2000; Srinivasan *et al.,* 2000; Hartke, 1999; Grier *et al.,* 1996; Mosekilde, 1995; Newman *et al.,* 1995; Barlet *et al.,* 1994; Miller *et al.,* 1995; Cesnjaj *et al.,* 1991). This chapter not only emphasizes many of their important points, but also widens the perspective by encompassing additional animal models and secondary osteoporoses. Its fundamental approach is to identify animal models that match tissue behaviors that can be assessed *in vivo* in osteoporotic humans.

GROWTH AND ADULT PHASES

An *in vivo* animal model of osteoporosis should exhibit both growing and adult skeletal phases of meaningful duration. Low peak bone mass is an established factor in osteoporosis etiology (Lofman *et al.,* 2000; Baylink, 2000). Its importance is clear because a single BMD measurement at menopause is the best quantitative predictor of future fracture in healthy persons (Hui *et al.,* 1989; Gärdsell *et al.,* 1989a; Ross *et al.,* 1988). That value approximates peak bone mass, because premenopausal bone mass changes are minor (Recker *et al.,* 1992; Rodin *et al.,* 1990).

Growth processes, principally bone modeling under the influence of nutrition, physical activity, and heredity (Johnston *et al.,* 1992; Jones *et al.,* 1977; Liel *et al.,* 1988; Smith *et al.,* 1973; Eisman, 1999; Ralston, 1999; Econs, 2000; Rubin *et al.,* 2000), determine peak bone mass. Adult skeletal processes, predominantly bone remodeling, but some modeling to effect shape changes in response to changing physical activity patterns, determine bone quantity after peak bone mass attainment (Frost, 1983). Accurate animal models of osteoporosis should have both growth and adult phases of sufficient duration to permit useful studies.

ROLE OF ESTROGEN

Menstrual/Estrus Cyclicity Humans not only have a menarche and regular, frequent ovulatory cycles, but also experience bone loss at cessation of ovarian function. The positive linkage of regular menses to bone mass is reinforced by amenorrheic individuals (Drinkwater *et al.,* 1984; Marcus *et al.,* 1985; Klibanski and Green Span, 1986; Dawood *et al.,* 1989) and the bone accumulation that occurs upon resumption of normal menses (Klibanski and Greenspan, 1986; Dawood *et al.,* 1989; Drinkwater *et al.,* 1986). Mammals with regular, frequent ovulatory cycles seem to experience estrogen-deficiency bone loss routinely. Some hold that regularly cycling female mammals integrate an estrogen-related component of bone into the skeleton at puberty (Garn, 1970; Gilsanz *et al.,* 1988) that is lost at menopause (Frost, 1987). Thus, animals with frequent cycles might tend to have the most estrogen deficiency bone loss.

Natural Menopause Women undergo a natural menopause of 2–7 years duration (Treloar, 1981). Only 25–30% of women experience surgical menopause, most with preserved ovarian function or prompt estrogen replacement (McMahon and Worcester, 1966). Though, aside from rate of bone loss, no differences in bone behavior between surgical and natural menopause are known (Cann *et al.,* 1980; Hartwell *et al.,* 1990), naturally postmenopausal women retain widely variant levels of circulating 17-β-estradiol long after their last period (Cummings *et al.,* 1998b; Langcope *et al.,* 1981, 1984; Canley *et al.,* 1999), which affect their fracture and breast cancer risk.

Bone Loss and Rise in Turnover Rate after Estrogen Deficiency Following estrogen deficiency, bone loss accelerates transiently in multiple sites and then decelerates and enters a semiplateau phase (Nordin *et al.,* 1976; Horsman *et al.,* 1977). The loss is most pronounced in cancellous regions and at endocortical surfaces (Nilas *et al.,* 1984; Keshawarz and Recker, 1984). Estrogen status plays a more important role in determining bone quantity in the adult woman than does age (Richelson *et al.,* 1984; Nilas and Christiansen, 1987). Estrogen-deficiency effects on Haversian remodeling in humans are poorly documented. The cancellous and endocortical bone loss is accompanied by kinetically and histomorphometrically increased bone turnover (Recker *et al.,* 1978; Stepan *et al.,* 1987; Coble *et al.,* 1994) and a marked, transient negative calcium balance (Heaney *et al.,* 1978). In an accurate animal model of postmenopausal osteoporosis, these behaviors should be easily monitored by similar methods and end points to those used in humans.

Skeletal Response to Estrogen Replacement Oophorectomized or menopausal women given prompt estrogen replacement experience a smaller rise in turnover (Recker *et al.,* 1978; Steiniche *et al.,* 1989), less bone loss (Jensen *et al.,* 1982; Wasnich *et al.,* 1983; Christiansen and Lindsay, 1990; Christiansen *et al.,* 1980; Ettinger *et al.,* 1985), and fewer fractures (Jensen *et al.,* 1982; Ettinger *et al.,* 1985; Lindsay *et al.,* 1976) than those who receive no estrogen replacement (Christiansen and Lindsay, 1990). This response is demonstrated well by histomorphometric techniques in humans and animals (Wronski *et al.,* 1988b; Coble *et al.,* 1994; Steiniche *et al.,* 1989; Turner *et al.,* 1987). This response is so certain in adult women that an accurate OVX animal model must have an identical response to estrogen replacement.

SKELETAL RESPONSE TO OSTEOPOROSIS TREATMENT AGENTS

The tissue level effects of agents proven to reduce osteoporotic fracture incidence in humans are known. These agents include alendronate (Cummings *et al.,* 1998a; Harris *et al.,* 1993; Liberman *et al.,* 1995), risedronate (Wallach *et al.,* 2000; Eastell *et al.,* 2000), and raloxifene (Ettinger *et al.,* 1999; Meunier *et al.,* 1999). Accurate animal models should yield data predictive of these agents' effects in humans.

Development of Osteoporotic Fractures and Steady-State Osteopenia

Functionally, human osteoporosis is marked by low trauma fractures of the spine and hip (Kanis and McCloskey, 1992; Cummings *et al.,* 1985). An animal model that developed estrogen deficiency fragility fractures could facilitate preclinical anti-fracture efficacy trials. Despite the existence of excellent animal models for detecting estrogen deficiency bone loss, none exhibit low trauma fragility fracture. The lack of fracture in the preclinical models may speak to the importance of low peak bone mass, falls (Tinetti *et al.,* 1994; Gärdsell *et al.,* 1989b), and extended observation periods (often ~5000 patient-years), as factors that contribute positively to the ability to use osteoporotic fracture as an end point in human studies. For example, the one animal model with low peak bone mass, the SAM/P6 mouse, has fragility fractures without estrogen deficiency (Matsushita *et al.,* 1986). Falls and long observation periods are difficult to implement in animal models.

Bone Loss and Decreased Formation after Decreased Mechanical Usage

Older humans experience both loss of cancellous and cortical bone and declines in bone formation unrelated to estrogen deficiency (Schaadt and Bohr, 1988; Riggs and Melton, 1983). These changes come during a life phase when a generalized decline in physical activity also occurs. While extreme physical inactivity, as during bedrest or paraplegia, causes marked bone loss (Donaldson *et al.,* 1970; Minaire *et al.,* 1974; Krolner and Toft, 1983), the impact of long-term, mild inactivity is neither well-understood nor easily assessed. Disuse osteopenia should also be easily demonstrable in an accurate animal model of osteoporosis, but is not crucial to its success.

Remodeling

Remodeling is the principal bone cellular process that influences bone quantity and strength in the adult skeleton (Parfitt, 1983). Osteoclasts, osteoblasts, and their progenitors function in sequence to first remove aged bone tissue and soon after replace it *in situ* with an approximately equal quantity of new bone tissue. Remodeling rate is quantitated in humans by both biomarkers of resorption and formation (Calvo *et al.,* 1996; Garnero *et al.,* 1994; Eyre, 1997; Garnero and Delmas, 1999) and histomorphometric methods in non decalcified sections of iliac crest biopsy specimens obtained after *in vivo* labeling with bone seeking fluorochromes (Parfitt, 1983). Remodeling activity in humans is important in a practical sense, as high remodeling rate tends to be a risk factor for osteoporotic fracture (Garnero *et al.,* 1996; VanDaele *et al.,* 1996). Most current agents that decrease osteoporotic fracture incidence also decrease remodeling rate (Cummings *et al.,* 1998a; Recker *et al.,* 1978, 1996; Steiniche *et al.,* 1989; Harris *et al.,* 1993; Liberman *et al.,* 1995; Wallach *et al.,* 2000; Eastell *et al.,* 2000; Ettinger *et al.,* 1999; Meunier *et al.,* 1999).

Adult humans have both cancellous and Haversian bone remodeling (Frost, 1983). Cancellous and the contiguous endocortical surfaces display relatively high remodeling rates, likely accounting for most remodeling rate variation in the skeleton. While cancellous remodeling plays an important role in renewing bone in the spine, wrist, and greater trochanter, Haversian remodeling is probably more important in the femoral neck. Remodeling rates among cancellous sites are likely to vary by an order of magnitude (Kimmel and Lee, 1982). Though the extent to which estrogen deficiency raises Haversian remodeling in humans has never been defined in quantitative histologic studies, cortical porosity is not a prominent feature of postmenopausal osteoporosis.

In theory, an accurate animal model should display considerable steady-state Haversian remodeling, because of its importance to the maintenance of cortical bone strength. It would be ideal if Haversian remodeling and cortical bone quantity changes caused by agents being considered for osteoporosis treatment were revealed first in animal studies. However, from a practical standpoint, Phase IV followup in humans remains the optimal way to assure long-term cortical bone safety of osteoporosis treatments. Thus, animal models that allow assessing cancellous bone remodeling and bone strength status provide most of the important information available from preclinical studies. Furthermore, it is now likely that anti-fracture efficacy results in Phase III trials will supersede cortical bone data from any preclinical large animal study.

Convenience

Convenience for animal models is denominated as time frame compression, purchase cost, availability, housing requirements, handling difficulties, and the necessity for designing/implementing/validating new analysis procedures.

In adult, estrogen-deplete women, the phase of accelerated estrogen-deficiency bone loss lasts 5–8 years. The time from attainment of peak bone mass until the development of fragility fractures is 30 or more years. An effective animal model known to experience peak bone mass followed by post-OVX bone loss should compress both times by an order of magnitude or more.

Using an animal model with full accuracy can be more difficult than doing a human study. For example, if an intervention requires active subject cooperation, animals may be unable to comply. On the other hand, in animal experiments, clinical research problems like recruitment, lost sampling units, and compliance to pharmaceutical regimens, are generally nonissues. Animals convenient for some researchers because of specialized facilities and expertise, are a poor choice for others lacking those tools. Investigators relying on outdated methods (e.g., whole bone ashing, simple radiography, etc.) will most certainly miss important findings in the name of "convenience."

Glucocorticoid Osteoporosis

The tissue level features of human glucocorticoid osteoporosis are cancellous osteopenia with vertebral and rib fractures (Need, 1987; Reid, 1989, 1997; Lukert and Raisz, 1990). The osteopenia is characterized by a normal number of thinned trabeculae (Bressot *et al.,* 1979; Chappard *et al.,* 1996; Aaron *et al.,* 1989). It occurs most frequently in postmenopausal women and in older persons with underlying gonadal hormone insufficiency (Reid, 1997; Reid *et al.,* 1985), during which both resorption and formation are elevated, though the elevation in resorption is greater (Heaney *et al.,* 1978; Dempster, 1989). Secondary hyperparathyroidism due to decreased intestinal calcium absorption is observed with both bone biomarkers and iliac crest biopsy (Dempster, 1989; Hahn *et al.,* 1979; Klein *et al.,* 1977; Suzuki *et al.,* 1983). Even mild doses of prednisone suppress bone formation. The ongoing rate of bone loss matches or exceeds that in women just after menopause (Heaney *et al.,* 1978; Laan *et al.,* 1993).

Male Osteoporosis

The clinical features, the treatment, and the tissue level mechanisms behind postmenopausal osteoporosis are well-known. Though its molecular mechanisms have not been conclusively identified, the tissue level pathogenesis is reasonably well characterized. Despite the fact that one-third of hip fractures occur in men, male osteoporosis has been less well-studied (Orwoll and Klein, 1995; Swartz and Young, 1988; Ebeling, 1998; Poor *et al.,* 1995; Jones *et al.,* 1994). Probably because of their higher peak BMD, men at comparable ages have a lower fragility fracture incidence than women, making prospective studies of male osteoporotic fracture logistically challenging (Blunt *et al.,* 1994; Jones *et al.,* 1994). Furthermore, gonadal insufficiency is rarely induced surgically and occurs naturally over a more protracted period than female menopause, making detection of its bone consequences more difficult. However, the negative effects of aging and hypogonadism on the adult male skeleton are well-documented (Francis *et al.,* 1986; Murphy *et al.,* 1993).

Animal Models Evaluation

Postmenopausal Osteoporosis

Animals that should receive initial consideration are bird, mouse, rat, rabbit, dog, pig, sheep, and nonhuman primate species. Because the above criteria are directed at tissue-level outcomes in humans, the animal models will be evaluated as to how they duplicate those outcomes. Dissimilarities from the human condition in molecular mechanisms of development of one or more conditions may well exist. The fit of each animal model to the criteria above is summarized in Table I.

Avian

Birds have time-compressed growing and adult skeletal phases when compared to humans. Adult female birds have daily egg-laying cycles that correspond, respectively, to

Table I Summary of *in Vivo* Animal Models for Postmenopausal Osteoporosis

Attribute	Human	Avian	Mouse	Rat	Dog	Pig	Sheep	Primate
Growth/adult phases	Yes	OK	OK	OK	Yes	Yes	Yes	Yes
Role of estrogen								
Cyclicity	28d	Daily	Inducible	4–5d	205d	21d	21d[c]	21–28d
Natural menopause	Yes	No	Yes[a]	Yes[a]	No	?	?	Yes
Estrogen deficiency turnover ↑ and bone loss	Yes	?	Yes	Yes	Not reliable	Poor	Fair	Yes
Estrogen response	Turnover ↓	Formation ↑	Turnover ↓ [d]	Turnover ↓	Not reliable	?	?	Turnover ↓
Response to proven anti-osteoporotic agents	Yes	?	Some	Yes	No	?	Some	Some
Development of osteoporotic fractures	Yes	No	No	No[b]	No[b]	?	?	No[b]
Remodeling								
Cancellous	Yes	No	No	Yes	Yes	Yes	Yes	Yes
Haversian	Yes[c]	No	No	Low levels; inducible	Yes	Yes	Yes	Yes
Timeframe compression	No	N/A	Yes	Yes	N/A	Some	Some	Some
Convenience	OK[e]	Yes	Yes	Yes	Weak	Poor	Fair	[e]

[a]perpetual diestrus
[b]biomechanical tests as surrogate
[c]anatomic study site difficult
[d]at low doses; formation rises with high doses
[e]faculty dependent

formation and resorption of medullary bone during cyclic oviposition and egg calcification (Bloom *et al.,* 1941). Estrogen status plays a major role in determining medullary bone mass (Wilson and Thorp, 1998). The bone accumulation phase occurs with rising serum 17-β-estradiol, while the removal phase accompanies falling 17-β-estradiol. Estradiol treatment of male birds also stimulates medullary bone formation (Wilson and Thorp, 1998; Miller and Bouman, 1981). Tamoxifen blocks the cyclic formation of medullary bone and the associated loss of cancellous bone, possibly through an indirect action (Wilson and Thorp, 1998).

The bird skeleton experiences localized bone loss during immobilization and increased bone mass during applied mechanical loads (Rubin and Lanyon, 1984, 1985; Matsuda *et al.,* 1986). Though avian models have helped in understanding bone cell origin (Kahn and Simmons, 1975; Jotereau and Ledouarin, 1978), bone loss during immobilization, and bone responses to increased loading (Rubin and Lanyon, 1984, 1985; Matsuda *et al.,* 1986), they are not accurate for studying tissue level aspects of the estrogen/fracture-centered disease of osteoporosis. Furthermore, current data suggest that birds have little cancellous or Haversian remodeling.

The bone response to estrogen in birds provides many opportunities for experiments that bear on bone biology (Miller, 1977), but the estrogen-related bone buildup suggests a fundamental dissimilarity to adult mammalian physiology. While avian hypoestrogenemia is associated with medullary bone *loss,* just as estrogen deficiency in mammals is associated with osteopenia, the course of bone mass following OVX is unknown. The estrogen-related bone accumulation may prove helpful in understanding peak bone mass accumulation in pubertal humans (Garn, 1970; Gilsanz *et al.,* 1988; Attie *et al.,* 1990; Bonjour *et al.,* 1991), but seems likely to hinder the proper interpretation of experiments about osteoporosis.

Avians are convenient, but, at this time, irrelevant for tissue level osteoporosis research, because their skeletal behavior does not mimick tissue-level features associated with adult human osteoporosis.

Mouse

Past success with mice in physiologic aspects of skeletal research is encouraging. Recently, strain-specific post-OVX bone loss and effects of estrogen replacement have begun to appear. It is excellent as a model for osteopetrosis (Walker, 1972; Barnes *et al.,* 1975; Marks and Lane 1976; Felix *et al.,* 1990), osteoclast and stromal cell ontogeny (Lennon and Micklem, 1986), and cytokine and marrow studies (Kyoizums *et al.,* 1992; Most *et al.,* 1995; Passeri *et al.,* 1993; Liu *et al.,* 2000; Masuzawa *et al.,* 1994; Nicola *et al.,* 1985; Takahashi *et al.,* 1994; Jilka *et al.,* 1992; Miyaura *et al.,* 1997; Simonet *et al.,* 1997; Hankenson *et al.,* 2000). The mouse has become even more popular for the ease with which its genome can be systematically manipulated (Cassella *et al.,* 1994; Yoshitake *et al.,* 1999; Lorenzo *et al.,* 1998; Ammann *et al.,* 1997; Ducy *et al.,* 1996; Korach *et al.,* 1996; Windahl *et al.,* 1999; Masuda *et al.,* 1997; Poli *et al.,* 1994). Mice have been used to identify and characterize genes responsible for osteopetrosis (Marks and Lane, 1976; Felix *et al.,* 1990). Mice with respectively low and high peak bone mass (Beamer *et al.,* 1996) lend themselves to genetic investigations using F2 cross and F1 backcross studies of extremes of a continuous phenotype (Lander and Botstein, 1989).

Data validating the mouse as an *in vivo* model for tissue-level osteoporosis research now exist. They suggest that, much like in the rat, cancellous (Jilka *et al.,* 1992; Yoshitake *et al.,* 1999; Lorenzo *et al.,* 1998; Ammann *et al.,* 1997; Masuda *et al.,* 1997; Poli *et al.,* 1994; Broulik, 1991; Onoe *et al.,* 2000; Ishimi *et al.,* 2000; Yamamoto *et al.,* 1998), but not cortical (Edwards *et al.,* 1992), bone loss occurs soon after OVX in popular strains like Swiss-Webster and C57BL. Genetically hypogonadal female mice are osteopenic (Smithson *et al.,* 1994). Estrogen-deficiency bone loss appears to be prevented by 17-β-estradiol (Jilka *et al.,* 1992). Ectopic bone ossicles are preserved by 17-β-estradiol (Hashimoto *et al.,* 1991). Mice also experience age-related cancellous and cortical bone loss in the vertebrae (Bar-Shira-Maymon *et al.,* 1989a,b) and femur (Weiss *et al.,* 1991) during the second year of life.

Estrogen administration increases bone formation with woven bone deposition through an estrogen receptor-mediated mechanism (Edwards *et al.,* 1992; Urist *et al.,* 1950; Liu and Howard, 1991; Bain *et al.,* 1993). However, estrogen-stimulated bone formation in mice occurs at 17-β-estradiol doses above 0.05 mg/kg/day subcutaneously (sc), about an order of magnitude above that which prevents bone loss in the OVX rat. When 17-β-estradiol doses $\sim$0.01 mg/kg/-day 2–3X/week sc have been used in the OVX mouse (Jilka *et al.,* 1992), estrogen replacement has effects like in the rat and human, reducing turnover and stopping bone loss. Additional work at these lower doses of 17-β-estradiol and $\sim$0.5 mpk oral 17-α-ethinyl estradiol is needed to confirm this important parallel of the mouse with rats, nonhuman primates, and adult humans.

Validation of the mouse as a model for tissue level osteoporosis seen in humans needs some targeted experimental work to document the appropriateness of its skeletal response to estrogen. Applications for which rats are not suitable, principally studies of transgenic and knockout animals, are now rapidly driving mouse skeletal experiments. The similar life span of mice to rats and the similar cost for experimentation, suggests that unique applications of the mouse will induce many investigators to choose the mouse in the future. Some mouse strains, including C57BL/6J used to make knockout and transgenic mice, appear to develop estrogen-deficiency bone loss that is prevented by low levels of estrogen replacement. The time course and site specificity for development of estrogen-deficiency osteopenia should be established in representative mouse strains, as it has been for the rat (Wronski *et al.,* 1988a, 1989a). The bone formation response at endocortical surfaces to estrogen seems to be a high dose phenomenon. However, its existence may make studies of bone formation responses in the mouse

questionable, since this formation pathway does not appear to exist in larger animals. Experiments with transgenic and knockout mice are the most likely method to find the molecular pathways by which estrogen influences tissue level behavior of the human skeleton.

Specimens adequate for histomorphometric study of cancellous bone mass, structure, and turnover in mice can best be obtained from the distal femoral metaphysis. Mouse bones contain such small amounts of mineral that conventional dual X-ray absorptiometry (DXA) lacks the necessary precision. Equipment like peripheral quantitative computed tomography (pQCT) is useful (Beamer *et al.,* 1996; Ferretti *et al.,* 1994), but provides a less direct link to human-applicable densitometry measurements than DXA. Specialized (slower speed, smaller collimator) peripheral DXA instruments or μCT (Kapadia *et al.,* 1998; Kinney *et al.,* 1995; Breen *et al.,* 1998) with high resolution and software modifications may hold an answer.

The SAM Mouse

The SAM/P6 (senescence accelerated mouse) mouse not only has low peak bone mass, but also develops fractures in middle and old age (Takahashi *et al.,* 1994; Okamoto *et al.,* 1995; Breen *et al.,* 1998). It is the *only* experimental animal with documented low peak bone mass and fragility fractures of aging. The SAM mouse needs full genetic (Tsuboyama *et al.,* 1993; Benes *et al.,* 2000), hormonal (Takahashi *et al.,* 1994), and biomechanical characterization, including site specificity for fractures. If it does not have collagen defects like those in osteogenesis imperfecta (Cassella *et al.,* 1994; Spotila *et al.,* 1991), it may, when combined with OVX and standard osteopenic prevention approaches like estrogen replacement or bisphosphonates, provide a model for studying the role of low peak bone mass in causing late-life fractures. It may also provide an opportunity to identify genes contributing to peak bone mass.

Rat

The rat has a long history of providing data relevant to the adult human skeleton. It gave the first evidence that osteoclasts ingest bone substance (Arnold and Lee, 1957) and early evidence about the hematogenous origin of osteoclasts (Gothlin and Ericsson, 1973; Andersen and Matthiessen, 1966; Marks and Schneider, 1978). The rat was once held unsuitable as an adult human skeletal model because many epiphyseal growth cartilages in *male* rats remain open past age 30 months (Dawson, 1925). However, bone elongation ceases and effective epiphyseal closure ensues at important sampling sites in *female* rats by age 6–9 months (Acheson *et al.,* 1959; Joss *et al.,* 1963; Spark and Dawson, 1928; Turner *et al.,* 1989; Kimmeh, 1992a). Periosteal expansion continues until about age 10 months in female rat, its age of peak bone mass (Schapira *et al.,* 1992; Li *et al.,* 1992). The healthy life span for intact female rats is 21–24 months. Thus the female rat has an appreciable life span both before and after attainment of peak bone mass. The lengthy bone accumulation period in the female rat presents ample opportunity to study both internal and external determinants of peak bone mass. Even the most vocal critics of the rat as a model of adult human skeletal disease because of its "continuous growth and lack of remodeling" now acknowledge both the inaccuracy of this statement and the presence of cancellous remodeling. They only caution to use female rats of at least 6–9 months age and avoid studying Haversian remodeling (Frost and Lee, 1992).

Adult female rats have a regular estrus cycle during which estrogen levels spike for 18 hr every 4 days (Butcher *et al.,* 1974). During ages 12–24 months, the fraction of rats found in constant diestrus rises gradually (Lu *et al.,* 1979), and cancellous bone loss is frequently observed (Schapira *et al.,* 1992). While this is not true menopause, peaks in estrogen cease as bone loss occurs, making a link of rat "menopause" to cancellous bone loss possible.

Following OVX, loss of cancellous bone mass and strength occurs and then decelerates in a site-specific fashion after 3–4 months, to enter a plateau phase (Wronski *et al.,* 1988a, 1989a,b; Kalu, 1991; Wronski, 1992; Kimmel and Wronski, 1990; Mosekilde *et al.,* 1993a). This bone loss is accompanied by an increase in bone turnover rate (Wronski *et al.,* 1986, 1988a; Black *et al.,* 1989). These events mimick well the bone changes that accompany estrogen deficiency in humans. Not all cancellous bone sites in the rat experience such bone loss (Ito *et al.,* 1993; Li *et al.,* 1996a), further tightening the parallel of the rat and human skeletons, since osteoporotic fragility fractures and osteopenia are limited to a few sites in humans (Wasnich *et al.,* 1983; Cummings, 1991). DXA, the most widely applied method for measuring bone mass in humans, works well in rats (Ammann *et al.,* 1992; Kimmel and Wronski, 1990), providing the best precision on excised specimens obtained at necropsy.

OVX rats given prompt estrogen replacement experience no rise in turnover (Wronski *et al.,* 1988b; Turner *et al.,* 1987; Kalu *et al.,* 1991) and no bone loss (Wronski *et al.,* 1988b; Garner *et al.,* 1992). Agents like bisphosphonates (Toolan *et al.,* 1982; Seedor *et al.,* 1991), SERMs (Evans *et al.,* 1994, 1996), and calcitonin (Nakatsuka *et al.,* 1990; Mazzuoli *et al.,* 1990) also block the rise in turnover and bone loss in both rats and humans (MacIntyre *et al.,* 1988; Szucs *et al.,* 1992). Rats exhibit no dose-related bone formation response to 17-β-estradiol.

The rat, like all other animal models of osteoporosis, has no fragility fractures associated with estrogen deficiency. This shortcoming can be partially overcome by assessments with servohydraulic test systems. Reliable assays now exist for the vertebral body (Mosekilde *et al.,* 1991, 1993a; Toolan *et al.,* 1992) and femoral shaft (Ferretti *et al.,* 1994). Rat vertebral body strength appears to be a reasonable surrogate for human vertebral body fracture risk. Such preclinical testing may avoid the problems revealed during the investigation of sodium fluoride as a treatment for osteoporosis (Kleerekoper *et al.,* 1991; Riggs *et al.,* 1990).

Rats lose bone following immobilization. Multiple methods of permanent and temporary immobilization are available (Wakley *et al.,* 1988; Svesatikoglou and Larsson, 1976; Thomaidis and Lindholm, 1976; Thompson and Rodan 1988; Li *et al.,* 1990; Lindgren and Mattson, 1977). Acute (Thompson and Rodan, 1988), chronic (Li *et al.,* 1990), and recovery phase (Lindgren and Mattson, 1977; Maeda *et al.,* 1993; Lane *et al.,* 1994; Mattson, 1972) bone changes related to disuse are easily studied.

Adult female rats have adequate amounts of cancellous bone remodeling to permit useful experiments (Frost and Lee, 1992; Vignery and Baron, 1980; Baron *et al.,* 1984). The remodeling : modeling ratio rises with age. Reversal lines at the base of cancellous osteons are absent in 4-month-old rats (Erber, 1996), suggesting a predominance of modeling at this age. However, studies of cement line morphology in older rats confirm that bone activity undergoes a transition to remodeling activity during adulthood (Erben, 1996; Wronski *et al.,* 1999). Many cancellous bone regions in intact adult rats maintain stable bone mass for months while showing abundant bone formation and resorption. This neutral balance for resorption and formation (Wronski *et al.,* 1989a,b) suggests the presence of remodeling.

In most cortical bone sites, levels of Haversian remodeling approximate zero in rats. However, processes resembling intracortical remodeling are induced by both anabolic agents (Lee *et al.,* 1990) and stressful metabolic conditions (Ruth, 1953; Baylink *et al.,* 1970; Morey and Baylink, 1978; Wronski *et al.,* 1987; deWinter and Steendijk, 1975). Unfortunately, it is not known whether these agents and conditions accelerate Haversian remodeling in humans. The rat has such low levels of Haversian remodeling that it cannot be used for analysis of Haversian remodeling behavior, especially of agents that suppress such remodeling. These data come from cross sections taken at the tibio–fibula junction or mid-femur (Baylink *et al.,* 1970; Morey and Baylink, 1978). Cortical bone regions surrounding cancellous bone, as in long bone metaphyses, are a reasonable, and currently uninvestigated, site to check to find higher levels of Haversian remodeling.

In 3-month-old OVX rats, the phase of accelerated estrogen deficiency bone loss lasts 3–4 months in the proximal tibial metaphysis (Wronski *et al.,* 1989a), a 20-fold time frame compression when compared with estrogen-deplete women. Experiments with 6- to 9-month-old OVX rats create extremely reliable estrogen deficiency bone loss within 6 weeks. The rat reaches peak bone mass by age 10 months, a 30-fold time frame compression when compared to the adult human. The rat is also among the most convenient of experiment animals to handle and house.

In executing OVX rat experiments, it is wise to restrict food to limit OVX-induced weight gain. This may be either pair-feeding to a sham-group (conservative) or weight-restriction to a sham-group (aggressive). Either type of food restriction speeds cancellous bone loss (Roudebush *et al.,* 1993), and possibly creates more reliable cortical bone loss. Investigators may be tempted to choose "retired breeders" because of their low cost and ready availability. However, the variable skeletal status of retired breeders is likely to influence negatively experimental outcomes. Virgin female rats that are more skeletally mature and not osteopenic in their long bone metaphyses are much more acceptable for prevention studies because they have considerably more cancellous bone in their long bone metaphyses whose loss can be prevented after OVX (Binkley and Kimmel, 1994).

The OVX rat is an excellent model that replicates the most important clinical features of the estrogen-deficient adult human skeleton. Its site-specific development of estrogen-preventable cancellous osteopenia in 6- to 9-month-old female rats is the most widely replicated and easily detected physiologic response in *in vivo* skeletal research. Ample time exists for experimental designs that either prevent estrogen-deficiency bone loss or restore bone lost after estrogen deficiency. The rat's low levels of Haversian remodeling present little problem when testing agents for their ability to prevent the loss of or rebuild lost cancellous bone. The lack of fragility fractures can be compensated by mechanical testing of vertebral bodies. Rats are convenient for most investigators; virgin females ages 6–9 months at the start of an experiment are the optimal choice. Six weeks is the optimal time post-OVX for necropsy sampling in prevention mode studies. Six to eight weeks post-OVX is an appropriate time for beginning treatment mode experiments in the osteopenic OVX rat (Li *et al.,* 1999), but responses in extremely osteopenic bone sites have been studied at up to 15 months post-OVX (Qi *et al.,* 1995). Existing laboratory measurement tools of biochemistry, densitometry, histomorphometry, and mechanical testing are readily applicable. Experimental situations may exist in which the extreme estrogen deficiency of the OVX rat may not be an adequate match to the variable estrogen deficiency seen in naturally postmenopausal women (Cummings *et al.,* 1998b; Langcope *et al.,* 1981, 1984; Cauley *et al.,* 1999).

Guinea Pig, Rabbit, Ferret, and Cat

Though occasional reports using guinea pigs, rabbits, ferrets, and cats in osteoporosis research have appeared (Duncan *et al.,* 1973; Jowsey and Gershan-Cohen, 1964; Vanderschueren *et al.,* 1992; Hirano *et al.,* 2000; Mori *et al.,* 1997; Mackey *et al.,* 1994; Li *et al.,* 1994a,b), few studies of estrogen deficiency bone loss exist. Too few experiments generally exist to properly assess their validity. Seven-month-old guinea pigs do not lose bone by 4 months post-OVX (Vanderschueren *et al.,* 1992). Adult rabbits have abundant Haversian remodeling and an appropriate parathyroid hormone response (Hirano *et al.,* 2000), but their estrus cycle is not like that in humans. Though calcium deficiency bone loss can be induced, estrogen deficiency bone loss does not seem to exist (Mori *et al.,* 1997). The ferret, weighing less than 1 kg, has Haversian remodeling (Mackey *et al.,* 1994). Its normal skeletal physiology, including the accumulation of estrogen-dependent bone that seems to accompany normal cyclicity in other mammals, is dependent on a regular light cycle (Li *et al.,*

1994b). Like the rabbit, it exhibits expected changes in bone remodeling rate and bone volume during treatment with parathyroid hormone (Li *et al.,* 1994a; Parfitt, 1976).

Dog

The adult dog is generally a reliable model for the adult human skeleton. Studies of ^{239}Pu-injected beagles not only identified adult cancellous bone remodeling (Arnold and Jee, 1957), but also pointed to the hematogenous origin of osteoclasts (Lee and Nolan, 1963). The ratio of cortical to cancellous bone is similar to humans (Gong *et al.,* 1964; Johnson, 1964). Both Haversian and cancellous osteons remodel with similar morphology, though more rapidly in dogs (Frost, 1969; Kimmel and Jee, 1982). Skeletal responsiveness parallels the adult human for glucocorticoids (Bressot *et al.,* 1979; Jett *et al.,* 1970), uremia (Ritz *et al.,* 1973; Malluche *et al.,* 1987), bisphosphonates (Delmas *et al.,* 1982; Flora *et al.,* 1981), and disuse (Donaldson *et al.,* 1970; Minaire *et al.,* 1974; Krolner and Toft, 1983; Unthoff *et al.,* 1985; Unthoff and Jaworski, 1978; Jaworski and Unthoff, 1986), and parathyroid hormone excess (Parfitt, 1976; Podbesek *et al.,* 1983).

In contrast to all other uses for the adult beagle as a model of the adult human skeleton, the oophorectomized beagle is controversial, yielding dissimilar results in multiple laboratories. Most studies lack significant findings (Kimmel, 1992b; Cumming, 1990; Drezner and Nesbitt, 1990; Barbier *et al.,* 1991; Shaw *et al.,* 1992; Lynch *et al.,* 1991; McCubbrey *et al.,* 1991; Boyce *et al.,* 1990; Malluche *et al.,* 1986, 1988; Faugere *et al.,* 1990; Nakamura *et al.,* 1992; Dannucci *et al.,* 1987; Karambolova *et al.,* 1985, 1986, 1987; Shen *et al.,* 1992; Snow and Anderson, 1985a,b, 1986; Snow *et al.,* 1984; Koyama *et al.,* 1984a,b). The most complete experiment suggests that annual estrogen-deficiency bone loss of ~6–9% occurs. The bone turnover pattern in one suggests a transient rise after OVX (Boyce *et al.,* 1990). Yet, several publications by one group (Malluche *et al.,* 1986, 1988; Faugere *et al.,* 1990) suggest that formation falls rapidly to 50% of sham, with no increased turnover phase. This suggests a marked dissimilarity to data from transmenopausal humans, where the transient rise in turnover has been well documented (Recker *et al.,* 1978; Stepan *et al.,* 1987; Coble *et al.,* 1994). The data also indicate that estrogen suppresses turnover with uncertain effects on bone mass (Snow and Anderson, 1985a,b, 1986; Snow *et al.,* 1984).

Estrogen exposure and menstrual/estrus cyclicity is much less than in other animals. 17-β-estradiol levels are usually very low in the dog, rising *twice yearly* for several weeks (Concannon *et al.,* 1975; Jones *et al.,* 1973; Bell *et al.,* 1971). In rats, 17-β-estradiol spikes for 18 hr every 4 days (Butcher *et al.,* 1994). Women spike 17-β-estradiol for 1–2 days monthly (Baird and Guevara, 1969; Reed *et al.,* 1986). The estrus cycle in monkeys has a similar frequency to humans (Longcope *et al.,* 1989). Estrogen exposure in dogs, though only marginally less than in rats, is only one-fourth that in humans, but lacks the frequency. It is similar to that in primates, except during the peak periods. This difference could contribute to the dog's developing only a small estrogen-dependent compartment of cancellous bone.

The adult beagle, an excellent model of the adult human skeleton except for estrogen deficiency, has Haversian remodeling. The age of peak bone mass is about 3 years. Though the OVX beagle seems to have estrogen-deficiency osteopenia, poor interlaboratory reproducibility makes it unreliable. A problem has been that most individual studies have inadequate methodology to detect the expected bone loss (Barbier *et al.,* 1991; Shaw *et al.,* 1992; Lynch *et al.,* 1991; McCubbrey *et al.,* 1991; Boyce *et al.,* 1990; Malluche *et al.,* 1986, 1988; Faugere *et al.,* 1990; Nakamura *et al.,* 1992; Dannucci *et al.,* 1987; Karambolova *et al.,* 1985, 1986, 1987; Shen *et al.,* 1992; Snow and Anderson, 1985a,b, 1986; Snow *et al.,* 1984; Koyama *et al.,* 1984a,b). Beagles are less estrogen-replete than women and may have a smaller estrogen-dependent compartment of bone in their skeleton. Despite its unreliable estrogen deficiency bone loss, the dog remains an excellent model for testing the effects on Haversian remodeling of agents that have anabolic effects on cancellous bone. However, using one animal model that combines both Haversian remodeling and consistent estrogen deficiency bone loss is possible and seems a better strategy.

Pig

The pig has both growing and adult skeletal phases and has a regular estrus cycle somewhat shorter than in humans. The OVX pig has been tested a few times (Mosekilde *et al.,* 1993b; Franks *et al.,* 1994). In one study, despite minor structural deterioration, no differences in bone mass, either by densitometry or histomorphometry, were seen. In a second study, minor bone loss in the fourth lumbar vertebra was seen at 3 months post-OVX. Pigs have been used successfully to study fluoride and exercise effects on the skeleton (Kragstrup *et al.,* 1989; Raab *et al.,* 1991). The age of peak bone mass is older than 3 years. Though existing data are limited, they do not encourage further development of this model.

Sheep

The ewe has both growing and adult skeletal phases with an age of peak bone mass of 5–7 years. Ewes have a regular estrus cycle during the short days of fall and winter, but have anestrus in spring and summer (Webb *et al.,* 1992; Malpaux and Karsch, 1990). Fluoride skeletal effects in the adult ewe also seem to parallel histologic changes in humans with frequent signs of increased formation, sluggish mineralization, and toxicity to bone forming cells (Charassieux, 1990; Eriksen *et al.,* 1985; Charassieux *et al.,* 1991a,b). Though early data on post-OVX skeletal behavior were not promising (Hornby *et al.,* 1994; Geusens *et al.,* 1996), recent data are more encouraging (Charassieux *et al.,* 2001). In particular, elevated bone remodeling is seen by

biochemical and histomorphometric measures in cortical and cancellous bone of 8-year-old ewes at 6 months post-OVX. Significant DXA-measured bone loss also occurs in the vertebra and femur. Glucocorticoid data seem consistent with findings in other large animal models and humans (Bressot *et al.,* 1979; Chavassieux *et al.,* 1993). Sheep can be housed readily at most vivariums and pose little problem for handling. Though wider interlaboratory validation is needed, the adult ewe seems to hold promise as an *in vivo* model of osteoporosis.

Nonhuman Primate

The above models focus on traditional skeletal studies that involve a few *in vivo* measurements and primarily post-mortem evaluations. With the nonhuman primate, both terminal and nonterminal studies relevant to the study of osteoporosis are possible.

Terminal Studies

The nonhuman primate has both growing and adult skeletal phases. Peak bone mass occurs at age 10–12 years in cynomolgus and rhesus monkeys and baboons (Jayo *et al.,* 1990, 1991; Pope *et al.,* 1989; Colman *et al.,* 1999a,b; Champ *et al.,* 1996). All non-human primates have a regular menstrual cycle with a duration of ~28 days, an excellent analog of adult women. Nonhuman primates experience a natural menopause early in the third decade of life.

Nonhuman primates show lower bone mass and strength with increased turnover (Binkley *et al.,* 1998; Jerome *et al.,* 1986) after OVX (Binkley *et al.,* 1998; Jerome *et al.,* 1986; Miller and Weaver, 1986; Miller *et al.,* 1986; Balena *et al.,* 1993; Thompson *et al.,* 1992; Lundon and Grynpas, 1993; Lundon *et al.,* 1994; Keller *et al.,* 2000; Jayo *et al.,* 1998; Mann *et al.,* 1992), premature menopause (Shively *et al.,* 1991), or GnRH agonist treatment (Mann *et al.,* 1990). Estrogen or bisphosphonate treatment prevents OVX-induced bone loss (Binkley *et al.,* 1998; Thompson *et al.,* 1992; Keller *et al.,* 2000). Nonhuman primates experience bone loss with age (Jayo *et al.,* 1990, 1991; Pope *et al.,* 1989; Colman *et al.,* 1999a,b; Champ *et al.,* 1996; Aufdemorte *et al.,* 1993; Grynpas *et al.,* 1993), menopause (Colman *et al.,* 1999b), and immobilization of long duration (Young *et al.,* 1983). Histomorphometric studies of primates and humans yield remarkably similar values (Pope *et al.,* 1989; Jerome *et al.,* 1986; Schnitzler *et al.,* 1993; Recker *et al.,* 1988). Though post-OVX bone loss is frequently masked by the use of animals at an age when they are still acquiring peak bone mass (Brommage *et al.,* 1999; Jerome *et al.,* 1997, 1999), when rhesus monkeys age 12 years or greater are used, elevated turnover markers and significant bone loss can be seen at 3 months, with a maximum spinal BMD decrease of 8.5% detected by DXA (Binkley *et al.,* 1998) within 9 months.

Late life spinal pathology in rhesus monkeys (Grynpas *et al.,* 1993; Carlson *et al.,* 1996) and baboons (Kimmel *et al.,* 1993; Hughes *et al.,* 1994) is mostly osteoarthritis. Baboons experience osteopenia (Aufdemorte *et al.,* 1993) with an age-related decline in anterior vertebral height that bears more similarity to that accompanying osteoarthritis than to vertebral crush fractures (Kimmel *et al.,* 1993; Hughes *et al.,* 1994; Degueker, 1985). Thus nonhuman primates are not likely to be a model of fragility fractures, because human osteoarthritis and osteoporosis tend to be mutually exclusive (Dequeke 1985; Ng *et al.,* 1984). Though spinal bone mass measurements in primates may be affected by the osteophytes that occur in osteoarthritis (Hopkins *et al.,* 1989; Drinka *et al.,* 1992), 12-year-old nonhuman primates can easily enter DXA-based studies for 2–3 years.

Nonterminal Studies

Biochemical markers of bone resorption, like urinary deoxypyridinoline show both age and gender effects in rhesus monkeys (Cahoon *et al.,* 1996). Early data suggest that nonhuman primates can be used to test the effects of agents and conditions on bone markers of resorption and formation. $\alpha V\beta 3$ integrin antagonists and cathepsin K antagonists reduce urinary *c*-telopeptide concentrations in GnRH-treated rhesus monkeys, after 5 days of treatment (Stroup *et al.,* 1999, 2000). Though it is not known whether such agents influence bone resorption in estrogen-deficient humans, this model has considerable conceptual relevance.

Summary

The adult nonhuman primate is an excellent model for all aspects of the adult human skeleton. Its extensive Haversian remodeling makes study of cortical bone relevant. The age of peak bone mass is 11–12 years. Its regular estrus cycles are an excellent analog of menstrual cycles in humans. High turnover with cancellous osteopenia develops within 3 to 6 months post-OVX. Requirements for housing and care of nonhuman primates limit their use to a small number of facilities. When handled by experienced staff in an appropriate environment, they present few care problems. In testing new anti-osteoporosis treatment agents that will require human anti-fracture efficacy for successful marketing, the need for terminal nonhuman primate studies must be carefully considered. The use of nonhuman primates in nonterminal studies using bone resorption markers may present an excellent opportunity for future investigations.

Glucocorticoid Osteoporosis

Small animal models generally reproduce important aspects of human glucocorticoid osteopenia poorly (Miller *et al.,* 1995). Most mouse and rat experiments use growing animals and relatively high doses of glucocorticoids (King *et al.,* 1996; Geusens *et al.,* 1990; Simmons and Kunin, 1967;

Turner *et al.,* 1995; Jowell *et al.,* 1987; Li *et al.,* 1996b; Ortoft and Occlude, 1988; Ferretti *et al.,* 1992, 1993; Ortoft *et al.,* 1992, 1999; Aerssens *et al.,* 1994; Altman *et al.,* 1992). They show *increased* metaphyseal cancellous bone mass accompanied by decreased bone elongation rate and lower rates of bone formation and resorption. The increased cancellous bone mass is due to the glucocorticoid influence on endochondral ossification. Glucocorticoids decrease both the disappearance rate of mineralized metaphyseal tissue and the bone elongation rate, thereby increasing cancellous mineralized tissue in a bone of reduced size.

Experiments in small growing animals also show cortical osteopenia and low bone strength with glucocorticoids (Ortoft and Occlude, 1988; Ortoft *et al.,* 1999; Ferretti *et al.,* 1992, 1993), due to inhibition of the rapid bone formation at periosteal surfaces. While the decreased bone formation data are pertinent, the overall picture differs physiologically from adult human glucocorticoid osteopenia, because of the relatively high glucocorticoid doses, the endochondral ossification in growing bones, and the gonadal hormone sufficient state of the rats. Cortical osteopenia and its associated low bone strength are due to reduced periosteal expansion, a minor process in the adult human skeleton. The growing rat is therefore a poor model for adult human glucocorticoid osteopenia.

Recent experiments indicate that mice may be useful for studying glucocorticoid-induced osteoblast apoptosis (Plotkin *et al.,* 1999; Weinstein *et al.,* 1998). Glucocorticoid experiments in older rats tend to show either a trend to or a significant, minor decrease in metaphyseal cancellous bone mass and cortical area (Nakamura *et al.,* 1996; Sjoden *et al.,* 1984; Larsson and Seuastikoglou, 1972; Goulding and Gold, 1988; Lindgren and DeLuca, 1983; Ortoft *et al.,* 1995; Wimalawansa *et al.,* 1997; Wimalawansa and Simmons, 1998). The usual dose and treatment time is subcutaneous 0.5–2.5 mg/kg/day prednisolone for 6–8 weeks. Metaphyseal bone density by Archimedes' principle or defatted bone weight was ~6% lower (Sjoden *et al.,* 1984; Larsson and Sevastikoglou, 1972; Goulding and Gold, 1988; Lindgre and DeLuca, 1983). Lumbar spine BMD by DXA was ~9–10% lower after 6 weeks (Wimalawansa *et al.,* 1997; Wimalawansa and Simmons, 1998). In 3-month-old female rats, total body calcium was ~12–15% lower after 12 weeks (Goulding and Gold, 1988). However, no glucocorticoid data in older rats have been uniformly reproduced in multiple laboratories.

Glucocorticoids in large animals cause increased remodeling activity and development of osteopenia (Duncan *et al.,* 1973; Charassieux *et al.,* 1993). Consistent with the small animal, the formation phase that follows remodeling's resorption phase proceeds slowly (Duncan *et al.,* 1973; Jett *et al.,* 1970). Studies in rabbits suggest that bones are weaker and BMD is lower after glucocorticoid treatment (Lindgren *et al.,* 1984; Waters *et al.,* 1995; Grardel *et al.,* 1994). In dogs, a 13% loss of lumbar spine BMD occurs over 12 months (Lyles *et al.,* 1993; Jett *et al.,* 1970). Bone formation is suppressed in sheep (Charassieux *et al.,* 1993, 1997; O'Connell *et al.,* 1993). Though these large animal results are consistent with data from humans and aged small animal models of glucocorticoid osteopenia, these large animals are poor models of the estrogen deficient skeleton (Kimmel, 1996). Given that glucocorticoid osteopenia has a hypogonadal component and frequently develops in the backdrop of postmenopausal estrogen deficiency, these large animals offer no possibility for studying this important interaction.

Summary

The clinical picture of glucocorticoid-induced osteopenia in humans is one of low bone mass accompanied by negative bone balance. The young, growing rat is a poor model for human glucocorticoid osteopenia. A relative shortage of data on adult small animals treated with glucocorticoids exists. Additional experiments documenting the influence of: (a) decreased food intake and (b) gonadal hormone status on the glucocorticoid effects on BMD, bone cell status, and bone strength are needed. The large animals showing glucocorticoid bone loss are poor models of the estrogen-deficient skeleton, limiting the degree to which they can mimick humans. At this time, there is no accepted small animal model for glucocorticoid osteopenia. The rabbit is the animal model of choice.

Male Osteoporosis

Small animal models of male osteoporosis are now sufficiently reliable to be employed in studies of male osteoporosis (Feldmann *et al.,* 1989; Wink and Felts, 1980; Gurkan *et al.,* 1986; Vanderschueren *et al.,* 1993, 1994, 1996, 2000a,b; Ke *et al.,* 2000; Erben *et al.,* 2000; Kapitola *et al.,* 1995). For instance, like female rats, male rats gonadectomized as young adults (age 4–6 months) soon become relatively osteopenic (Wink and Felts, 1980; Gurkan *et al.,* 1986). Though significant cancellous bone *loss* occurs in female rats OVX at this age, the osteopenia of young adult orchidectomized (ORX) male rats represents failure to achieve normal peak bone mass, rather than hypogonadism-induced bone loss in the adult skeleton.

The female and male rat skeletons age at different rates. The female skeleton stops growing and reaches peak bone mass by age 6–9 months, some 6 months before that of male rats. Considering their later age of peak bone mass, male rats should be at least age 12–15 months to demonstrate the prevention and treatment of osteopenia intended to mimic that in adult human males.

Orchidectomy induces high turnover and bone loss in cancellous regions (Wink and Felts, 1980; Garkan *et al.,* 1986; Vanderschueren *et al.,* 1993, 1994, 2000a,b; Ke *et al.,* 2000; Erben *et al.,* 2000). These changes are reversed by androgen replacement (Vanderschueren *et al.,* 1994). At longer times,

cortical osteopenia occurs. Age-related bone loss also occurs in male rats between 1 and 2 years of age. The 12- to 15-month-old male rat is an accurate model of the adult male human skeleton. In order to use rats of this age in studies of sufficient length, consideration should be given to restrict feeding to extend healthy life span (Weindruch, 1996).

Summary

When an *in vivo* osteoporosis research project cannot be done in humans, the 6- to 9-month-old female rat is the animal model of choice to mimick tissue level findings in humans. It is near peak bone mass and can be manipulated to simulate accurately most clinical findings of osteoporosis in the adult female skeleton. Serum biochemistry, histomorphometry, and densitometry, routinely used in humans, can be used. Like all animal models, the rat develops no fragility fractures. However, mechanical testing of rat bones is a reasonably accurate predictor of bone fragility.

The OVX mouse is an accurate model for tissue-level bone changes when low doses of 17-β-estradiol ($<$0.01 mg/kg sc) are used for replacement. Appropriately prepared transgenic and knockout mice will be the most direct route to identifying molecular mechanisms behind estrogen deficiency bone loss.

The rat's low levels of Haversian remodeling do not permit accurate evaluation of cortical bone behavior, making additional studies of cortical bone, either in large animals or in humans, a necessity. Though the adult dog is an accurate model of the adult human skeleton, its hormonal dissimilarity seems to cause it to yield inconsistent results in the acutely estrogen-deficient state that differ from those in humans. Estrogen deficient nonhuman primates are currently the large animal of choice for quantitative histologic evaluation of Haversian remodeling.

Small animals are poor models of glucocorticoid-induced osteopenia. Larger animals like rabbits and dogs are acceptable, but do not replicate well the usually coexistent condition of estrogen deficiency. Aged male rats model all aspects of male osteoporosis well except for aspects connected with cortical bone remodeling.

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CHAPTER 96

Defining the Genetics of Osteoporosis

Using the Mouse to Understand Man

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Introduction—Historical Perspective

Low bone mineral density (BMD) has become the most established and identifiable risk factor for osteoporotic fractures. The proliferation of newer tools for measuring bone mass has resulted in widespread testing and has also led to the realization that BMD is a complex trait normally distributed across various populations. In addition, the data produced by these tools also provided the first clues that a syndrome once characterized as an age-related disorder associated with back pain and fractures is, in fact, a heritable disease. More recently, BMD studies of mother–daughter pairs, twins, and large sib cohorts estimated the heritability of this trait to be between 50 and 70% (Eisman, 1999). This finding led most investigators somewhat hastily to conclude that the genetic influences on BMD were "oligogenic"; i.e., the phenotypic variation in BMD was caused by the actions of a limited number of genes with discrete effects. Fueled by this concept, the past 10 years have been characterized by a flood of candidate-gene association studies in both small and large unrelated cohorts (Eisman, 1999). Although data from these papers were conflicted, and failed to yield major genes that defined osteoporotic risk, such studies, combined with genome-wide scanning of multigenerational families, served to reinforce the complex and polygenic nature of the genetic influence on bone acquisition. Having entered a new millenium, we are now left with the daunting task of defining the role of multiple genes that individually, or in concert, moderate the acquisition and maintenance of peak bone mass.

In addition to the complex multifactorial nature of genetic influences, three other factors have emerged that further complicate our search for osteoporosis genes. First, it is now clear that individual genetic determinants of BMD are strongly influenced by other genes that do not have direct effect themselves on BMD; i.e., epistasis (Eisman, 1999; Frankel, 1995). Second, there are numerous environmental factors that may modulate expression of one or more genes (Eisman, 1999). Sorting these genetic and environmental interactions will require complex modeling that must control for nutritional, hormonal, mechanical, and lifestyle factors. Third, most investigators have come to recognize that the BMD phenotype is only a surrogate for fracture. Defining "bone density genes" provides limited information with respect to prediction of future fractures, in part because there are other contributing factors that affect the eventual health of any given bone. The emergence of measurement tools with superb resolution of bone microstructure has further heightened this awareness. Such technology has resulted in a better understanding of what bone "quality" represents, and how this is related to skeletal frailty. Finally, and probably most importantly, there is now a growing realization that BMD represents the sum of several temporally related processes beginning with skeletal development and including modeling, remodeling, and consolidation. Decomposition of bone mass into intermediate phenotypes, such as BMD, cross-sectional area, shape, or a biochemical marker such as insulin-like growth factor (IGF)-I, is likely to yield more mechanistic insights not only into the overall processes of peak acquisition, but also into the

determinants of skeletal strength. Most certainly, finding genes that predict the risk of fractures in humans is going to require novel strategies and will remain a challenging endeavor for the foreseeable future.

Enter the mouse! Rodent models for testing hypotheses related to skeletal disorders are not new. The ovariectomized rat is a well-established tool for testing new therapies for osteoporosis, as well as for understanding how estrogen deprivation affects the bone remodeling unit. Adding to data from the rat are new models of laboratory mice that carry specific gene deletions (knockouts), gene additions (transgenic), or spontaneous mutation. These mice are currently at a frontier of basic research, specifically to test how a known gene may regulate diverse skeletal actions For example, targeted overexpression of IGF-I in transgenic mice using the osteocalcin promoter is characterized by a marked increased in both cortical and trabecular bone density at 6 weeks of age (Zhao *et al.,* 2000). Similarly, mice globally lacking expression of the *Cbfa1* gene (i.e., null mutation) are characterized by the absence of osteoblast differentiation, failure to mineralize bone, and lethality at birth (Komori *et al.,* 1997). Finally, spontaneous mutants such as the osteopetrotic mouse (gene symbol, *op*) which lacks a functional *mcsf* gene and its product, fail to exhibit differentiated osteoclasts which are required for normal bone resorption (Suda *et al.,* 1999). Such gene mutations, induced or spontaneous, help to elucidate the potential rule of single gene action in bone biology.

An entirely different approach utilizes the power of the mouse as a genetic tool to uncover genes whose normal allelic variation regulates BMD. Over the past 30 years, inbred strains of mice have helped identify genetic determinants of various disease states with both single and polygenic bases (Silver and Nadeau, 1997). Although investigators in the bone field have been a little late in recognizing these models, several factors have hastened their utilization. First, technology was developed to measure BMD accurately, easily, and relatively inexpensively in mice. Use of peripheral quantitative computed tomography (pQCT), peripheral dual X-ray absorptiometry (DXA), and full body DXA, by both *ex vivo* and *in vivo* methods, now allow investigators to measure BMD and appreciate large differences among knockouts, transgenics, mutants, and healthy inbred strains (Beamer *et al.,* 1996). Micro-CT has, for the first time, provided an opportunity to define three-dimensional microstructural aspects of bone, and in conjunction with newer methods of measuring bone strength, have opened the door for identifying determinants of bone quality (Turner *et al.,* 2000). Finally, the power of breeding strategies to isolate quantitative trait loci (QTL), and to test their effects either singly or in combination with other genetic determinants, has permitted hypothesis testing for individual or clusters of genetic loci (Zeng, 1994).

There are several confounding factors that have plagued human genetics studies in the past two decades, making gene identification exceedingly difficult. These have included the complex nature of the phenotype regulated by numerous genes, significant gene × gene and environment × gene interaction, and the multifactorial nature of bone quality (Eisman, 1999; Kelley *et al.,* 1990). Mouse studies have made these much more amenable to resolution. Furthermore, the homology between human and mouse genomes, as well as the intense efforts to map every gene in both species, provides more impetus to use this animal as a tool for defining the heritable determinants of osteoporotic risk. In this paper, we will describe the role of several mouse model systems for determining the polygenic basis of osteoporosis. In addition, we will define their relevance for subsequent human studies. We will not examine mutant, transgenic or knockout models, in part because we want to deemphasize the role of single genes in producing extreme pathology. Rather this review will focus on normal allelic variation in inbred strains of mice, an animal model more directly applicable to understanding the BMD trait in humans.

Inbred Strains of Mice

There are many types of mice available for genetic and biologic studies. In general, mice have become the workhorses of biomedical research because of their ease of breeding and reproductive capacity, their short life span, and the availability of large numbers of genetic markers in the mouse genome. Probably more importantly, though, and unlike the rat, dozens of different inbred mouse strains have been available since the early decades of the 20th century. These inbred strains of mice were developed by repeated matings between siblings for at least 20 consecutive generations (Silver and Nadeau, 1997). This resulted in nearly 100% homozygosity at all alleles across the mouse genome. By continuing the process until the 60th generation, inbred mice eventually became 100% homozygous at all loci (except for any spontaneous mutations which arise), thereby providing researchers with a plethora of genomically identical mice. More than 700 pure inbred strains are currently available at The Jackson Laboratory alone, including C57BL/6J (B6), the standard strain for many laboratories doing genetic as well as biologic analyses.

The second feature of inbred strains that makes them powerful genetic and physiologic tools is that an individual inbred strain differs from all other strains. Each strain has its own set of phenotypic characteristics that make it unique and allow innumerable differences in physiologic behavior. One such difference is in the wide variation in BMD among inbred strains. Thus, by choosing two inbred strains that differ in a trait of interest, a cross can be made to enumerate, locate, and define heritability of the genes that contribute to that trait.

Crossing two inbred strains of mice results in hybrid F1 mice that are genetically identical with each other and heterozygous at all loci. As illustrated in Fig. 1, intercrossing F1 mice results in F2 progeny in which genetic alleles for BMD have randomly reassorted into new combinations, such that at any given locus an F2 mouse will be homozygous for either progenitor strain alleles (i.e., *b6*/*b6* or *c3*/*c3*)

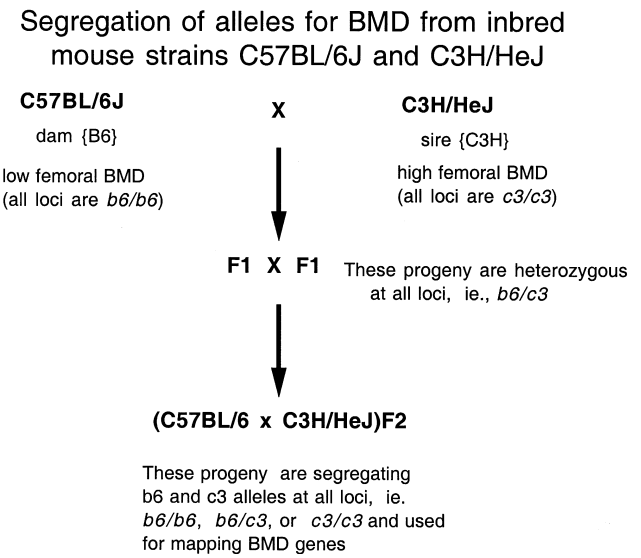

Figure 1 The use of two inbred mouse strains for analysis of a genetically regulated phenotypic trait. The C57BL/6J and C3H/HeJ strains characterized by low and high volumetric bone mineral density (BMD) are depicted. F2 progeny analyses are equally effective for mapping single gene traits and polygenic traits that are either quantitative or qualitative in nature.

or heterozygous (i.e., *b6/c3*). BMD regulatory loci that are not genetically linked to each other will independently segregate in these F2. The net effect of alleles at all BMD regulatory loci yields the BMD for each mouse.

Since there are now more than 8000 genotypic markers that are variably polymorphic across inbred strains, investigators can identify QTLs by genotyping and phenotyping the F2 progeny (Lander and Botstein, 1989). Analyses of the F2 progeny in the extremes of the phenotypic distribution allows rapid identification of major effect loci, whereas analyses of all F2 progeny yields major and minor effect loci, as well as opportunity to assess trait variance accounted for by each locus and gene × gene interactions. Genetic linkage is established by testing for association of progenitor alleles with high or low expression of a particular phenotype using various computer software programs (Lander and Kruglyak, 1995; Lander and Botstein, 1989). The initial QTLs may reside in chromosomal regions up to 40 centiMorgans (cM, recombination distances between specific markers), areas of the chromosome with hundreds of potential candidate genes. Fine mapping and congenic construction (see below) define narrower regions of the chromosome and allow for positional cloning and gene sequencing to be undertaken. This type of QTL strategy has been successful in identifying genes associated with obesity, atherosclerosis, epilepsy, and cancer susceptibility in the mouse.

Beamer *et al.,* in 1996 first described large differences in femoral and vertebral BMD, as measured by pQCT, among various inbred strains of mice (Beamer *et al.,* 1996). Subsequently, other investigators reported similar findings using planar radiography, whole-body BMD by DXA, and site-specific regions of interest (i.e., vertebrae, femur, and tibia) by peripheral DXA technology (Benes *et al.,* 2000; Klein *et al.,* 1998; Shimizu *et al.,* 1999). More recently, micro-CT has been utilized to detect differences in trabecular bone structural parameters in vertebrae, femorae, and tibia among nbred strains (Turner *et al.,* 2000). For each pair of strains differing in a bone phenotype, the strategy has been to map QTLs by crossing the progenitor strains and then intercrossing their F1 hybrids to produce large numbers of F2 progeny (range 250–1000 males and females). Data from several sets of such F2 progeny have now been reported for the BMD phenotype and include C3H/HeJ (C3H:) vs C57BL/6J (B6: low BMD), Castaneus/EiJ (CAST: high BMD) vs B6 (low BMD), SAMR1(high BMD) vs SAMP6 (low BMD), AKR/J (high) vs SAMP6 (low BMD), and B6 (high BMD) vs DBA/2J (D2 low BMD) (Beamer *et al.,* 1999, 2001; Benes *et al.,* 2000; Klein *et al.,* 1998; Shimizu *et al.,* 1999).

As noted earlier, bone mass is a complex phenotype that includes mineral content, size (length, width, cross-sectional area), trabecular connectivity, and shape. Bone strength is determined by these dimensions as well as other variables affecting overall bone quality. Depending on the exact measurement, and the instrument used to define it, a bone density phenotype varies within a given strain as well as between inbred strains. For example, B6 is a low bone density strain compared to C3H, when defined by volumetric measurements of the femur such as pQCT, but B6 is a high density strain in comparison to D2 when whole body BMD using DXA technology is the bone density phenotype of choice (14,17). In part, this can be related to the shape as well as the size of bone and its individual components. The B6 femurs have thinner cortices, a more elliptical shape, and lower volumetric BMD than C3H animals. However, in comparison to D2 mice, the periosteal circumference of B6 is greater, hence areal measurement of the femur by DXA can actually show relatively greater apparent BMD for this strain. Since size, shape and mineral are all critical components of strength, the phenotype under study becomes critical not only for assigning QTLs, but also for attempting to understand the biomechanical mechanisms that ultimately define both bone morphology and strength.

Mapping "bone density genes" can be extremely productive in F2 mice because there is independent segregation of unlinked genes for this polygenic phenotype. Surprisingly, progenitor differences are not mandatory before performing genetic analyses of polygenic traits, as the F2 population, with its independent assortment of loci, will reveal genetic determinants whose actions might not to be evident in the progenitor inbred strains or may be hidden by actions of epistatic loci (Soller *et al.,* 1976). Moreover, QTLs in the F2s can display alleles with actions that appear contrary to expectations based on the pprogenitor strains' phenotype. For example, the high BMD C3H inbred strain has been found in one QTL analysis to be carrying two genes yielding a "low density" in F2 progeny (Beamer *et al.,* 2001). Similarly, Benes *et al.* found that two AKR QTLs (i.e., Chr 7, 11) were associated with low areal BMD when these alleles were homozygous in F2 mice from an AKR (high density) × SAMP6 (low density) cross (Benes *et al.,* 2000).

Thus, F2 mice provide an invaluable tool for locating and enumerating QTLs, as well as delineating allelic effects. Moreover, the phenomena of transgenesis (gene recombinations in the F2s resulting in phenotypic values that are greater or lesser than the progenitor strain phenotype) can offer critical insight into the complex genetic influence on a specific phenotype (Benes *et al.,* 2000).

Another exciting aspect of gene mapping in mice is the evolving story surrounding trabecular structure and strength. Newer technology with micro-CT has provided investigators with the opportunity to study trabecular spacing, number, connectivity, and three-dimensional structure, in the vertebrae and femorae of mice. These determinants are likely to produce even more phenotypes for QTL analyses, especially in respect to understanding "bone quality." Recent work by Turner *et al.,* suggest that data on vertebral and femoral neck breaking strength in one inbred strain can differ considerably from that at the mid-diaphysis of the femur in the same strain (Turner *et al.,* 2000). For example, C3H femurs have a high BMD and thick cortex and therefore are stronger than B6 femurs by three-point bending in the mid-diaphysis. However, vertebral strength by compression testing is reduced in C3H compared to B6. Micro-CT analyses has revealed a markedly reduced number of trabeculae in C3H vertebrae (Turner *et al.,* 2000). These data are consistent with the fact that although C3H has higher apparent BMD (even in the vertebrae!) by projectional methodology, trabecular BMD, when quantitated properly, is actually reduced. This would suggest that there are distinct genetic determinants within a given strain that define cortical vs trabecular BMD. Moreover, these findings support long-standing clinical observations that different bone compartments acquire peak bone mass at different times, and that there can be very disparate BMD values between the spine and hip in an individual. Furthermore, response to both anti-resorptive and anabolic treatment clearly differs from one skeletal site to another. Hence, there has to be a multitude of osteoporosis genes, some of which regulate both trabecular and cortical components, some which must determine distinct skeletal compartments, and some which must be temporally activated or suppressed during the acquisition and consolidation phase of acquiring bone mass.

Finally, it should be noted that the search for mouse "bone density" genes permits a more in-depth analysis of the biology related to acquisition and maintenance of BMD. For example, C3H mice have high cortical bone density, in part because, their bone marrow stromal cells proliferate more rapidly, their osteoblasts are programmed to synthesize more IGF-I and alkaline phosphatase than B6 bone cells, and their rate of bone formation is much higher than B6 mice. By histomorphometry, C3H bone shows high rates of bone formation and modest bone resorption rates compared to B6 mice (Rosen *et al.,* 1997, 2000). These data combined with biochemical markers of bone turnover reflecting similar alterations in bone remodeling, demonstrate that the genetic determinants of bone density affect the basic cellular processes of bone. Intermediate skeletal phenotypes, such as IGF-I, N-telopeptides, or alkaline phosphatase, can also be examined in F2 mice for QTL analysis, thereby providing additional approaches to understanding the cellular mechanisms responsible for acquisition of peak bone mass.

Recombinant Inbred Strains

Recombinant inbred (RI) strains are generated by outcrossing two progenitor strains and then intercrossing these F1 hybrids to produce F2 progeny, as illustrated in Fig. 2. Then, pairs of sister–brother F2 mice are selected at random to serve as founders for each RI strain. These F2 mice are mated to produce F3 generation mice and this process of sibling mating within each RI line is repeated 20 generations. The result is new inbred strains nearly 100% homozygous at all loci, yet of which has a different combination of genes from the same original progenitor strains (Silver and Nadeau, 1997). This constitution can be maintained indefinitely by continual brother-sister matings. Since the original founders were selected at random, many distinct RI strains can be generated from the original progenitors (more than 12 such sets of RI strains exist at The Jackson Laboratory). These RI strain sets are named by the capital letter of each strain separated by "X" (e.g., B6 and D2 gave rise to BXD) plus a number for the particular strain (e.g., BXD-16) (Silver and Nadeau, 1997). Unlike classic inbred strains, the genotype of a RI strain is somewhat limited by the fact that there are only two possible alleles (e.g., B6 or D2) at each locus. More importantly, there is limited recombination because homozygosity sets in relatively quickly during the intercrosses. In addition, because both D2 and B6 inbred strains were originally derived from the same founder animals more than 70 years ago, a significant number of loci are not polymorphic. However, the strength of the RI strategy is that hundreds of genomic markers for each strain are identified, and publicly available databases can be utilized for rapid linkage studies and for determining map distances, with as few as one to two mice phenotyped from each RI strain. To establish genetic linkage, the investigator simply phenotypes each of the strains in a RI set (e.g., 12 BXD strains) to obtain a strain distribution pattern for the phenotype. The strain distribution pattern for the new phenotype is compared with strain distribution patterns of known polymorphic loci previously established in that RI set. When congruence between strain distribution patterns is found, linkage of the phenotype to a specific chromosomal region is established. By using known recombination sites in the mouse RI stains, one can mate RIs to parental strains (e.g., BXD-8 to DBA/2 and B6) to obtain new F1 progeny. Then by linkage between the genotype and phenotype, the QTL can be placed either above or below the recombination break point. This approach is called the recombinant inbred segregation test strategy (RIST) and has been successfully used by one group (see below) to help refine QTLs locations for bone density genes (Darvasi, 1998).

Klein *et al.,* was the first group to employ RI strains to define BMD QTLs. Their group utilized 19 BXD RI strains to show that: (1) whole-body areal BMD is heritable

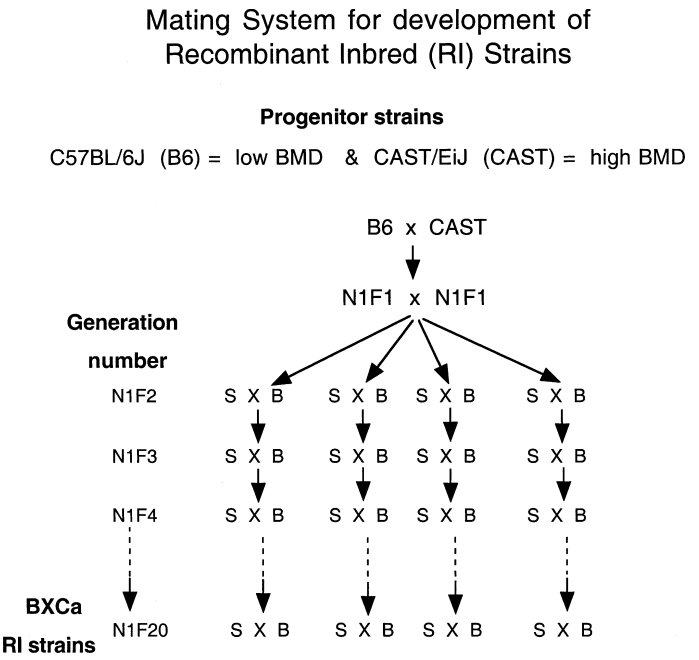

Figure 2 The method for developing a set of recombinant inbred (RI) strains. The C5&BL/6J and CAST/EiJ inbred strains are presented as the progenitors. Randomly selected sister (S) and brother (B) sibling N1F2 pairs are mated to produced N1F3 progeny, which in turn are mated to produce N1F4 progeny, etc. At the N1F20 generation, the mice within a strain will be inbred, with better than 99% of all loci homozygous for either B6 or CAST alleles. Each RI strain will have inherited on average one half its genes for each of the progenitor strains.

($h2 = 0.50$); (2) gender- and strain-specific effects are present, and (3) BMD differed by 30% among individual RI strains, suggesting that combinations of genes played a big part in overall peak bone acquisition (Klein *et al.,* 1998). Using published data bases containing more than 1500 genetic markers, Klein *et al.* (2001) identified QTLs on Chrs 2, 7, and 11 (see Table I) with major effects. Interestingly, some QTLs were present only in males, while others only in females, and three QTLs (Chrs 1, 18, 19) appeared to be gender independent (Orwoll *et al.,* 2001). Moreover, several of these QTLs were found to be similar to those identified independently by other groups using different strategies (i.e., F2 matings). Finally, from this same group, Klein *et al.,* recently reported that RIST allowed them to narrow the QTLs on Chrs 2 and 11 by more than 10 cM (Klein *et al.,* 2001). Hence, RI strains have provided a tool for rapidly establishing linkage of whole-body areal BMD QTLs, as well as for further resolution of large QTL regions into smaller segments.

Congenic Strains

Congenic strains are generated to test the effect of individual or multiple linked loci from a donor strain placed on the genetic background of a recipient strain (Silver, 1995). As illustrated in Fig. 3, the strategy is based on repetitive backcrossing to the recipient strain while genotyping for the donor strain's alleles in the subsequent backcross generations. Procedurally, the donor and recipient inbred strains are mated; the F1 progeny are then backcrossed to the recipient successively for 10 generations (N10). With each generation, the homozygosity of the recipient background increases from 50% at N1F1 to 99.9% at N10F1. The residual heterozygosity resides at the region of interest carrying the donor alleles. Hence, the genetic background of the chromosomal region of interest has been switched from donor to recipient. Congenic strains are particularly useful for confirming the QTL existence, fine mapping of the QTL, and for testing the quantitative effect of individual QTLs. For example, if there is a strong BMD QTL on Chr 1 from C3H mice, as found by F2 analysis, congenic strain mice in which the Chr 1 QTL is now placed on a B6 background, allows the investigator to test the effect of this single genetic locus on a low-bone-density background. The congenic strain would be named B6.C3H-1 (recipient, donor, and chromosome). Multiple QTLs can be combined to test for gene $\times$ gene interactions. Congenic strain construction takes about 18 months and may be labor intensive, but it provides an essential means of evaluating the biology regulated by the QTL, as well as refining the map position of individual loci. However, there are two caveats to this approach: (1) "passenger" loci adjacent to the QTL of interest travel with the donor QTL may affect the phenotype and affect mapping precision; (2) the QTL phenotype may be

Table I Summary of QTLs for BMD in Mice from Four Different Laboratories

Chromosome	Marker	Map position	Method and bone	Human homologous chromosomal region	Ref.
Chr 1	Mit14	81.6	pQCT femur, L5	1*q*21–*q*31	*a*
Chr 1	Mit15	95.8	pQCT femur	1*q*21–*q*31	*b*
Chr 2	Mit456	86.3	pQCT femur	20*q*11	*a*
Chr 2	Ncvs42	87.0	DEXA whole body	20*q*11–*q*12	*d*
Chr 2	Mit464	10.9	DEXA spine	10*p*13–*p*11; 2*q*14; 9*q*34	*c*
Chr 3	Mit23	4.6	pQCT femur	1*q*24–*q*32; 8*q*12–*q*22	*b*
Chr 4	Mit51	82.7	pQCT femur, L5	1*p*36	*a*
Chr 4	Mit124	57.4	pQCT femur, L5	13*q*14–*q*21	*a*
Chr 5	Mit112	42.0	pQCT femur	4*p*14–*p*12; 4*q*11–*q*13	*b*
Chr 6	Mit150	51.0	pQCT femur	3*p*26–*p*25; 3*q*21–*q*24; 19*q*13; 10*q*11	*a*
Chr 7	Mit332	65.6	pQCT L5	10*q*25–*q*26	*a*
Chr 7	Mit234	44.0	DEXA whole body	15*q*24–*q*26; 11*q*13–*q*21	*d*
Chr 7	Mit210	11.0	DEXA spine	19*q*12–*q*13	*c*
Chr 9	Mit196	48.0	pQCT L5	6*q*12–*q*16; 15*q*24	*a*
Chr 11	Mit242	31.0	pQCT femur	5*q*31–*q*32; 17*p*12–*p*11	*a*
Chr 11	Mit90	43.0	CTI femur	17*p*–*p*ter; 17*q*–*q*ter	*e*
Chr 11	Mit284	52.0	DEXA spine	17*q*21–*q*22	*c*
Chr 12	Mit215	2.0	pQCT femur	2*p*25–*p*22	*a*
Chr 13	Mit135	10.0	CTI	7*p*15–*p*13; 6*p*22; 9*q*22	*e*
Chr 13	Mit266	16.0	pQCT femur	6*p*25–*p*21	*a,b*
Chr 13	Mit20	35.0	DEXA spine	6*p*24–*q*22	*c*
Chr 13	Mit13	35.0	pQCT femur, L5	5*pq*22–*q*35	*a*
Chr 14	Mit160	40.0	pQCT femur, L5	13*q*14–*q*21	*a*
Chr 14	Ptprg	2.0	DEXA whole body	3*p*14; 10*q*21–*q*24; 8*p*23	*c*
Chr 15	Mit29	42.8	pQCT femur	8*q*24; 22*q*12–*q*13	*b*
Chr 16	Mit12	27.6	pQCT femur	3*q*13–*q*29	*a*
Chr 16	Mit39	28.4	DEXA spine	3*q*13–*q*29	*d*
Chr 18	Mit36	24.0	pQCT femur, L5	5*q*21–*q*33	*a*

Note. Map position given in centiMorgans (cM); human homologous regions drawn from ± 3 cM of published best marker.

[a] Beamer *et al.* (2001).
[b] Beamer *et al.* (1999).
[c] Benes *et al.* (2000).
[d] Klein *et al.* (1998).
[e] Shimizu *et al.* (1999).

the net result of linked genes with different effects within the QTL region. In each case, fine mapping and generation of nested congenics can overcome these problems (Silver and Nadeau, 1997).

Congenics have recently become extremely valuable tools for bone biologists, not only for studying the quantitative effect of individual QTLs, but also for more completely understanding the phenotype and its underlying physiology. Thus, one moves from breeding strategies and QTL analysis to individual congenic construction in order to define the locus of interest, as well as to test precisely how that locus could affect the phenotype. Two groups have reported generation of congenics using their most promising QTLs for either whole-body BMD or femoral BMD. These loci include Chrs 1, 2, 4, 5, 6, 11, 13, 14, and 18 (see Table I (Beamer *et al.,* 2001; Klein *et al.,* 2001)). In addition, one group has developed congenic strains for the serum IGF-I phenotype on Chrs 6 and 15. The power of this strategy is illustrated by some very recent studies (Rosen *et al.,* 2000).

Klein and colleagues constructed congenic mice for the Chr 2 areal BMD QTL and reported that after the fourth generation, D2.B6-2 mice (i.e., mice carrying a B6 QTL) on a nearly 93% homozygous D2 background had a difference of nearly one standard deviation in whole-body BMD compared to inbred D2 mice (Klein *et al.,* 2001). Moreover, this effect occurred only in female mice. Beamer *et al.,* reported

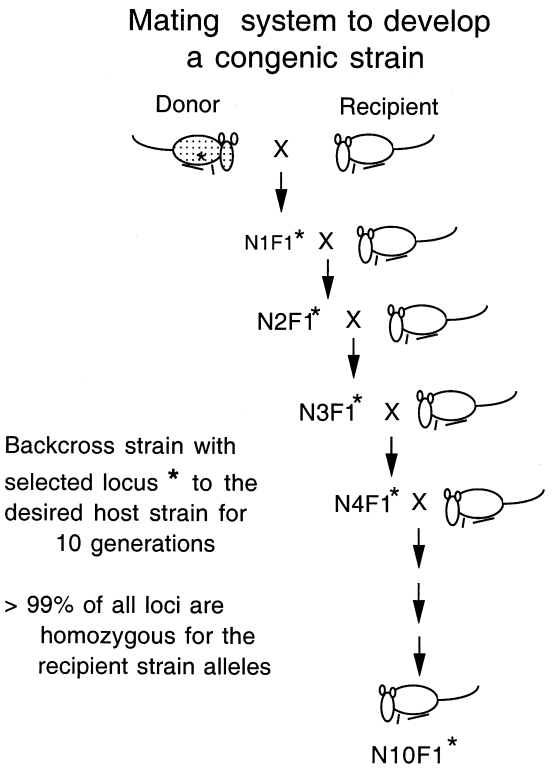

Figure 3 The method for producing a congenic strain that carries a segment of a chromosome transferred from a donor strain to a recipient strain. The transfer is accomplished by at least 10 cycles of backcrossing and F1 to a recipient strain. The region containing the gene or QTL of interest is found by genotyping each generation of progeny and mating the identified carrier of the donor segment to the recipient strain.

that the Chr 1 QTL from C3H mice had an approximately 8% increase in femoral BMD when placed on a B6 background in N6 congenics (Beamer *et al.,* 2000). Similar findings were also noted for the Chr 4 QTL. On the other hand, a "low bone density QTL" from C3H in Chr 6 was associated with a 3.5% reduction in BMD, when donated to a B6 background. Not unlike the studies from Orwoll *et al.,* this effect was noted only in female mice (Orwoll *et al.,* 2001). Hence, congenic strains can provide insight into the effect of individual QTLs in respect to bone and to the mechanism of such action.

The power of the congenics extends beyond simple tests for QTL effects. For example, Beamer *et al.,* reported that although the congenic on Chr 1 had a statistically significant effect on femur BMD, micro-CT analysis revealed a much greater effect on vertebral trabecular bone (Beamer *et al.,* 2000). In fact, vertebral bone density (measured as BV/TV) was nearly 35% greater in the Chr 1 congenic compared to progenitor B6 mice at 16 weeks. These findings, also noted for the Chr 4 QTL congenic, support the thesis that bone microstructure may be altered dramatically while BMD may change only modestly. If confirmed these data provide more impetus for defining aspects of bone quality and their relationship to skeletal fragility, an issue very relevant when considering the skeletal response to long-term anti-resorptive therapy. Finally, Rosen *et al.,* have noted that one of the strongest QTLs for serum IGF-I in B6C3F2 mice is likely to be the Chr 6 QTL noted for BMD (Rosen *et al.,* 2000). Moreover, the congenic B6.C3H-6 mice at N10 generated from this QTL, show not only reduced femoral and vertebral BMD, but also markedly lower serum IGF-I concentrations. This model provides further proof that the congenics can offer insight into the mechanisms whereby peak bone mass is acquired.

Recombinant Congenic Strains

A fourth system available for genetic and biological studies of polygenic traits, such as BMD, is illustrated in Fig. 4. Recombinant congenic (RC) Strains represent a combination of the attributes found in RI strains and congenic strains of mice. As can be discerned from Fig. 4, two backcrosses are made to a recipient strain to achieve progeny that carry 12.5% of genes from the donor strain. Sibling progeny from the N3F1 cross are then incrossed to inbred status as shown. The intent of this system is to isolate small subsets of genes that regulate a complex trait within distinct inbred strains. Demant and colleagues have successfully used RC strains to genetically analyze both colon and lung cancer in mice (Fijneman *et al.,* 1996; Moen *et al.,* 1991). In addition, in preliminary studies, Blank *et al.* (1999) has reported that

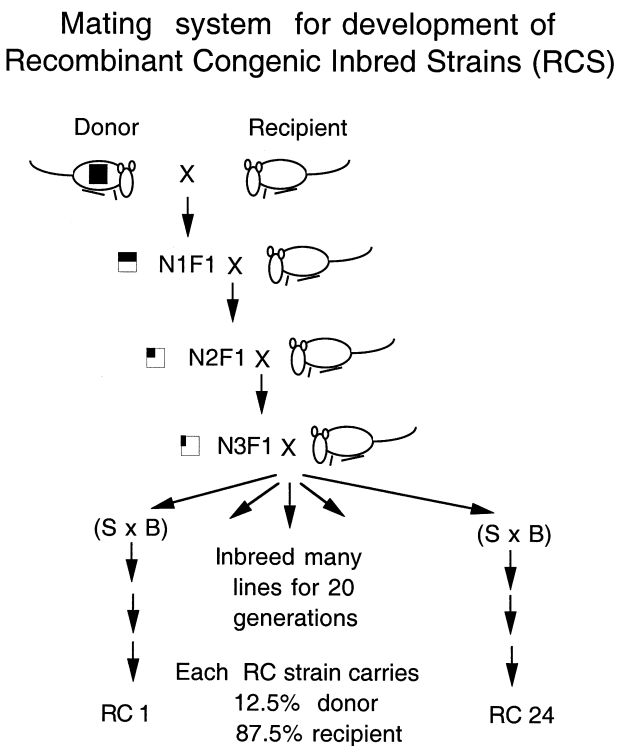

Figure 4 The method for development of a set of recombinant congenic strains, each of which carries 12.5% of it genes from the donor strain and 87.5% of the genes from the recipient strain. The desire is to capture subset of genes that regulate a complex trait in a series of related but distinct inbred strains. This method is particularly useful for analyses of phenotypes that depend on modifier genes for expression.

genetic linkage to regions on 11 different chromosomes could be demonstrated for bone strength, ash percentage, and morphological parameters using the set of 27 HcB/Dem RC strains. These strains were derived from donor C57BL/10Sc-SnA and recipient C3H/DiSnA strains. Thus, RC strains are very suitable for analyses of complex traits and may be most valuable for assessment of gene that interact in subtle ways not easily identified by standard statistical means.

Mouse to Man—The Present and Future

This review has pointed out some of the models investigators have used to define the genetic determinants of bone density in mice. However, it is quite obvious that despite major efforts by several groups, no mouse "bone density" gene has yet been cloned. Still, inbred, recombinant inbred and congenic strains, as model systems, offer a wealth of information related to acquisition and maintenance of peak bone mass. With the advent of more rapid genotyping and congenic generation techniques, it seems certain that these putative QTLs will yield numerous genes that contribute to the variance in bone density within a mouse strain. Moving from mouse genes to human genes using published maps, as well as with the ongoing genome sequencing projects, may actually turn out to be easier than once thought (Blake *et al.,* 1997). More of a challenge, however, will be to understand the full effects of a particular gene on bone cell function, the interactions with environmental factors, and perhaps even more importantly, the regulators of such genes. Notwithstanding those tasks it has become clear that the power of the mouse for bone biologists lies in several relevant factors:

(1) the strong homology (60–70%) between human and mouse genomes (see Table I);
(2) the ongoing efforts to completely map the mouse genome, thereby permitting more rapid identification of putative bone density genes;
(3) the ease and rapidity of conducting crosses among various strains of mice;
(4) the relative control that investigators have over environmental factors that modulate genetic determinants of bone density; and
(5) the rapid acceleration in knockout and transgenic technology permiting functional testing of putative bone density "genes."

Clearly, the last two factors are the most appealing and compelling. In contrast to human studies, strict regulation of dietary factors, physical activity, life style, and environment, is relatively straightforward in the mouse. Moreover, except for the sex chromosomes, each of the mice in an inbred is an identical twin to the next, carrying the same genome as all others within that strain. This makes it considerably easier to refine the search for various bone density genes, and more importantly, to be able to test their responsiveness to various perturbations, all within a defined life cycle. Finding "osteoporosis" genes in mice provides an unmatched opportunity to test their role in all aspects of bone biology, and indeed, such findings can then be used to further our understanding of the pathophysiology of this debilitating disease.

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Index for Volumes 1 and 2

A

Activated leukocyte cell adhesion molecules, 278
Activator proteins, 87–88
Activin receptor-like kinases, 930–931
Adenosine triphosphate, 1367–1369
Adhesion molecules. *see* Cell adhesion molecules
Adipocytes, role in remodeling, 311
Adrenal gland
  abnormalities MEN syndrome-associated, 1049–1050
  medullary, 634–635, 649
AER. *see* Apical ectodermal ridge
Age/Aging
  effects of cortical wall thickness, 27–28
  effects of GH/IGF-I, 1442
  effects on
    bone turnover, 1550
    collagen, 214–215
    GH treatment, 1445
    previtamin D_3 photosynthesis, 587
    skeletal adaptation, 1483–1484
  stem cell changes, 73–74
Aggrecan, 226
AHO. *see* Albright's hereditary osteodystrophy
Albright's hereditary osteodystrophy
  characteristics, 42, 1140
  clinical features, 1151–1152
  molecular defect in, 1143–1145
  phenotypic variability, 1148–1149
  treatment, 1156
  vitamin D therapy, 1411–1412
Albright's hypothesis, 1079
ALCAM. *see* Activated leukocyte cell adhesion molecules
Alkaline phosphatase. *see also* Hypophosphatasia
  biology, 1231–1232
  as bone formation marker, 1544–1545
  discovery, 1229
  effects of PTH/PTHrP, 498
  effects of thyroid hormones, 709–710
  history, 1229–1231
  levels in
    metastatic bone disease, 1562–1564
    Paget's disease, 1551, 1553
    PHPT, 1551
  physiological role, 1229–1231
  replacement therapy, 1238–1239
  role in hypophosphatasia, 1240–1243
Allele-sharing studies, osteoporosis genes, 1261–1264
ALP. *see* Alkaline phosphatase
Aluminum-bone, interaction, 360–361
AMG 073, 1352–1353
Amino acids, aromatic, 382
γ-Aminobutyric acid, as Ca sensor, 383
Amylin
  characterization, 634
  distribution, 641
  effects on
    bone mass, 648
    Ca metabolism *in vivo,* 649–650
    osteoblasts, 647–648
    osteoclasts, 645–646
  extraskeletal actions, 642–643
  production, 641
  receptors, 649–650
Amyloidosis, cutaneous lichen, 1070
Anabolic steroids. *see* Androgens
Androgens
  description, 1455
  effects on
    bone
      formation, 764–766
      metabolism, 766–767
      resorption, 764–766
    bone cell apoptosis, 161
    chondrocytes, 767–769
    osteoblasts, 762–764
  mode of action, skeletal, 1462
  nuclear receptors, 1459–1461
  osteoporosis pathophysiology and, 1280

Androgens (*continued*)
receptors
characterization, 1461
coactivators, 1461–1462
effects of osteocytes, 99
expression
localization, 760–761
regulation, 761–762
function, 1462
molecular mechanisms, 757–758
response element, 1461
signaling pathway, 758–760
in women
animal models, 1458–1459
levels, 1455–1456
skeletal role, 1456–1457
treatment effects, 1457–1458
Angiofibroma, 1059–1060
Angiogenesis
effects of OPN, 243–244
effects of VEGF, 893
Animal models
androgen studies, 1458–1459
CaR therapy, 1348–1350
diabetic studies, 741–742, 746–747
FGF, 831–833
glucocorticoids, 1644–1645
gonadal hormone-deficient, 659
hyperresorptive diseases, 1362–1364
IFG-I bone effects, 1446–1448
MEN-1 syndrome, 1060
OI, 1180–1181
osteopetrosis, 1222–1223
osteoporosis
congenic/recombinant strains, 1650–1651
congenic strains, 1651–1653
convenience, 1639
criteria, 1636
glucocorticoid, 1639
inbred strains, 1648–1650
male, 1645–1647
Mg studies, 352–353
postmenopausal, 1637–1640
recombinant/congenic strains, 1650–1651
recombinant strains, 1650–1651
regulatory recommendations, 1635
specific species, 1639–1644
in vivo experimentation, 1635
ovariectomized, effects of HRT, 659–660
PTH anabolic properties, 1307–1308
skeletal effects of thyroid hormones, 712–713
vitamin D deficiency, 1205
Anorexia nervosa, 1450
Antisense, inhibition of PTHrP, 1087
AP-1. *see* Activator proteins
Apical ectodermal ridge, 38
Apoplipoprotein E, 1267
Apoptosis
chondrocytes, 155–156
effects of PTH/PTHrP, 497
general features, 151, 153
osteoblasts
glucocorticoid-induction, 161
PTH regulation, 161–162
role of
androgens, 762–763
bisphosphonates, 162–163
estrogens, 665
sex steroid regulation, 160–161
osteoclasts
description, 156–159
glucocorticoid-induction, 161
role of bisphosphonates, 162–163
sex steroid regulation, 160–161
osteocytes
description, 95–96, 159–160
glucocorticoid-induction, 161
PTH regulation, 161–162
role of bisphosphonates, 162–163
sex steroid regulation, 160–161
regulation, 151, 153–155
TGFβ-induced, 905
Arachidonic acid derivatives, 198–199
Areal moment of inertia, 27
Aromatase
deficiency in men, 1469–1472
genes, knockout models, 1472
Arthritis
osteo
stem cell changes and, 73–74
stem cell therapy, 74
tissue engineering for, 74
psoriatic, 598
rheumatoid, 895–896
Aspartic proteinases, 258
Assays
calcium phosphate, 1510
CHIP, 1514–1515
DEAE-dextran, 1510
DNAseI footprinting, 1513–1514
electroporation, 1510
EMSA, 1513
ICTP, 220
lipopfection, 1510
nuclear run-on, 1508
viral vectors, 1510–1511
in vivo footprinting, 1514
Association studies, 1262
ATP. *see* Adenosine triphosphate
ATPases, bisphosphonate inhibition, 1366–1367
AUF1 protein, 414–415
Autosomal-dominant hypophosphatemic rickets, 330–331
Avian models, 1639–1640

B

BABMI, 933
Bapx1, 86
Barakat syndrome, 1111
Basic multicellular unit
biopsy, 1628
definition, 303
in osteoporosis, 1364
remodeling at

activation, 307–309
formation, 309–310
mineralization, 310–311
origination, 304–307
osteoporosis, 314
Basic structural unit, biopsy, 1628
Bcl-2, 153–155
Biglycan (PG-I), 226–227
Biochemical markers
bone formation, 1544–1547
bone resorption, 1547–1549
clinical use
osteoporosis, 1558–1560
Paget's disease, 1551, 1553–1554, 1556
primary hyperparathyroidism, 1556–1558
induced bone metabolism changes, 1561
variability, 1549–1551
Biomechanics
properties, 17–18
special properties, 22–24
stress-strain relationships, 18–20
Biopsy. *see* Transilial bone biopsy
Bisphosphonates. *see also specific drugs*
animal toxicology, 1377–1378
chemistry, 1361
development, 1361
distribution, 1374–1375
effects on
bone cell apoptosis, 162–163
collagen fragment processing, 217
fracture risks, 1376
OI, 1185–1186
osteolysis, 1088–1089
osteoporosis
glucocorticoid-induced, 1376
postmenopausal, 1375–1376
Paget's disease, 1376
remodeling, 315
tumor bone disease, 1376–1377
efficacy, 1375
general characteristics, 1361–1362
inhibition of
calcification, 1373–1374
osteoclasts, 1372–1373
intestinal absorption, 1374
nitrogen-containing
cellular level actions, 1369–1372
description, 1367
FPP synthase as target, 1367–1369
inhibition of
mevalonate pathway, 1369
osteoclast recruitment, 1372
osteoclasts, 1372–1373
metabolism to ATP analogues, 1367–1369
protein isoprenylation blocking, mechanism, 1369–1371
sterol synthesis blocking mechanism, 1369–1371
osteoporosis therapy models, 1362–1364
pharmacological effects
action *in vivo,* evidence, 1371
inhibition of bone resorption
general concepts, 1366
models, 1362–1365
molecular level, 1366–1367
relative potency, 1365–1366
physicochemical effects, 1362
PTH therapy and, 1314
renal clearance, 1375
response, genetic determinants, 1269
Blomstrand's lethal chondrodysplasia
affected infants, 1111–1112
characterization, 1125–1127
PTH-Rc mutation in, 440
Blood-calcium homeostasis, 99
BM40, 228–229
BMD. *see* Bone mineral density
BMP. *see* Bone morphogenetic proteins
BMU. *see* Basic multicellular unit
Bone
-Al, interaction, 360–361
architecture
CT imaging
coherent-scatter, 1611
&grm;, 1612–1615
high-resolution, 1600–1604
peripheral quantitative, 1611
ultra-high-resolution, 1611
volumetric, 1609–1610
gonadal hormone-deficient models, 659
microradiography, 1605–1606
MRI microscopy, 1615–1618
radiography, 1600–1604
biology, cells affecting, 1497–1498
biomechanics
mechanotransduction, 25–26
properties, 17–18
special properties, 22–24
stress-strain relationships, 18–20
biopsy, transilial
in clinical research, 1633
complications, 1617
histology, 1628–1629
morphometric methods, 1629, 1631
in patient care, 1632
practical considerations, 1633
procedure, 1615–1616
reference data, 1631–1632
regions evaluated, 1629, 1631
specimen processing, 1627
timing, 1633
-boron, interaction, 361
calcium
function, 1327–1328
storage, 1326–1327
cartilage to, 12
-Cd, interaction, 361–362
cells (*see specific bone cells)*
cellular interactions, studies, 1536
composition, 3, 24
connexin expression, 292–293
cortical (*see* Cortical bone)
-Cr, interaction, 362–363
CSF-1 expression in, 947
degradation, 135–136
diabetic nephropathy in, 746

Bone (*continued*)
discs, etopically implanted, 243–244
effects of
androgens, 1458–1459
CGRP, 784–786
C-type natriuretic peptides, 1009
glucocorticoids, 731–733
GM-CSF, 950–952
IL-4, 965
IL-6, 966–971
IL-7, 966
INF-γ, 964
leukotrienes, 988–989
LIF, 966–971
mechanical loading, 1482–1483
neuropeptide Y, 786
neurotransmitters, 787–789
neurotrophins, 789–790
NSAIDs, 988
opioid peptides, 787
PACAP, 784
PTH therapy, 1315–1316
SERMs, 681
somatostatin, 787
substance P, 786
TNF, 963–964
VEGF, 893–896
VIP, 781–783
evolution, Ca and, 1325–1326, 1328–1329
formation
basic aspects, 1544
biopsy, 1628
description, 12
effects of
androgens, 764–766
FGF, 836–838
glucocorticoids, 727–729
prostaglandin receptors, 986–987
PTH/PTHrP, 499–500
factors effecting, 1550–1551
markers, 1544–1547
nodule, 711
in osteoporosis, 1277–1278
in perichondrium, 52
rates, 1631
resorption, coupling to, 1277, 1543–1544
role of PTH/PTHrP, 1120–1122
fractures (*see* Fractures)
GH mechanisms, 1442–1443
growth
appositional, 43–44
description, 9–11
effects of IGF-I, 1446–1447
homeostasis, role of CaR, 377–379
innervation, 781
intramembranous, formation, 42–43
loss
after decreased mechanical usage, 1638
animal models, 1637
effects of IL-1, 962
as fracture risk marker, 1296–1298
glucocorticoid-induced, 724–727
ovariectomy-induced, 699–702
posttransplantation, 1561–1562
prediction, biochemical markers for, 1558
rate of, 1275–1276
role of prostaglandins, 987–988
T-4 therapy, 714–715
mechanical loading, 987
metabolism
biochemical markers, 1316
effects of
androgens, 766–767
kinins, 776–778
leptin, 790
metastases
osteoblastic, 1097–1098
osteolytic, 1099–1101
role of TGFβ, 911–912
mineral phase, 359
NO in
associated pathology, 1007
isoform occurrence, 998–999
molecular targets, 1006
therapeutic implications, 1007–1008
noncollagen proteins of, 731–732
organ cultures (*see* Tissues, cultures)
organic matrix
ectopic calcifications, 235
gene expression control by, 233–235
glycoproteins, 228–232, 235
hyaluronan, 228
proteoglycans, 225–228
serum proteins, 232–233
Pb content, 359
-Pb interaction, 363–365
physiology
dynamics of, 303–304
progesterone receptors, 668–669
plasminogen activators in, 257–258
proteases, effects of PTH/PTHrP, 498
PTH in, carboxy-terminal specific receptors, 389–400
PTH/PTHrP expression in, 493–494
puriergic receptors, 296–297
remodeling (*see* Remodeling, bone)
resorption (*see* Resorption, bone)
-Si, interaction, 365
-Sr, interaction, 365–366
strength, fracture correlation, 28–29
structural macromolecules
analysis, 1491–1493
Cis regulation, 1493–1494
target tissues, role of 1α,25(OH)$_2$ vitamin D$_3$, 574–575
tissue, effects of neurons, 780
trabecular (*see* Trabecular bone)
turnover (*see* Turnover, bone)
woven, bone remodeling *vs.,* 315
Bone-forming cells
cadherin association, 290
effects of TGFβ, 905
role of connexin43 gap junctions, 294
Bone-lining cells. *see* Lining cells
Bone marrow cells, role in remodeling, 311

Bone mass
  effects of
    amylin, 648
    CGRP, 648
    IDDM, 744–745
    insulin, 743–746
    Mg, 349–350
    NIDDM, 745
    raloxifene, 682–683
    sex steroids, 658
    tamoxifen, 685
    TNF, 963–964
  hyperinsulinemia and, 745
  maintenance genes affecting, 1494–1496
  peak, 1275–1276
  regulation, inheritance patterns, 1261
Bone matrix proteins, 498
Bone mineral density. *see also* Osteoporosis
  anabolic agents, 1306
  anti-resorptive agents, 1306
  genetic determinants
    effects on
      bisphosphonate response, 1269
      calcium absorption, 1269
      clinical practice, 1269–1270
      HRT response, 1269
      vitamin D absorption, 1269
  measurement
    densitometry techniques
      clinical use, 1582–1583
      conflicting findings, 1583–1584
      descriptions, 1574–1580
      discrepancies in, 1587–1588
      DXA, peripheral, 1577–1578
      ethnic-specific databases, 1590–1593
      evaluating, 1580–1581
      gender-specific databases, 1590–1593
      for osteoporosis diagnosis, 1589–1590
      peripheral measurements, interpretation, 1583
      reference ranges, 1580–1582
      relationship between, 1580
      reporting prevalence *vs.* risks, 1589–1590
    effects of
      estrogen therapy, 1401
      GH, 1443–1446
      NSAIDs, 988
      PTH therapies, 1313
    genetic determinants
      apoplipoprotein E, 1267
      calcitonin receptor, 1267–1268
      COLIA1, COLIA2 genes, 1265–1266
      estrogen receptor genes, 1266
      evidence for, 1260–1261
      interleukin genes, 1267
      TGFβ genes, 1266
      VDR gene, 1264–1266
    -habitual loading, relationship
      athletes, 1478–1479
      estimating, 1479–1480
      nonathletes, 1479
    high-resolution CT, 1607–1609
    MRI, 1615–1618
    serial, 1593–1596
    ultrasound, 1292–1293
  osteoporosis
    associated fractures, 1292–1293
    diagnosis, 1573–1574
    risk factor
      epidemiology, 1305
      history, 1647–1648
  pQCT, 1611
  volumetric CT, 1609–1610
Bone morphogenetic proteins
  biology, 922–924
  connexin regulation by, 293–294
  extracellular regulators, 938–939
  identification, 929
  intracellular signaling
    SMADS
      in cytoplasm, 935–936
      in nucleus, 936
      regulation, 937–938
      structure, 933–935
      target genes, 936–937
  mechanism, 919–920
  osteoblastic bone metastases and, 1098
  pseudoreceptors, 933
  receptors
    activation mechanism, 932
    mutations *in vivo,* 933
    serine/threonine kinase, 929–930
    type I, 930–931
    type II, 931–932
  therapeutic applications, 920–922
Bone sialoprotein
  description, 231–232
  levels in metastatic bone disease, 1584
  levels in PHPT, 1557–1558
  as resorption marker, 1549
Boron-bone, interaction, 361
Brandykinin receptors
  characterization, 776
  effects on resorption, 778
Breast. *see also* Mammary gland
  cancer cells
    biochemical bone markers for, 1583
    effects of calcitonin, 1428
    VDR activity, 592
  effects of
    raloxifene, 684
    SERMs, 682
    tamoxifen, 686
BSP. *see* Bone sialoprotein

C

Cadherins
  association with bone-forming cells, 290
  cell–cell contact, 288
  characterization, 268–269
  role in
    cartilage, 271–272
    osteoblast functions, 278
    osteoclastic resorption, 271–272

Cadherins (*continued*)
osteogenic cells, 289–290
skeletal development, 288–289
Cadmium-bone, interaction, 361–362
Calbindin, effects of vitamin D-3, 577–578
Calcifications
ectopic, 235
inhibition by bisphosphonates, 1373–1374
Calcinosis, tumoral, 334
Calcipotriol, 1412–1413
Calcitonin
associated clinical conditions, 629–633
cellular actions, 1426–1428
discovery, 1423
effects on
osteoblasts, 147
osteoclasts, 1009
remodeling, 315
genes
bioactions, 624–628
biochemistry, 619–621
carboxyl-terminal peptides, 629
distribution, 624
family members, 634–635
postranslational processing, 621–622
related peptides I and II, 633–634
structure, 1423–1424
mature
description, 624
measuring, 628–629
molecular actions, 1431–1432
osteoporosis pathophysiology and, 1283
peptide structure, 1424–1426
pharmacology
analgesic effects, 1434
clinical applications, 1432
general concepts, 1431
side effects, 1434
therapeutics, 1434–1435
physiopathology, 619
receptor family, 1429, 1431
role in osteoclast apoptosis, 159
secretion, 622–624, 628
Calcitonin gene-related peptides
bone microenvironment access, 643
characterization, 641, 784–786
effects on
bone mass, 648
Ca metabolism *in vivo,* 649–650
osteoblasts, 646–647
osteoclasts, 643–645
extraskeletal actions, 642–643
production, 1423–1424
receptors, 649–650
regulation, 1424
structure, 1424–1426
Calcitonin receptors
downregulation, homologous, 613–614
function, 603–604
genes
bioactivity, 635
organization, 609–611
regulation, 611–613
role in BMD, 1267–1268
identification, 603
proteins
isoforms, 607–609
signaling, 604–607
structure, 604–607
Calcitriol
levels, effects of vitamin D-3, 1204
PTH therapy and, 1314–1315
Calcium
absorption, genetic determinants, 1269
balance, effects of PTH therapy, 1316–1317
-blood homeostasis, role of osteocytes, 99
in bone
evolution and, 1325–1326
function, 1326–1327
storage, 1326–1327
channels, 538–539
effects of calcitonin, 624–625
effects of fluoride, 1388–1390
effects on PTH mRNA, 414–415
environmental, 1325
excretion
regulation by PTH, 487–488
regulation by PTHR signal transduction, 488–489
extracellular, sensing receptor
biochemical properties, 373–374
cloning, 371–372
effects on Mg, 381, 382
GABA as, 383
gene regulation, 374–375
G protein-coupled receptor association, 372–373
intracellular signaling by, 374
as nutrient sensor, 381–383
predicted structure, 372–373
role in
Ca homeostasis, 375–379
Ca metabolism, 379–381
water metabolism, 379–381
homeostasis, effects of PTH, 1339
intake
manipulating, 1329–1331
nutritional factors, 1333
osteoporosis therapy and, 1335
intercellular signaling
description, 295–296
in skeletal tissue, 297–298
metabolism *in vivo,* effects of
amylin, 649–650
CGRP, 649–650
pseudohypoparathyroidism therapy, 1154–1156
reabsorption, 485–487
requirements
defining, 1331–1332
evolutionary factors, 1328–1329
insurance of, 1328
studies, interpreting, 1334–1335
role in PTH gene regulation, 412–413
serum, effects of PTH therapy, 1317
systemic plasma regulation, 1035–1036
therapy, for hypophosphatasia, 1238

toxicity, 1333–1334
transport
effects of glucocorticoids, 725–726
placental, 522–523
urinary, 1317
Calcium phosphate, 1510
Calcium receptors
description, 1340
expression, 1340
pharmacology
calcilytics, 1342–1344
calcimimetics, 1341–1342
effects on circulating PTH, 1353–1354
effects on skeletal tissue, 1355
for hyperparathyroidism, 1345
ligand selectivity, 1344–1345
primary hyperparathyroidism, 1345–1348
secondary hyperparathyroidism
animal models, 1348–1350
clinical studies, 1350–1353
Calcium-sensing receptor sensor gene
abnormalities, 1108
function, 1024
mutations, role in FBHH, 1038–1039
Calreticulin, 411–412
Camaruti-Englemann disease, 1266
cAMP, signaling response, 391–392
Canalicular fluid flow, 100–101
Cancellous bone
bone remodeling and, 313
description, 3
effects of ovariectomy, 659
Cancer
bone metastasis
pathologic models, 1536
role of TGFβ, 911–912
breast cells
effects of calcitonin, 1428
VDR activity, 592
cells, characterization, 1017–1018
medullary thyroid
calcitonin-associated, 629–630
genetic pathogenesis, 1025
MEN2 syndrome-associated, 1069
parathyroid, 1025
prostate, 1098
vitamin D therapy and, 1412
Carcinoids, MEN1 syndrome-associated, 1051
Cardiovascular system. *see also* Heart; Vasculature
effects of
amylin, 642
CGRP, 642
PTH, 473–475
SERMs, 681–682
PTH regulation, 531–532
Cartilage
articular, 51–52
to bone, 12
Ca homeostasis, role of CaR, 377–379
connexin expression, 292–293
growth capacity, 43–44
immunoglobulins, 279–281
role of nonintegrin adhesion molecules, 279–281
structural macromolecules, 1493–1494
Caspases, 153–155
CaSR. *see* Calcium-sensing receptor sensor gene
CASTing method, 1518
CAT. *see* Escherichia coli CAT gene
Catecholamines, effects on bone, 787–788
Cathepsin K, role in bone resorption, 216
Cat models, 1642–1643
Cbfa 1
characterization, 85
expression, 85–87
identification, 83–84
regulation, 69–71
regulator factors, 177–183
role in bone formation, 234
role in osteoblast differentiation, 83–85
transgenic models, 1494–1496
c-Cbl, role in osteoclast adhesion, 145–146
C cells
Ca homeostasis, 375–376
effects of calcium, 1341–1342
neoplastic transformation, 1067–1068
CD44
cartilage expression, 280
characterization, 97, 269
expression, 98
osteoblast expression, 278
Cell adhesion molecules. *see also specific molecule*
cell–cell communications, 291–295
cell–cell contact via, 288–289
effects on osteoclasts, 290–291
families, 266, 268–269
role in
cartilage, 279–281
osteoblast function, 276–279
osteoclast clear zone, 272–274
osteoclast development, 272
role of VEGF-A, 890–891
Cell cultures
coculture systems, 1536
current techniques, 1531–1534
Cell line hierarchies, 60–61
Cell organization, control of, 287
Cell proliferation, effects of PTH/PTHrP, 497
Cell shape, effects of PTH/PTHrP, 497
Cell survival, role of VEGF-A, 890
Central nervous system
effects of calcitonin, 626
PTH/PTHrP expression in, 537–540
c-fos knockout mutation, 1223
CFU. *see* Colony forming factors
CGRP. *see* Calcitonin gene-related peptides
CHIP. *see* Chromation immunoprecipitation
Chondrocytes
apoptosis, 155–156
effects of androgens, 767–769
effects of thyroid hormones, 712
growth plate, proliferation/differentiation
articular cartilage, 51–52
mediators, 46–51
intercellular Ca signaling, 297–298

Chondrocytes (*continued*)
role of integrins, 279
Chondrodysplasia. *see* Blomstrand's lethal chondrodysplasia; Jansen's metaphyseal chondrodysplasia
Chondrodystophies, FGF in, 834
Chondrogenesis
coupling, 52–53
effects of VEGF, 893
Chromatin organization, 171–174
Chromation immunoprecipitation assays, 1514–1515
Chromium-bone, interaction, 362–363
Circadian, effects on bone turnover, 1550–1551
Cloning, transcriptional regulatory molecules, 1515–1518
Collagenases
characterization, 253–257
genes in bone, 838–839
type IV (*see* Gelatinases)
Collagen-binding proteins, role in osteoclast functions, 274–275
Collagenoma, *MEN1* gene and, 1059–1060
Collagens
definition, 189
fibril-associated, 189–190
forming sheets, 190
receptors, integrin-binding, 277
type I
in bone, percentage, 3
Cis regulation, 1491
cross-links
changes, age-related, 214–215
component release *in vitro,* 213–214
formation, 211–212
immunoassays, 1548–1549
location, 213–214
spectrum, 213–214
structure, 212–213
degradative pathways, 216–217
effects of glucocorticoids, 731
effects of thyroid hormones, 709–710
fibrillognenesis, 193
genes
mutations, consequences, 193
posttranslational modifications, 192–193
prevalence, 189
role in BMD, 1265–1266
structure, 190
transcriptional regulation, 200–204
translational modifications, 192–193
metabolism, 218–220
mutant gene transgenic models, 1492–1493
phase biomechanical effects, 24
promotors
function analysis, 1494–1496
role in osteoblast differentiation, 87
sequence analysis, 1493
structural integrity, 1491
synthesis
cytokine regulation of, 197–199
description, 190–192
effects of INF-γ, 964
effects of TNF, 963–964
growth factor regulation of, 194–197
hormone regulation of, 199–200
PGE regulation of, 198–199
vitamin regulation of, 199–200
type II
function analysis, 1493–1496
promotors, role in osteoblast differentiation, 87
sequence analysis, 1493
transportation factors, 1494
type IX, transgenic models, 1493
type X, analysis, 1492–1493
Colony forming factors, CFU-F
assays, 60–61
effects of aging, 73–74
regulation, 69–71
Colony-stimulating factors. *see also* Granulocyte-colony stimulating factor
CSF-1
biochemistry, 944
description, 943–944
expression, 944–945, 947
molecular forms, 947–948
regulation, 944–945
role in
osteoclast formation, 946–947
osteoporosis, 944–945, 1279
Communications
cell–cell, 291–295
intercellular
by CaR, 374
cell–cell adhesion, 298
description, 295–298
Compression
causes, 19
forces, 26–28
Computed tomography
coherent-scatter, 1611
μ, 1612–1615
high-resolution, 1607–1609
peripheral quantitative, 1611
ultra-high-resolution, 1611
volumetric, 1609–1610
Connexins
in bone, 292–293
in cartilage, 292–293
Cx43
location, 293
modulation, 294–295
effects on bone-forming cells, 294
in osteoclastogenesis, 295
regulation, 293–294
in skeletal development, 293
Cortical bone
bone remodeling and, 313
description, 3
effects of fluoride, 1394
strength of, 22–23
thickness, effects of aging, 27–28
Corticosteroids
effects on bone turnover, 1550
induced osteoporosis, 314
role in collagen synthesis, 199
Cortisone, hypophosphatasia-associated, 1238
COX. *see* Cyclooxgygenases

Cranial sutures, 42–43
Cranial vault, 38
Craniofacial disorders, 43
Craniosynostosis, FGF in, 834–835
Creep, 23
Cre-ERT system, 1498–1499
Cre/loxP recombinase system, 1498–1499
Critical functions rule, 12
Crouzon syndrome, 43
CSF. *see* Colony-stimulating factors
c-Src
  effects on osteoclasts, 143–144
  -Pyk2, role in osteoclast adhesion, 144–147
CT. *see* Computed tomography
C-terminal peptides, as collagen formation markers, 218
C-terminal telopeptide of collagen type I
  assay, as bone resorption marker, 220
  circadian variability, 1550–1551
  description, 1549
  levels in immobilization, 1550
  levels in pregnancy, 1550
CTR. *see* Calcitonin receptors
CTX-1. *see* C-terminal telopeptide of collagen type I
Culture shock proteins, 228–229
Cyclin D1/*PRAD1,* 1019–1021
Cyclooxgygenases
  COX-2
    expression, importance, 982
    mouse model, 984–985
    transcriptional regulation, 983
  isoforms, 980–981
  nomenclature, 979–980
  substrate, 981
Cyclosporines, 1561
Cysteine
  PTH1–Rc, characterization, 439–440
  rich FGF receptors, 831
Cysteine proteinases, 258
Cytochrome P450, 1413, 1415–1416
Cytokines. *see also* specific cytokines
  effects on bone cell function, 964
  inhibitors, role in ovariectomy-induced bone loss, 699–702
  -kinins, interactions, 780
  -neuropeptides, interactions, 780
  osteclastogenic
    effects of
      estrogen, 695–697
      menopause, 697–698
      ovariectomy, 699
    role in formation, 693–695
  role in osteoblast development, 71–73
Cytoskeleton
  osteoclasts
    organization, 143–144
    in resorption cycle, 132–133
  osteocytes, 98

D

DEAE. *see* Diethylaminoethyl-dextran
Deafness, hypoparathyroidism-associated, 1110
Death receptors, 153
Decorin (PG-II), 226–227
Degenerative joint disease, 156
5α-Dehydrotestosterone. *see* Androgens
Denervation, experimental, 790–791
Dentin Matrix protein-1, 232
Dentition, hypophosphatasia-associated, 1236
Dent's disease, 333
Deoxypyridinoline
  levels in PHPT, 1548
  as resorption marker, 1548
Desitometry. *see also* specific techniques
  conflicting findings, 1583–1584
  evaluating, 1580–1581
  peripheral measurements, interpretation, 1583
  reference ranges, 1580–1582
  relationship between, 1580
  serial measurements, 1593–1596
  T scores
    definition, 1583–1584
    discrepancies in, 1587–1588
    ethnic-specific databases, 1590–1593
    gender-specific databases, 1590–1593
    for osteoporosis diagnosis, 1589–1590
    reporting prevalence *vs.* risks, 1589–1590
  types, 1574–1578
Diabetes mellitus
  associated bone disease, 750–751
  insulin dependent (type 1)
    animal models, 746–647
    effects on
      bone mass, 744–745
      fractures, 745–746
      remodeling, 743–744
      skeletal growth, 741–743
  noninsulin dependent (type 2)
    animal models, 746–647
    effects on
      bone mass, 745
      fractures, 745–746
      remodeling, 744
Diethylaminoethyl-dextran, 1510
Differentiation, sclerotome, 37
DiGeorge syndrome, 1109–1110
1,25–Dihydroxyvitamin D. *see* Vitamin D
Dlx5, role in osteoblast differentiation, 86
DMP-1. *see* Dentin Matrix protein-1
DNA
  binding
    domains, 1516–1518
    site selection, 1518–1519
  genomic, packaging, 171–174
  probes for expression library screening, 1515–1516
  -protein
    incubation, 1518–1519
    interaction at PTH mRNA, 414–417
    interactions *in vitro* and *in vivo,* 1512–1515
DNAseI footprinting assay, 1513–1514
Dog models, 1643
Dopamine transporters, 789
Dual X-ray absorptiometry
  BMD, 1291–1293

Dual X-ray absorptiometry (*continued*)
general description, 1574–1576
and other techniques, comparisons, 1580
peripheral measurements, 1577–1578, 1583
reference ranges, 1581–1583
serial measurements, 1593–1596
Ductility, definition, 22
DXA. *see* Dual X-ray absorptiometry
Dynein light chain, 415–416
Dysplasias, skeletal, 40

E

ECD. *see* Extracellular ligand binding motif
Edothelin, 1009
Ehlers-Danlos syndrome, 193
Elasticity, 21–22
Electric fields, 1483
Electrophoretic mobility gel shift assays, 1513
Electroporation, description, 1510
Embryos
extraembryonic tissues, 523–524
mammary development, 517–518
membranes, 524
skeletal development
axial, 34–38
chondrogenesis, coupling, 52–53
intramembranous, formation, 42–52
limb initiation, 38–40
mesenchymal condensation, 40–42
morphology, 33–34
osteogenesis, coupling, 52–53
patterning, 40–42
role of BMP, 922–923
EMSA. *see* Electrophoretic mobility gel shift assays
Endocrine system
tumors, *MEN1* gene and, 1055, 1058–1059
vitamin D
description, 548–549
genetics, 556–558
Endothelin-1, osteoblastic bone metastases and, 1098
Endothelin-A receptor, in bone formation, 38
Epidermal growth factor
effects on VEGF-A expression, 891
phosphate transport regulation, 324–326
Epiphysis, vascular invasion, 52–53
Epithelial cells-mesenchymal interactions, 1122
ERK. *see* Estrogen receptor knockout
Escherichia coli CAT gene, 1511
Estrogen receptor knockout, role in osteoclast apoptosis, 158–159
Estrogens
coregulators, 657
deficiency, role in
as fracture risk marker, 1295
osteoporosis pathophysiology, 1278
disruption, rodent models, 660–661
effects of osteocytes, 99
effects on
bone mass, 658
chondrocytes, 46–47
osteoblasts, 661–665
osteoclasts, 665–686
prostaglandin production, 984
PTH, 417–418
remodeling, 315
TNF, 963–964
VEGF-A expression, 892
gene expression in
bone cells, 657–658
skeletal tissue, 658
and growth spurts, 1469–1472
-mechanical loading, relationship, 1484
mechanism of action, 655–656
menstrual/estrus cyclicity, 1637
molecular actions, 1403–1405
osteoporosis pathophysiology and, 1280
pharmacology (*see also* Hormone replacement therapy)
administration routes, 1402–1403
biochemistry, 1401
BMD, 1401–1402
fracture risks, 1402
pseudohypoparathyroidism therapy, 1156
PTH therapy and, 1314
receptors
deficiency in men, 1468–1469
effects on skeletal phenotypes, 660
genes, knockout models, 1472
genes, role in BMD, 1266
isoforms, 656
response elements
mechanism, 677
molecular mechanisms, 663–664
role in bone cell apoptosis, 160–161
VDR_{nuc} response, 553–554
Ethnicity, effects on bone turnover, 1550
Evolution, bone, 1325–1326, 1328–1329
Exercise
-BMD, relationship
athletes, 1478–1479
estimating, 1479–1480
effects on bone turnover, 1551
postmenopausal bone loss and, 1484
skeletal adaptation
constraints, 1484–1485
effective mechanical loading, 1481–1482
models, 1480–1481
Extracellular ligand binding motif, 375
Extracellular matrix, 1182–1184
Extrathyroidal neuroendocrine cell hyperplasia, 629
Extrathyroidal neuroendocrine tumors, 630

F

Facial skeleton, 38
Fanconi's syndrome, 333–334
Farnesyl diphosphate synthase
cellular level actions, 1369
inhibition by N-BP, 1367
as N-BP target, 1369
protein isoprenylation blocking, mechanism, 1369–1371
sterol synthesis blocking mechanism, 1369–1371
Farwestern blot, 1516
Fatigue failure, 28

Ferret models, 1642–1643
FGF. *see* Fibroblast growth factors
Fibrillins, description, 231
Fibrillognenesis, collagen, 193
Fibroblast growth factors
  basic, role in collagen synthesis, 197
  bioactive polypeptides, 826–827
  effects on
    bone formation, 836–837
    bone resorption, 836–837
    collagenase gene, 838–839
    limb development, 39
    osteoclastogenesis, 839
  extracellular, regulation, 830–831
  FGF-23 gene, 331
  -heparin, interaction, 839–840
  osteoblastic bone metastases and, 1098
  production, 835–836
  receptors
    in chondrodystophies, 834
    in craniosynostosis, 834–835
    effects on chondrocytes, 50–51
    expression in bone, 833–834
    types, 830–831
  regulation, 835–838
  role in osteoblast apoptosis, 160
  signaling, 838
  synthesis *de nova,* 827–830
  trangenic animal model systems, 831–833
Fibronectin, description, 231
Fibrous dysplasia
  clinical presentation, 1165–1166
  description, 1165
  genetics, 1168
  management, 1173
  pathogenesis, 1170–1173
  pathology, 1169–1170
  radiological findings, 1168–1169
Fluid flow
  cell stretch *vs.,* 102–103
  in vitro, osteocytes response, 101–102
Fluoride
  characterization, 1387
  effects on remodeling, 315
  pharmacology
    BMD trials, 1306
    bone cells, 1389
    calcium effects, 1388–1390
    food effects, 1388
    GI absorption, 1387–1388
    molecular mechanisms of action, 1389
    osteoporosis prevention, 1390–1392
    renal excretion, 1388–1389
    serum levels, 1388
    side effects, 1389–1390
  stress fractures and, 1389–1390
  therapy
    combined regimes, 1394–1395
    osteoporosis
      corticoid-induced, 1394
      male, 1394
      postmenopausal, 1392–1394
      problems/perspectives, 1396–1397
  in water, 1391
Fluorochrome labels, 1629
Focal adhesion kinase, 1431
Footprinting assay, 1514
Foregut carcinoid, 1049, 1055
FosB regulation, 71
FPP. *see* Farnesyl diphosphate synthase
Fractures
  bone behavior and, 26–28
  effects of tamoxifen, 685
  effects on bone turnover, 1550
  hip, fluoride-induced, 1394
  osteoporosis-associated
    animal models, 1638
    bone turnover in, 1558–1559
    genetic factors, 1260–1261
    prediction, 1559
    risk factors
      BMD, 1292–1293
      bone turnover, 1296–1298
      clinical, 1291–1292
      combined assessment, 1298
      hormonal markers, 1293–1294
      menopause, 1294–1296
  preventing, 29–30
  repair
    BMP application, 920–921
    role of FGF, 838
    role of PDGF, 821
  risks
    bone strength correlation, 28–29
    effects of
      bisphosphonates, 1376
      estrogen therapy, 1402
      PTH therapies, 1309–1310
  role of diabetes, 745–746
  stress
    fluoride-induced, 1389–1390
    occurance, 28
  -stroke, association, 791–792
FSD. *see* Functional secretory domain
Functional secretory domain, 130–132

G

GABA. *see* γ-Aminobutyric acid
β-Galactosidase/LacZ, 1512
Galactosyl hydroxylysine, 1549
Gap junctions
  in bone, 94
  effects of PTH/PTHrP, 497
  formation by bone-lining cells, 278
  as intercellular channels, 291–292
  role in bone remodeling, 294–295
  in skeletal cells, 292–293
Gastrinoma, MEN1 syndrome-associated, 1051
Gastrointestinal tract
  bisphosphonate absorption by, 1374
  effects of calcitonin, 627
  fluoride absorption by, 1387–1388
  phosphorus absorption by, 322

Gelatinases, 252–253
GEM2, abnormalities, 1108–1109
Genes. *see also specific genes*
  chromatin organization, 171–174
  expression, nuclear architecture link, 176–179
  sequence organization, 170
Gene therapy
  BMP, 921–922
  methods, 74–76
  for OI, 1186–1187
Germline DNA-based tests, 1057
GFP. *see* Green fluorescent protein
GH. *see* Growth hormone
GHD. *see* Growth hormone, deficiency
Glucocorticoids
  effects on
    bone formation, 727–729
    bone loss, 724–727
    calcium transport, 725–726
    chondrocytes, 47
    gene expression in bone, 730–733
    osteoblasts, 727–730
    phosphorus transport, 725–726
    PTH function, 725–726
    resorption, 729–730
    vitamin D metabolism, 725–726
  induced bone metabolism changes, 1561
  induced osteonecrosis, 725–726
  induced osteoporosis
    animal models, 1639, 1644–1645
    description, first, 723
  pharmacology, 723–724
  phosphate transport regulation, 324–326
  receptors, effects of osteocytes, 99
  role in bone cell apoptosis, 161
  target cell metabolism, 730
Glucose, effects of calcitonin, 627
Glutamate
  effects on bone, 788–789
  receptors, effects on bone, 788–789
  transporter, effects on bone, 788–789
Glycoproteins
  description, 228
  RDG-containing, 229–230
  types, 228–232
GM-CSF. *see* Granulocyte-macrophage colony stimulating factor
GNA11, role in FBHH, 1038
Gonadal status, GH/IGF-I secretion and, 1442
G proteins
  characterization, 1170–1171
  gp130, role in osteoclast apoptosis, 158–159
  heterotrimeric, 1138–1140
  receptors
    CaR association, 372–373
    characterization, 981–982
  role in
    fibrous dysplasia, 1171–1172
    McCune-Albright syndrome, 1171–1172
Granulocyte-colony stimulating factor
  biochemistry, 952
  function, 952
Granulocyte-macrophage colony stimulating factor
  biochemistry, 950
  in bone, effects of PTH/PTHrP, 496
  effects on bone, 950–952
  in osteoblasts, effects of estrogen, 662–663
  role in HHM, 1095
Green fluorescent protein, 1496–1497, 1512
Growth factor receptors, 155
Growth hormone
  deficiency, effects of GH treatment, 1443–1445
  effects on
    chondrocytes, 46
    OI, 1186
    PTH, 468–469
  function, 1441
  osteoporosis treatment
    administration, 1445
    clinical trials, 1445–1446
    effects on BMD, 1443–1446
    mechanism on bone, 1442–1443
    physiology, 1441–1442
  as reporter gene, 1512
  treatment, in GHD patients, 1443–1445
Growth plate
  chondrocyte processes
    articular cartilage, 51–52
    mediators
      local, 47–51
      systemic, 46–47
  description, 45
  vascular invasion, 52
Growth spurts, estrogen and, 1469–1472
Guinea pig models, 1642–1643

H

Hageman factor, 774
HAT. *see* Histone acetyltransferases
Heart
  effects of PTHrP, 536–537
  transplantation, associated bone loss, 1561–1562
Heparin
  effects on bone turnover, 1550
  -FGF, interaction, 839–840
Hereditary hypophosphatemic, 329–331
HHM. *see* Humoral hypercalcemia of malignancy syndrome
High-affinity receptors, 830–831
Hirschsprung's disease, MEN2 syndrome-associated, 1069–1070
Histone acetyltransferases, characterization, 174
HOB-01–CI, characterization, 97
Homeodomain proteins, role in *Cbfa 1* expression, 85–87
Homeostasis, phosphorus
  clinical disorders of, 326–334
  regulation, 321–326
Hormone replacement therapy
  effects on bone, 667–668
  effects on ovariectomized models, 659–660
  fluoride therapy with, 1394–1395
  response, genetic determinants, 1269
  skeletal response, animal models, 1637
HPA. *see* Hypothalamic-pituitary-gonadal axis
HRT. *see* Hormone replacement therapy

Humoral hypercalcemia of malignancy syndrome
associated cytokines, 1094–1095
discovery, 1093–1094
estrogen series prostaglandin association, 1095
PTH association, 1094
PTH-RP association, 1093–1095
Hyaluronan, description, 228
Hydrogen excretion, 491–492
1α-Hydroxylase, 1413
25–Hydroxylase, 558, 1413
Hydroxylysine glycosides, urinary, 219
Hydroxyproline
levels in heart transplants, 1561
as resorption marker, 1547–1548
urinary, as bone resorption marker, 219
Hypercalcemia
calcitonin therapy, 1432
effects of IL-1, 962
familial hypocalciuric
biochemistry, 1033–1034
characterization, 1024
clinical studies, 1033–1035
discovery, 1031–1032
genetic studies, 1037–1040
history, 1032–1033
management, 1040
molecular bases, 1039
pathophysiologic studies, 1035–1036
history, 1079
malignancy-associated
characteristics, 1079–1080
humoral (*see* Humoral hypercalcemia of malignancy syndrome)
leukocytosis-associated, 1101
RANKL/RANK/OPG system and, 1085–1087
rickets link, 328–329
role of PTHrP
approaches to controlling, 1086–1087
cytokine mediation, 1087–1088
mechanisms of action, 1083–1086
molecular aspects, 1080–1082
osteolysis, 1088–1089
Hypercalciuria, hereditary hypophosphatemic rickets with, 329–330
Hyperinsulinemia, 745
Hyperparathyroidism
neonatal severe
animal models, 1041
clinical characteristics, 1040–1041
discovery, 1032
genetic basis, 1041
management, 1041
primary
CaR therapy, 1345–1348
characterization, 1031
MEN1 syndrome and, 1048–1049
treatment, 1050–1051
secondary
CaR therapy
animal models, 1348–1350
clinical studies, 1350–1353
role of PTH, 416–417
Hyperplasia, extrathyroidal neuroendocrine cell, 629
Hyperresorptive diseases, 1362
Hypertension, effects of PTHrP, 536–537
Hyperthyroidism
animal models, 712–713
human studies, 713–714
Hypocalcemia, clinical features, 1150–1152
Hypoparathyroidism
associated syndromes, 1109–1112
autosomal dominant, 1107
autosomal recessive, 1107–1108
isolated, 1105–1106
pluriglandular autoimmune, 1112
x-linked recessive, 1109
Hypophosphatasia. *see also* Alkaline phosphatase
adult, 1234
biochemical defects, 1237–1238
childhood, 1233
clinical features, 1232–1234
diagnosis, 1234–1237
fibroblast studies, 1242–1243
genetic defects, 1237–1238
history, 1231
infantile, 1233
odonto, 1234
prenatal, 1232–1233, 1239
prognosis, 1239
pseudo, 1234
role of ALP, 1240–1243
treatments, 1238–1239
Hypothalamic-pituitary-gonadal axis, 725
Hypothyroidism
animal models, 712–713
human studies, 713
Hypoxia, 892
Hysteresis, 22

I

Iatrogenic fluorosis, 1390
ICTP assays, as bone resorption marker, 220
IDDM. *see under* Diabetes mellitus
Immobilization, effects on bone turnover, 1550
Immune system
effects of
PTH therapy, 1318
VDR, 592–594
vitamin D_3, 577
vitamin D therapy, 1412
Immunoglobulins, 269. *see also* specific molecules
Immunophenotyping, SCM, 62–65
Implantations, bone discs, 243–244
Indian hedgehog, 47–50
Inflammation
associated bone loss, role of prostaglandin, 987–988
process, 773
-sepsis, calcitonin-associated, 630–631, 633
systemic, calcitonin-associated, 633
Inflammatory joint disease, 156
Innervation, bone, 781
Insulin
as BMD marker, 745

Insulin (*continued*)
effects of vitamin D_3, 577
effects on
bone
animal models, 746–747
cells *in vitro,* 748–749
function, 743–746
skeletal growth, 741–743
Insulin-like growth factor
in bone, effects of FGF, 837
effects of glucocorticoids, 732
effects of PTH/PTHrP, 495
effects on
chondrocytes, 46
OI, 1186
PTH, 468–469
remodeling, 810–811
genes, 801–802
osteoporosis pathophysiology and, 1279
peptides, characterization, 801
regulation in bone cells, 802–803
role in
collagen synthesis, 196–197
osteoblast apoptosis, 160
secretion, age and, 1442
type I
as anorexia nervosa treatment, 1450
function, 1441
gonadal status and, 1444
osteoporosis treatment
animal models, 1446–1448
availability, 1446
clinical trials, 1448–1449
regulation in bone cells, 803–804
type II, regulation in bone cells, 804–806
Insulin-like growth factor-binding proteins
effects of thyroid hormones, 710
effects on pituitary function, 725
expression in bone cells, 806–809
osteoporosis pathophysiology and, 1279
proteases, 809–810
Insulinoma, MEN1 syndrome-associated, 1051
Integrins
binding, role in osteoclast apoptosis, 158
chondrocyte expression, 279
fibronectin expression, 277
$\alpha_v\beta_3$
effects of vitamin D_3, 578
role in
osteoblast apoptosis, 160
osteoclast function, 142–143, 274–275
osteoclast organization/motility, 143–144
resorption, 270–271
structure, 268
survival signaling via, 155
as therapeutic target, 275–276
osteoblast expression, 277
osteoclast expression, 144–145
Interaction cloning strategies, 1516
Interferon-γ
effects on bone, 964
role in collagen synthesis, 198
Interleukins
IL-1
effects on bone, 961–962
gene clusters, role in BMD, 1267
in osteoclasts
effects of estrogen, 662–663
effects on apoptosis, 158–159
effects on differentiation, 121
osteoporosis pathophysiology and, 1278–1279
production, effects of menopause, 698–699
role in
collagen synthesis, 198
MAH, 1087–1088
ovariectomy-induced bone loss, 699–702
IL-2, role in vitamin D_3 systems, 579
IL-3 (*see* Multicolony-stimulating factor)
IL-4
effects on bone, 965
osteoporosis pathophysiology and, 1278–1279
role in collagen synthesis, 198
IL-6
effects of FGF, 837
effects of PTH/PTHrP, 495
effects on bone, 966–971
gene, role in BMD, 1267
in osteoblasts
effects of estrogen, 495
role in apoptosis, 160
osteoporosis pathophysiology and, 1278–1279
role in
HHM, 1094–1095
MAH, 1087–1088
ovariectomy-induced bone loss, 699–702
IL-7, effects on bone, 966
IL-8, role in MAH, 1087–1088
IL-10, role in collagen synthesis, 198
IL-13, osteoporosis pathophysiology and, 1278–1279
Intestine
Ca homeostasis, role of CaR, 376–377
role of vitamin D_3, 574–575
In vivo footprinting assay, 1514
Ions
channels, effects of PTH/PTHrP, 497
excretion, effects of FBHH, 1034–1035

J

Jackson-Weiss syndrome, 43
Jansen's metaphyseal chondrodysplasia
characteristics, 1122–1123
role of PTHrP, 440

K

Kallikrein-kinin system, 774–776
Kallikreins, 774–775
Kearns-Sayre syndrome, 1111
Kidney
anomalies syndrome, 1110–1111
bisphosphonate clearance by, 1375
cells, PTHR signaling, 484–485

effects of
calcitonin, 627
PTH, 1118
1α,25(OH)$_2$ vitamin D$_3$, 575–576
fluoride excretion, 1388–1389
function, effects of FBHH, 1034–1035
osteodystrophy, vitamin D therapy, 1411–1412
phosphate transport, effects of PTH, 469–471
phosphorus excretion, 322–326
PTHrP expression, 483–485, 493
role in
FBHH, 1036–1037
Mg homeostasis, 341–342
processing collagen fragments, 216–217
transplantation, associated bone loss, 1562
Kininases, 775–778
Kininogens, 775
Kinins
characterization, 775
clinical observations, 791–792
-cytokines, interactions, 780
effects on bone metabolism, 776–778
-neuropeptides, interactions, 780
receptors, 778–779

L

Lactate, effects of calcitonin, 627
Lactation, 520–522
LacZ gene-β-galactosidase, 1512
Lead-bone, interaction, 359, 363–365
Leptin, 790
Leukemia inhibitory factor, 966–971
Leukocytosis, hypercalcemia-associated, 1101
Leukotrienes
effects on bone, 988–989
osteoporosis pathophysiology and, 1279
LIF. *see* Leukemia inhibitory factor
Limb
development, 155–156
initiation, 38–42
Lining cells
biopsy, 1629
function, 5
gap junction forming by, 278
strain sensing role, 277
structure, 5
Linkage analysis, 1261, 1263–1264
Lipomas, MEN1 syndrome-associated, 1050
Lipopfection, description, 1510
Liver
role in processing collagen fragments, 216–217
vitamin D clearance, 1416
Loading
effective mechanical
cellular basis, 1482–1483
characteristics, 1481–1482
habitual (*see* Exercise)
Long bone failures, 26–27
Low-affinity receptors, 830–831
Luciferase, 1511
Lysine, derived cross-links, 214–215

M

Macrophage colony-stimulating factor
effects on osteoclasts, 121
osteoporosis pathophysiology and, 1279
role in
osteoclast apoptosis, 157–159
osteoclast formation, 693–695
osteoclastogenesis, 111–112
ovariectomy-induced bone loss, 699–702
vitamin D$_3$ systems, 579
Magnesium
content, 339
depletion
effects on
parathyroid gland, 345–346
PTH, 347–348
vitamin D metabolism, 348–349
osteoporosis association, 349–351
effect on mineral formation, 353
excretion
regulation by PTH, 487–488
regulation by PTHR signal transduction, 488–489
homeostasis, renal role, 341–342
intestinal absorption, 339–341
intracellular, 343–344
osteoporosis association
animal models, 352–353
mechanism, 353
risk factors, 351–352
treatment, 351
RDA, 340
reabsorption, sites/mechanisms, 485–487
role in PTH secretion, 344–345
Magnetic resonance imaging, 1615–1618
MAH. *see* Hypercalcemia, malignancy-associated
Mammary gland. *see also* Breast
effects of PTH/PTrP
adolescent development, 518–520
embryonic development, 517–518
lactation, 520–522
pregnancy, 520–522
MAPKs. *see* Mitogen-activated protein kinases
Marrow stromal cells cultures, 1534–1535
Mass spectrometry, transcription factor analysis, 1519–1520
Matrix metalloproteinases. *see also specific types*
activation, 251–252
classification, 251
description, 52
MMP-1, role in organic matrix degradation, 134–135
role in osteoblast apoptosis, 160
subdivisions, 252–257
McCune-Albright syndrome
characteristics, 1166–1167
genetics, 1168
management, 1173
pathogenesis, 1170–1173
pathology, 1169–1170
radiological findings, 1168–1169
MCP-1. *see* Monocyte chemotactic peptide-1
M-CSF. *see* Macrophage colony-stimulating factor
MC3T3–E1 cells, 1506–1507

Mechanosensing, role of osteocytes, 100–101
Mechanosensory cells, osteocytes as, 99–100
Mechanotransduction, 25–26, 294
Megalin, 473
Melanin, 588
Melanoma cells, 245–246
MEN. *see* Multiple endocrine neoplasia
Men
  aromatase deficiency in, 1469–1472
  α-ER deficiency in, 1468–1469
  osteoporosis, animal models, 1639, 1645–1647
  skeleton
    interventional studies, 1474–1475
    observational studies, 1472–1473
Menopause
  associated-bone loss, effects of exercise, 1484
  associated osteoporosis
    animal models, 1637–1638
    biochemical markers, 1558–1560
    characterization, 693
    effects of bisphosphonates, 1375–1376
    fluoride treatment, 1392–1394
    PTH therapy for, 1315
    role of CSF-1, 95–96
    role of PTH, 1354–1355
  effects on
    bone-resorbing cytokines, 697–698
    cytokine production, 697–698
    IL-1 production, 698–699
  fracture risks and, 1484–1486
  natural, 1637
Menstrual cycle, 1551, 1637
Mental retardation, 1148
Mesangial cells, 817–818
Meschymal stem cells
  age-associated changes, 73–74
  disease-associated changes, 73–74
  -epithelial interactions, role of PTHrP, 1122
  immunophenotyping, 62–65
  nomenclature, 59
  therapeutic use, 74
Mesenchymal condensation, 40–42
Metalloproteinases, membrane type, 253
Metastatic bone disease, markers for, 1562–1564
Mevalonate pathway, inhibition by N-BP, 1369
MG63 cells, 1506
Microprojectile particle bombardment, description, 1511
Microtubules, effects of mynein light chain, 415–416
Mineral
  homeostasis, impaired mechanism, 347–348
  opposition rate, 1631
Mineralization
  effects of Mg, 353
  lag time, 1631
  mechanism, 4–5
  osteoid, formation, 1182–1184
  remodeling-associated, 310–311
  skeleton, effects on analysis, 12–13
  surface, 1631
Mitochondrial trifunctional protein deficiency syndrome, 1111
Mitogen-activated protein kinases, 428–429
Mitogenesis, VEGF-A, 889–890
MLO-Y4, 97
MMPs. *see* Matrix metalloproteinases
Monocyte chemotactic peptide-1, 964
Mouse
  COX-2 null model, 984–985
  ER $\alpha\beta$ knockout, 660–661
  human link, 1654
  knockout, 1472
  NOS-gene deficient, 1004–1006
  NSHPT models, 1041
  OPN-deficient, 242
  osteopetrotic phenotype, resolution, 948–949
  osteoporosis models, 1640–1641
  PR gene disruption in, 661
  RANKL/RANK deficient, 115–116
  TGFβ knockout, 904
  transgenic
    bone cell analysis, 1497–1498
    bone mass maintenance, 1494–1496
    osteoblast lineage, 1496–1497
    skeletal biology, 1498–1499
    skeletal development, 1494–1496
    structural macromolecules
      analysis, 1492–1493
      Cis regulation, 1493–1494
MRI. *see* Magnetic resonance imaging
mRNA. *see* RNA, messenger
Msenchymal cells, 962
Msx-1, role in osteoblast differentiation, 85–86
Msx-2
  function, 1496
  regulation, 69–71
  role in osteoblast differentiation, 85–86
MTPDS. *see* Mitochondrial trifunctional protein deficiency syndrome
Multicolony-stimulating factor, 949–950
Multiple endocrine neoplasia syndrome
  type 1
    clinical aspects, 1047–1048
    description, 1047
    gene
      characterization, 1021–1022
      endocrine tumors and, 1058–1059
      localization, 1057–1058
      mutations, 1048, 1058
      normal, 1058
      phenocopies, 1048
      sequence, 1058
    pathophysiology
      animal models, 1060
      associated organs, 1053–1056
      biochemical/radiological tests, 1056–1057
      general considerations, 1052–1053
      germline DNA-based tests, 1057–1060
    treatments, 1050–1052
  type 2
    components, 1067–1068
    with cutaneous lichen amyloidosis, 1070
    description, 1067–1068
    gene, characterization, 1021–1022
    Hirschsprung's phenotype, 1069–1070
    role of *RET* mutations

correlations, 1070–1071
management, 1072–1073
mechanism, 1071–1072
Myeloma
bone disease, 1095–1097
multiple, 1583

N

Natriuretic peptides, C-type, 1009
N-BP. *see* Bisphosphonates, nitrogen-containing
N-CAM. *see* Neural cell adhesion molecules
Nephropathy diabetic, in bone, 746
Nervous system
central (*see* Central nervous system)
development, role of *RET,* 1072
effects of amylin, 642
effects of CGRP, 642
role in remodeling, 311
Neural cell adhesion molecules, 278
Neuroendocrine system, diffuse, 1055
Neuromodulation, 539
Neuropeptides. *see also* specific neuropeptide
-cytokines, interactions, 780
-kinins, interactions, 780
Y, 786
Neurotransmitters. *see* specific neurotransmitters
Neurotrophins, 789–790
NIDDM. *see under* Diabetes mellitus
Nitric oxide
associated pathology, 1007
characterization, 995
donors, 1003
effects on osteoclasts, 999–1003
isoform occurrence, 998–999
molecular targets, 1006
osteoporosis pathophysiology and, 1279
synthesis
inhibition, 1003–1004
mouse model, 1004–1006
therapeutic implications, 1007–1008
Nonhuman primate models, 1644
Nonneuroendocrine tumors, calcitonin-associated, 630
Nonsteroidal anti-inflammatory drugs, 988
Notch/Delta signaling, 35–36
NPS 2143, 1343–1344
NPS R-568
characterization, 1341–1342
effects on PTH, 1342
primary hyperparathyroidism, 1346–1348
secondary hyperparathyroidism
animal models, 1348–1350
clinical studies, 1351–1352
N-terminal peptides, as collagen formation markers, 218
N-terminal telopeptide of collagen type I
assays, as bone resorption marker, 220
circadian variability, 1550–1551
description, 1548–1549
levels in immobilization, 1550
levels in pregnancy, 1550
NTX-I. *see* N-terminal telopeptide of collagen type I
Nuclear
architecture
skeletal gene expression link, 176–183
three-dimensional context, 170–176
extracts, preparation, 1513
receptors, 1459–1461
run-on assays, 1508

O

OCIF. *see* Osteoprotegerin
OI. *see* Osteogenesis imperfecta
Oncogenes. *see also specific genes*
effects of VEGF-A, 891
putative, 1021, 1024
Oncostatin M, 198
OPG. *see* Osteoprotegerin
Opioid peptides, 787
OPN. *see* Osteopontin
Oral contraception, 1550
OSE1, 87
Osf1, 87
Osseous systems, 625–626
Ossification
endochondral
embryonic, 33–34, 43–45
patterning, 12
process, 8–9
role of PTH, 156
intramembranous, 8
Osteoarthritis
stem cell changes and, 73–74
stem cell therapy, 74
tissue engineering for, 74
Osteoblasts
apoptosis
description, 159–160
effects of androgens, 762–763
effects of bisphosphonates, 162–163
glucocorticoid-induction, 161
PTH regulation, 161–162
sex steroid regulation, 160–161
associated bone metastases, 1097–1098
biopsy, 1628
connexin expression, 292
cultures, current techniques, 1531–1534
development
control of, 61–62
role of
cytokines, 71–73
growth factors, 71–73
hormones, 71–73
osteoprogenitor cells, 67–69
transcription factors, 69–71
differentiation
Cbfa 1 role, 83–85
effects of androgens, 763–64
effects of FGF, 836–837
transcriptional factors, 85–88
effects of
amylin, 647–648
calcitonin, 147

Osteoblasts (*continued*)
CGRP, 646–647
estrogen *in vitro,* 661–665
FGF signaling, 838
fluoride, 1389
gap junction communication, 294–295
G-CSF, 952–953
IGF, 802–803
IGFI, 803–804
IGFII, 804–806
IL-1, 962
INF-γ, 964
insulin *in vitro,* 748–749
kinin receptors, 778–779
mechanical loading, 1482
NO, 1001–1003
OPN, 241
PDGF, 818–820
progesterone receptors, 668
PTH/PTHrP, 495–498
TGFβ, 905
VEGF, 894
estrogen distribution, 657
formation, role of estrogen, 664–665
function, role of
adhesion molecules, 276–279
cadherins, 278
CD44, 278
description, 4–5
immunoglobulins, 278–279
integrins, 277–278
hormone action in, 729–730
IGFBP expression in, 806–809
intercellular Ca signaling, 297–298
-like cell cultures, current techniques, 1531–1534
lineage, GFP to assess, 1496–1497
-stromal cells, role in
OPG expression, 116–118
osteoclast differentiation, 109–111
osteoclast regulation, 111–112
RANKL regulation, 116–118
transcriptional complexes
culture models, 1504, 1506–1507
OI
intrinsic properties, 1183
physiological demands, 1183–1184
ontogeny, 60–65
osteosarcomas, culture models, 1504
PDGF expressing by, 817–818
phenotypic markers, 709–710
precursor cells, 494–495
primary cultures, 1507
progenitor cells, 657
proliferation, effects of
androgens, 762–763
estrogens, 664
FGF, 836
thyroid hormones, 711
prostaglandin production, 982–984
PTH regulation, 465–467
regulation, 7–8
-stromal cells, M-CSF production by, 111–112
structure, 4–5
transgenic models, 1498
Osteocalcin
as bone formation marker, 1546–1547
effects of thyroid hormones, 710
effects of vitamin D_3, 578
gene, regulatory factors, 176–183
levels in metastatic bone disease, 1561, 1562–1564
levels in PHPT, 1551
as osteoporotic fracture predictor, 1559
Osteoclastogenesis
control of, 141
effects of FGF, 839
inhibitory factor (*see* Osteoprotegerin)
role of M-CSF, 111–112
Osteoclasts
activation
effects of thyroid hormones, 711
inhibition by bisphosphonates, 1373
adhesion, Pyk2/c-Src regulation of, 144–145
apoptosis
description, 156–159
effects of bisphosphonates, 162–163
glucocorticoid-induction, 161
sex steroid regulation, 160–161
biopsy, 1628–1629
cells, degradative effects, 216
clear zone, function, 272–274
cultures, current techniques, 1535
cytoskeletal organization, 143–144
development, role of adhesion molecules, 272, 290–291
differentiation, role of
osteoblasts/stromal cells, 109–111
RANKL, 114–115
differentiation factor (*see* Receptor activator of nuclear factor $\kappa\beta$ ligand)
dysfunction, in osteopetrosis, 1221
effects of
amylin, 645–646
calcitonin, 1009, 1426–1428
CGRP, 643–645
CSF-1, 948–949
edothelin, 1009
estrogens, 665–686
G-CSF, 952–953
IGF, 806
IL-1, 961–962
NO, 999–1001
OPN, 241
progesterone receptors, 668
prostaglandins, 984–986
PTHrP, 1084
sex steroids, 265
TGFβ, 905
VEGF, 894–895
estrogen distribution, 657
formation
cytokine regulation, 693–695
role of CSF-1, 946–947
function
-$\alpha_v\beta_3$ integrin-mediated, 142–143
osteoblast/stromal cell regulation by, 112

role of RANKL, 114–115
intercellular Ca signaling, 297–298
motility, $\alpha_v\beta_3$ integrin-mediated, 143–144
in resorption cycle
cytoskeletal changes, 132–133
degradation product removal, 135–136
life span, 128–129
membrane domains, 130–132
polarization, 130–132
sealing zone formation, 129–130
surface attachment, 129–130
role in
bone degradation product removal, 135–136
degradation of organic matrix, 134–135
dissolving bone mineral, 133–134
role of
integrin receptors, 270–271
nonintegrin receptors, 271–272
signal transduction, adhesion-dependent, 143–144
transgenic models, 1498
Osteocytes
apoptosis
effects of bisphosphonates, 162–163
glucocorticoid-induction, 161
PTH regulation, 161–162
sex steroid regulation, 160–161
biopsy, 1629
cell lines, 97
cell-matrix adhesion, 98
cultures, current techniques, 1535
cytoskeleton, 98
death of, 94–96
effects of
NO, 1001–1003
PTH/PTHrP, 498–499
fluid flow response, *in vitro,* 101–102
formation, 94–96
function, 3–5
hormone receptors, 98–99
isolation, 96
location, 93
markers, 97
matrix synthesis, 97–98
as mechanosensory cells, 99–100
mediated-mechanotransduction, 25
role in
blood-calcium homeostasis, 99
canalicular fluid flow, 100–101
functional adaptation, 99
structure, 5
syncytium, 93–94
Osteodystrophy. *see* Albright's hereditary osteodystrophy
Osteogenesis imperfecta
animal models, 1180–1181
characterization, 1177
coupling, 52–53
deforming
classification, 1177–1179
molecular basis, 1181–1182
diagnosis, 1180
effects of VEGF, 893–894
management, 1180
mild nondeforming
classification, 1179–1180
molecular basis, 1182
producing mutations, 1182–1185
role of BMP, 923–924
role of collagen mutations, 193
stem cell therapy, 74
tissue engineering for, 74
treatments
anabolic agents, 1186
antiresorptive agents, 1185–1186
cell/gene, 1186–1188
Osteogenic cells, 288–289
Osteolysis
focal, role in MAH, 1088
inhibition by bisphosphonates, 1088–1089
Osteomalacia
characterization, 1209
clinical features, 1195–1196
oncogenic
differential diagnosis, 1211–1212
pathophysiology, 1210–1211
treatment, 1212–1213
tumors, 1209–1210
tumor-induced, 331–333
vitamin D therapy for, 1411
Osteonecrosis, 726
Osteonectin, 228–229
Osteopenia, 1638
Osteopetrosis
adult forms, 1220–1221
animal models, 1222–1223
avian, 1223
development, 1217
infantile forms
intermediate, 1218–1220
malignant, 1217–1218
transient, 1220
pathophysiology, 1221–1222
phenotype, 12
treatment, 1224–1226
Osteopontin
cell attachment, 241
deficient mouse, phenotype, 242
-dependent intracellular signaling, 242
description, 231
effects of vitamin D_3, 578
effects on angiogenesis, 243–244
effects on resorption, 243–245
metastatic disease and, 245–246
receptors, 241
role in mediating mechanical stress, 246–248
role in postmenopausal bone loss, 242–243
structure, 239–241
Osteoporosis
animal models
congenic/recombinant strains, 1650–1651
congenic strains, 1651–1653
convenience, 1639
criteria, 1635
inbred strains, 1648–1650
postmenopausal, 1637–1640

Osteoporosis (*continued*)
recombinant/congenic strains, 1650–1651
recombinant strains, 1650–1651
regulatory recommendations, 1635
in vivo experimentation, 1635
BMD association
epidemiology, 1305
history, 1647–1648
Ca intake and, 1335
corticoid-induced, fluoride therapy, 1394
definition, 1573–1574
diagnosis, T scores, 1589–1590
effects of
bisphosphonates
mechanisms of action, 1364–1365
models, 1362–1364
remodeling, 313–314
ethnic differences, 1590–1593
fractures associated with
risks factors
BMD, 1292–1293
bone turnover, 1296–1298
clinical, 1291–1292
combined assessment, 1298
hormonal markers, 1293–1294
menopause, 1294–1296
gender differences, 1590–1593
genes
allele-sharing studies, 1261–1262
association studies, 1262
candidates
apolipoprotein E, 1267
calcitonin receptor, 1267–1268
collagen, type I, 1264
estrogen receptor, 1266
interleukins, 1267
TGFβ, 1266–1267
VDR, 1264
linkage analysis, 1261
genetic determinants
effects on
bisphosphonate response, 1269
calcium absorption, 1269
clinical practice, 1269–1270
HRT response, 1269
vitamin D absorption, 1269
evidence for, 1260–1261
identifying, 1259–1260
quantitating, 1259–1260
glucocorticoid-induced
effects of bisphosphonates, 1375–1376
pharma, animal models, 1639, 1644–1645
growth hormone therapy
administration, 1445
BMD and, 1443–1446
clinical trials, 1445–1446
mechanisms, 1442–1443
physiology, 1441–1442
idiopathic, IGF-I treatment for, 1449
IGF-I treatment
animal models, 1446
availability, 1446
clinical trials, 1448–1449
male
animal models, 1639, 1645–1647
fluoride therapy, 1394
Mg association
animal models, 352–353
epidemiological studies, 349–350
mechanism, 353
risk factors, 351–352
treatment, 351
pathophysiology
bone formation, 1277–1278
bone resorption, 1276–1277
calcitonin, 1283
cytokines and, 1278–1279
estrogen and, 1278
growth factors, 1279
leukotrienes and, 1279
local factors, 1278
nitric oxide and, 1279
parameters, 1275–1276
parathyroid function, 1281–1283
prostglandins and, 1279
systemic factors, 1280
vitamin D, 1280–1281
pharmacogenetics and, 1268–1269
postmenopausal
animal models, 1637–1640
biochemical markers, 1558–1560
characterization, 693
effects of bisphosphonates, 1375–1376
fluoride treatment, 1392–1394
PTH therapy for, 1315
role of CSF-1, 945–946
role of PTH, 1354–1355
prevalence *vs.* risks, 1590
role of
FGF, 840–841
gene-gene interactions, 1268
heparin, 840–841
IL-1, 962
PTH, 1354
TGFβ, 913
stem cell changes and, 73–74
therapies
anabolic agents, 1306
anti-resorptive agents, 1306
calcitonin, 1433
fluoride, 1390–1392
$\alpha_v\beta_3$-intergin as target, 275–276
stem cell, 74
vitamin D, 1411
tissue engineering for, 74
WHO classification, 1573–1574
Osteoprogenitor cells
age-associated changes, 73–74
differentiation, 67–69
disease-associated changes, 73–74
function, 65–67
Osteoprotegerin
in bone, effects of PTH/PTHrP, 496–497
discovery, 112

expression, 116–118
identification, 1084–1085
role in MAH, 1086–1087
Osteosarcomas
effects of IGFPB, 809–810
effects of insulin *in vitro,* 748–749
osteoblastic, culture models, 1504, 1506
Osteprogenitors, PTH regulation, 465–467
Ostoclasts
adhesion, inhibition of, 1372
lifespan, shorting of, 1372–1373
recruitment, inhibition of, 1372
Ovariectomy
effects of osteclastogenic cytokines, 699
effects on cancellous bone, 659
effects on turnover, 1614
induced bone loss, 699–702

P

Paget's disease
biochemical markers for, 1551, 1553–1554, 1556
biochemistry, 1251
calcitonin therapy, 1433
cellular biology, 1253–1255
characterization, 1249
complications, 1249
diagnosis, 1250
effects of bisphosphonates, 1376
etiology, 1255–1256
histopathology, 1250–1251
molecular biology, 1253–1255
occurrence, 1249
paramyxoviruses in, evidence, 1251–1253
therapy, 1251
Pancreas
effects of PTH/PTHrP, 525–527
MEN1 syndrome and, 1049, 1055
pathology, FBHH and, 1033
Paramyxoviruses, Paget's disease in, 1251–1253
Parathyroid cytosolic proteins, 414–415
Parathyroidectomy, 1050–1051
Parathyroid gland
abnormalities, MEN2 syndrome-associated, 1069
effects of
FBHH, 1036
Mg depletion, 345–346
vitamin D_3, 576
MEN1 syndrome and, 1053–1054
PTH gene expression, 408
tumors
clonality, 1018–1019
genetic derangements, 1019–1021
genetic pathogenesis, 1024–1025
molecular pathogenesis, 1025
Parathyroid hormone
anabolic properties
advantages, 1305
potential mechanisms, 1306–1307
biological action, 389
bone cell apoptosis regulation by, 161–162
cardiovascular activity, 531–532
cellular actions
cation/anion transport, 471–472
cytokine-mediation, 468–469
description, 465–469
hormone-mediation, 468–469
in collagen synthesis, 199
connexin regulation by, 293
ectopic secretion, 1025–1026
effects of
CaR therapy, 381, 1353
glucocorticoids, 725–726
Mg depletion, 347–348
NPS 2143, 1343–1344
NPS R-568, 1342, 1344
osteocytes, 98–99
protein kinases, 418
sex steroids, 417–418
vitamin D-3, 579
effects on
bone, 495–498
Ca homeostasis, 1339
cardiovascular system, 473–475
kidney
metabolism, 472–473
molecular mechanisms, 1118
phosphate transport, 469–471
OI, 1186
osteocytes, 498–499
pituitary function, 725
remodeling, 315
vasculature, 473–475
in endochondral ossification, 156
expression in bone, 493–494
genes
abnormalities, 1106–1109
characterization, 407
localization, 407
in hyperparathyroidism, 416–417
induced resorption, role of OPN, 244–245
levels in PHPT, 1557
mutations
characterization, 409
role in chondrodysplasias, 1127–1129
role in hypoparathyroidism
associated syndromes, 1109–1112
autosomal dominant type, 1107
autosomal recessive type, 1107–1108
isolated type, 1105
x-linked recessive type, 1109
role in PHP, 1112–1113
osteoblast regulation by, 465–467
osteoclast regulation by, 467–468
osteoporosis pathophysiology and, 1281–1283
peripheral metabolism, 463–465
phosphate transport regulation, 323–326
promoter sequences, 408
-PTHrP interactions
amino-terminal receptor domain, 393–395
characterization, 389–391
in CNS, 537–540
conformational changes, 397
conformation based design, 434–435

Parathyroid hormone (*continued*)
effects on
bone, 499–500
kidney, 473
mammary gland, 517–522
pancreas, 525–527
reproductive tissues, 522–525
skin, 515–517
integrated studies
advantages, 440–441
approaches, 441–442
contact sites, 442–445
molecular modeling, 445–450
photoreactive analogs, 442
interaction, 395–396
juxtamenbrane region, 395–396
ligand tethered, 445–450
mechanisms, 391–393
mid- and carboxy-terminal specific, 398–400
-PTH, interaction, 396–397
regulation, 397–398
signaling pathways
downstream activities, 428–429
dual intracellular, 427–428
selective ligands, 429–430
structure-activity relations, 424
subtype specificity switch, 426
target-specific ligands, 430
two-site dynamic model, 451–452
in vasculature, 522–525
regulation of
Ca excretion, 487–488
Ca in, 412–413
calcitriol in, 409–412
calreticulin in, 411–412
H excretion, 491–492
Mg excretion, 487–488
Na excretion, 491–492
Ph excretion, 489–491
phosphate in, 413–414
vitamin D metabolism, 492–493
role in
FBHH, 1035–036
humoral hypercalcemia of malignancy syndrome, 1094
osteoblast development, 71–73
secondary hyperparathyroidism, 416–417
type I osteoporosis, 1355
secretion, role of
CaR, 375
Mg, 344–345
signal transduction, 1137–1140
structure, primary, 424–426
therapy
adverse effects, 1318–1319
animal models, 1307–1308
bone metabolism markers, 1316
clinical studies, 1308–1309
delivery systems, 1318
effects on
BMD measurements, 1310, 1313
bone histology, 1315–1316
calcium balance, 1316–1317
fracture risk, 1309–1310
immunological response, 1318
with other treatments, 1313–1315
pharmacokinetics, 1317–1318
TIP39 receptor
characterization, 389–391
identification, 393, 426–427
tissue-specific expression, 407–408
Parathyroid hormone receptors
effects on kidney, 1119–1122
regulation of
Ca excretion, 488–489
H excretion, 491–492
Mg excretion, 488–489
Na excretion, 491–492
renal functions
cell signaling regulation, 484
signal transduction, 484
role in bone formation, 1120–1122
Parathyroid hormone-related peptides
amino-terminal receptor domain, 393–395
biological action, 389
bone, 493–494
characterization, 389–391
chimera, 437–439
cloning, 1080
conformational changes, 397
discovery, 1119
ectopic domain, cysteins characterization, 439–440
effects on
bone, 495–498
chondrocytes, 47–50
heart, 536–537
kidney, 483–485, 493
osteocytes, 498–499
identification, 393
juxtamenbrane region, 395–396
ligands, 1119–1122
mechanisms, 391–393
mid- and carboxy-terminal specific, 398–400
mutations
associated-genetic disorders, 440
role in Blomstand's lethal chondrodysplasia, 1127–1129
role in Jansen's metaphyseal chondrodysplasia, 1123–1125
as neuroprotective, 539–540
-PTH interactions
in CNS, 537–540
conformation based design, 434–435
description, 396–397
effects on
bone, 499–500
kidney, 473
mammary gland, 517–522
pancreas, 525–527
reproductive tissues, 522–525
skin, 499–500
integrated studies
advantages, 440–441
approaches, 441–442
contact sites, 442–445

molecular modeling, 445–450
photoreactive analogs, 442
ligand tethered, 450–451
signaling pathways
downstream activities, 428–429
dual intracellular, 427–428
selective ligands, 429–430
structure-activity relations, 424
subtype specificity switch, 426
target-specific ligands, 430
two-site dynamic model, 451–452
in vasculature, 522–525
purification, 1080
receptors, role in
bone formation, 1120–1122
epithelial-mesenchymal interactions, 1122
regulation, 397–398
role in
humoral hypercalcemia of malignancy syndrome, 1093–1094
malignancy-associated hypercalcemia
approaches to controlling, 1086–1087
mechanisms of action, 1083–1086
molecular aspects, 1080–1082
osteolysis, 1088–1089
osteolytic bone metastases, 1100
site-directed mutagenesis studies, 439
subtypes, 436–437
types, 435–436
in vasculature
effects on, 532–533
effects on anatomy, 532–533
expression, 533–534
mode of action, 533–534
Paraxis, 36
PDGF. *see* Platelet-derived growth factor
Peptide assay, as bone resorption marker, 219–220
Periodontal disease, BMP application, 921
Peroxisome proliferator-activated receptors, 982
Pfeiffer syndrome, 43
Pharmacogenetics, 1268–1269
Phenylalkylamines
NPS 2143, 1343–1344
NPS R-568
characterization, 1341–1342
effects on PTH, 1342
primary hyperparathyroidism, 1346–1348
secondary hyperparathyroidism
animal models, 1348–1350
clinical studies, 1351–1352
PHEX gene
rickets link, 328–329
tumor-induced osteomalacia link, 331–333
Phosphate
effects of calcitonin, 624–625
effects on PTH mRNA, 414–415
excretion, role of PTH/PTHR, 488–489
renal transport, effects of PTH, 469–471
role in PTH gene regulation, 412–414
therapy, for osteomalacia, 1212
Phosphoethanolamine, 1235, 1240
Phosphorus
deficiency, 321–332
function, 321
GI absorption, 322
impaired reabsorption, 326–334
renal excretion, 322–326
transport
effects of glucocorticoids, 725–726
mechanisms, 323–326
Photoreactive analogs, 442
Photosynthesis, previtamin Dd_3D
description, 587
in skin, 587–590
PHP. *see* Pseudohypoparathyroidism
PHPT. *see* Hyperparathyroidism, primary
Phytoestrogens, 667
PICP. *see* Procollagen type I propeptides
Pig models, 1643
PINP. *see* Procollagen type I aminoterminal propeptide
Pituitary adenylate cyclase-activating peptides, 784
Pituitary gland
function, effects of glucocorticoids, 725
tumors, MEN1 syndrome-associated
description, 1049–1050
pathology, 1055
treatment, 1052
P13 kinase, 146–147
Placenta, effects of PTH/PTHrP, 524
Plasminogen activator
in bone, 257–258
characterization, 257–258
gene expression in bone, 733
sequences, 1098
tissue-type, characterization, 257
Platelet conversion, 311–313
Platelet-derived growth factor
binding proteins, 820–821
clinical relevance, 821
effects on skeletal cells, 818–820
function, 821
osteoblastic bone metastases and, 1098
osteoporosis pathophysiology and, 1279
receptors, 820–821
synthesis by skeletal cells, 817–818
Polar moment of inertia, 27
Polymerase chain reaction
ligation mediated, 1514
reverse transcriptase
nascent transcript level analysis, 1508
transcription factor characterization, 1519–1520
PPARs. *see* Peroxisome proliferator-activated receptors
Pregnancy
effects of PTH/PTHrP, 524–525
effects on bone turnover, 1550
mammary epithelial cells and, 520–522
Preosteoclast cultures, current techniques, 1535–1536
Procollagen type I aminoterminal propeptide, 1547
Procollagen type I propeptides
circadian variability, 1551
description, 1547
levels in Paget's disease, 1553

Progesterone
effects onf VEGF-A expression, 892
receptors, effects on bone, 668–669
replacement therapy, 667–668
VDR_{nuc} response, 553–554
Progestins
coregulators, 657
disruption, rodent models, 661
effects on bone mass, 658
mechanism of action, 655–656
PRO-α genes
cis-acting elements binding, 200–201
first intron elements, 203–204
posttranscitptional regulation, 204
transcription factors binding, 200–201
upstream elements, organization, 201–203
Prostaglandins. *see also specific types*
connexin regulation by, 293
effects of
estrogen, 984
FGF, 838
glucocorticoids, 733
effects on
bone loss, 987–988
HHM, 1095
osteoblasts, 982–984
osteoclasts, 983–986
resorption, 778
VEGF-A expression, 892
metabolic pathways, 979–980
osteoporosis pathophysiology and, 1279
production, inhibition of, 983–984
receptors
characterization, 981–982
effects on
bone formation, 986–987
mechanical loading, 987
resorption, 986
Prostate cancer, role of TNFβ, 1098
Proteases of bone, 498
Proteinases, aspartic, 258
Protein-DNA incubation, 1518–1519
Protein isoprenylation, 1369–1371
Protein kinase A, 418
Protein kinase C
calcitonin and, 1431
CaR and, 375
PTH and, 418
Proteins. *see also specific proteins*
-DNA, interactions at PTH mRNA, 414–417
-DNA, interactions *in vitro* and *in vivo,* 1512–1515
leucine-rich, 228
Protein tyrosine phosphatases, 1366
Proteoglycans, 225–228
Proteolysis, bone cells, 809–810
Protooncogenes, expression in bone, 731
Proximal tubular
Na/H excretion, regulation by PTH, 492
Ph reabsorption, regulation by PTH, 487–488
Pseudohypoparathyroidism
definition, 1137
diagnosis, 1152–1154
genetics, 1152
identification, 1143
molecular classification, 1142–1143
pathophysiology, 1140–1142
patients with, 1112–1113
treatment, 1154–1156
type 2, 1150
type 1a
identification, 1143
multiple hormone resistance in, 1145–1149
neurological abnormalities, 1148
sensory abnormalities, 1146–1148
type 1b, 1149–1150
type 1c, 1150
Psoriasis, 595–597, 1412
PTPs. *see* Protein tyrosine phosphatases
Pyk2
-c-Src, role in osteoclast adhesion, 144–147
effects of calcitonin, 1431
Pyridinium
as bone resorption marker, 219
cross-links, 212
levels in heart transplants, 1561
levels in metastatic bone disease, 1563
Pyridinoline
levels in PHPT, 1548
as resorption marker, 1548
Pyridoxal 5′-phosphate, 1235, 1240–1242
Pyrophosphate, inorganic, 1235, 1242
Pyrroles cross-links, 212–213

Q

QCT. *see* Quantitative computed tomography
Quantitative computed tomography
general description, 1574–1576
and other techniques, comparisons, 1580
peripheral, 1577–1578
serial measurements, 1593–1596
Quantitative ultrasonometry
advantages, 1292–1293
description, 1578–1580
identifying patients for, 1583
QUS. *see* Quantitative ultrasonometry

R

Rabbit models, 1642–1643
Radiography
absorptiometry
description, 1577–1578
and other techniques, comparisons, 1580
description, 1600–1604
Raloxifene
effects on
bone, 682–683
breast, 684
cardiovascular system, 683–684
uterus, 684–685

studies, 681
thrombeoembolism association, 685
RANK. *see* Receptor activator of nuclear factor κβ
RANKL. *see* Receptor activator of nuclear factor κβ ligand
Rat models, 1641–1642
Receptor activator of nuclear factor κβ
-RANKL interaction, discovery, 112–114
regulation, 1085
role in
MAH, 1086–1087
osteoclast apoptosis, 157–159
osteoclast formation, 693–695
role in MAH, 1086–1087
signal transduction mechanism, 118–121
-TGF-β, cross-communication between, 121
Receptor activator of nuclear factor κβ ligand
in bone, effects of PTH/PTHrP, 496–497
deficient mice, 115–116
description, 109
effects on osteoclasts, 114–115
identification, 1084–1085
mutations, activating, 116
-RANK interaction, discovery, 112–114
role in postmenopausal osteoporosis, 945–946
Remodeling, bone
animal models, 1638
at BMU level
activation, 307–309
formation, 309–310
local factors, 311
mineralization, 310–311
origination, 304–307
direct activation, 315–316
effects of
calcitonin, 1431
IDDM, 743–744
IGF, 810–811
IL-4, 965
insulin, 743–746
NIDDM, 744
pharmacological agents, 314–315
pseudohypoparathyroidism, 1151
thyroid hormones, 712
effects on bone structure, 311–313
marrow spaces and, 315
microcallus formation and, 315–316
in osteoporosis, 313–314
physiology, 304
role of osteoclast apoptosis, 157
woven bone *vs.,* 315
Reporter genes, types, 1511–1512
Reproductive system
effects of
calcitonin, 627
raloxifene, 684–685
SERMs, 682
tamoxifen, 685–686
VDR in, 594–595
Resorption, bone
associated cell-matrix interactions, 266
biopsy, 1628–1629
coupling to formation, 1277
critical functions rule, 12
cycle, osteoclast role
cytoskeletal changes, 132–133
degradation product removal, 135–136
life span, 128–129
membrane domains, 130–132
polarization, 130–132
sealing zone formation, 129–130
surface attachment, 129–130
effects of
androgens, 764–766
FGF, 838–839
glucocorticoids, 727–729
IL-4, 965
IL-7, 966
INF-γ, 964
OPN, 243–244
PTH/PTHrP, 499–500
TGFβ, 905
thyroid hormones, 711–712
TNF, 963–964
effects on prostaglandin receptors, 986
factors effecting, 1550–1551
IL-1 stimulation, 961–962
inhibition by bisphosphonates
mechanisms of action level, 1366–1367
models, 1362–1365
relative potency, 1365–1366
markers, 218–220, 1547–1549
in osteoporosis, 1276–1277
phases, 307–309
PTH-induced, role of OPN, 244–245
role of
brandykinin receptors, 778
cathepsin K, 216
integrins, 270–271
kinin receptors, 778–779
septoclasts, 127–128
tumor-induced, effects of bisphosphonates, 1365
Respiratory system, effects of calcitonin, 626–627
RET protooncogene
characterization, 1024, 1067
mutations
role in MEN2 syndrome
correlations, 1070–1071
mechanism, 1071–1072
role in neural development, 1072
Rheumatoid arthritis, 156, 894–895
Rickets
clinical features, 1196–1197
diagnosis, 1204
hereditary hypophosphatemic, 329–331
therapy, 1204–1205, 1411
vitamin D-dependent
type I, description, 1197–1198
type II
biochemical features, 1198–1200
cellular defects, 1200–1204
clinical features, 1198–1200
mode of inheritance, 1199–1200
molecular defects, 1200–1204

Rickets (*continued*)
occurrence, 1197–1198
X-linked hypophosphatemic, 326–329
X-linked recessive hypophosphatemic, 333
RNA, messenger
PTH degradation, 414
splicing, CTR proteins, 607–609
stability, transcriptional control *vs.,* 1507–1508
Rod conversion, 311–313
ROS 17/2.8 cells, 1504
Ruffled border, 130–132

S

Saos-2 cells, 1506
Sclerotome differentiation, 37
SEAP. *see* Secreted alkaline phosphatase
Seasonal variability, effects on bone turnover, 1551
Secreted alkaline phosphatase, 1512
Selectins
characterization, 269
role in osteoblast functions, 278
Selective estrogen receptor modulators
function, 666–667
mechanism of action, 677–681
physiologic effects on
bone, 681
breast, 682
cardiovascular system, 681–682
reproductive tract, 682
Sepsis, calcitonin-associated, 630–631, 633
Septoclasts, 127–128
Serine kinase
activation, 932–933
characterization, 929–932
intracellular signaling
in cytoplasm, 935–936
in nucleus, 936
regulation, 937–938
structure, 933–935
target genes, 936–937
SERMs. *see* Selective estrogen receptor modulators
Serotonin
receptors, effects on bone, 789
transporter, effects on bone, 789
Serum proteins, description, 232–233
Sheep models, 1643–1644
SIBLING. *see* Small integrin-binding ligand with N-linked glycosylation
Signal transduction
adhesion-dependent, in osteoclasts, 143–144
androgen receptors, 758–760
BMP, SMAD
in cytoplasm, 935–936
in nucleus, 936
regulation, 937–938
structure, 933–935
target genes, 936–937
CSF-1, 949
$\alpha_v\beta_3$-mediated, 274
mechanical, electric fields in, 1483
microdomains, 539
NO, 966–968
protein binding, 889–890
PTH-PTHrP
downstream activities, 428–429
dual intracellular, 427–428
function, 1137–1140
selective ligands, 429–430
PTHR in
Ca excretion, 487–488
Mg excretion, 487–488
Na/H excretion, 491–492
Ph excretion, 490–491
renal cells, 484
RANK, 118–121
$1\alpha,25(OH)_2$ vitamin D_3, 549–551
Silicon-bone, interaction, 365
Skeleton
adaptation
belief in, 1477
cellular basis, 1482–1483
effects of age, 1483–1484
effects of exercise, 1480–1483
exercise constraints, 1484–1485
role of electric fields, 1483
androgen mode of action, 1462
biology, endogenous gene disruption, 1498–1499
development
cellular divisions, 5–6
effects of connexins, 293
embryonic
axial, 34–38
chondrogenesis, coupling, 52–53
intramembranous bone, 42–52
limb initiation, 38–40
mesenchymal condensation, 40–42
morphology, 33–34
osteogenesis, coupling, 52–53
patterning, 40–42
role of BMP, 922–923
formation, 8–11
genes affecting, analysis, 1494–1496
molecular regulation, 11–12
role of cadherins, 288–289
studying, 12–13
effects of estrogens, 655–656, 658
growth, effects of IDDM, 741–743
hypophosphatasia, 1236
intercellular Ca signaling, 297–298
male
interventional studies, 1474–1475
observational studies, 1472–1473
malignancy (*see specific diseases)*
osteoporosis treatment response to, 1637–1638
response
curvilinear nature of, 1481–1482
to HRT, animal models, 1637
role of androgens, 1456–1457
thyroid hormones
animal studies *in vivo,* 712–713
cell phenotypic responses, 711–712

gene products, 709–711
membrane actions, 709
nuclear receptors, 707–709
tissues, role of CaR, 1355
Skin
effects of PTH/PTHrP, 515–517
lipoma, *MEN1* gene and, 1059–1060
previtamin D_3 photosynthesis in, 587
Small integrin-binding ligand with N-linked glycosylation, 231
Sodium
effects of CaR, 381
excretion, role of PTH/PTHR, 491–492
-phosphate cotransporter, 413
Somatic cells therapy, 74–76
Somatostatin
characterization, 787
MEN1 syndrome treatment, 1051
Somitogenesis, 34–37
Southwestern blot, 1515–1516
SPARC, description, 228–229
SRC. *see* Steroid receptor coactivators
Stem cells. *see* Meschymal stem cells
Steroid receptor coactivators, 580–581
Sterol synthesis, blocking, role of FFP, 1369–1371
Strain
curve, 21–22
minimum effective, 1480
rate of, 1481
-stress, relationship, 18–20
Strength, tensile, 22
Stress
definition, 18
fractures
fluoride-induced, 1389–1390
occurence, 28
mediating, role of OPN, 246–248
patterns, 19
relaxation, 23
standard unit, 18
-strain
curve, 21–22
relationship, 18–20
Stroke-fracture association, 791–792
Stromal cells
-osteoblasts
M-CSF production by, 111–112
role in
OPG expression, 116–118
osteoclast differentiation, 109–111
osteoclast regulation, 111–112
RANKL regulation, 116–118
Stromelysin, 252
Strontium-bone interaction, 365–366
Substance P, 786
Sunscreens, 588
Surgery, orthopedic, 920
Syndecans
characterization, 269
expression in
cartilage, 281
syndecans, 279

T

Tamoxifen
effects on
bone, 685
breast, 685–686
cardiovascular system, 685–686
reproductive tract, 682, 685–686
thrombeoembolism association, 687
Tartrate-resistant acid phosphatase
assays, 1536
expression, 109–110, 1495
levels in Paget's disease, 1554
levels in PHPT, 1557
as resorption marker, 1549
T cells, role in RANKL regulation, 118
Telopeptides, cross-linked, 1548–1549
Tensile strength, 22
Tension, definition, 18–19
Testosterone. *see* Androgens
Tetracycline labeling, 1615–1616
Tetranectin, 229
Thiazide, effects on bone turnover, 1550
Threonine kinase
activation, 932–933
characterization, 929–932
intracellular signaling
in cytoplasm, 935–936
in nucleus, 936
regulation, 937–938
structure, 933–935
target genes, 936–937
Thrombospondin, description, 230–231
Thymus, PTH gene expression, 408
Thyroid gland
medullary cancer, 629–630
PTH gene expression, 408
Thyroid hormones
bone loss and, 714–715
effects on
chondrocytes, 46
skeleton
animal studies *in vivo,* 712–713
cell phenotypic responses, 711–712
gene products, 709–711
in humans, 713–714
membrane actions, 709
nuclear receptors, 707–709
osteoporosis pathophysiology and, 1280
phosphate transport regulation, 324–326
role in collagen synthesis, 199–200
Tissue-nonspecific alkaline phosphatase
biology, 1231–1232
circulating, 1242
deficiency, 1237
description, 1231
discovery, 1229
function, hypophosphatasia as model, 1243
history, 1229–1231
physiological role, 1229–1231
structural defects, 1238

Tissues
cultures
current techniques, 1530–1531
in vitro systems, 1529–1530
engineering, 74
TNALP. *see* Tissue-nonspecific alkaline phosphatase
TNF-related activation-induced cytokine, 113
Toremifene, 687
Torsion effects, 19–20
Trabecular bone
connectivity, 1631
strength of, 23
volume, 1631
TRAF. *see* Tumor necrosis factor receptor-associated factors
TRANCE. *see* TNF-related activation-induced cytokine
Transcription factors
collagen genes
mode of action, 203
proximal promoter binding, 200–201
function, 69–71
in osteoblast differentiation, 85–88
PTH, 495
regulatory complexes
analysis, 1507–1508
cloned or purified, characterization, 1518–1519
cloning, 1515–1518
DNA-protein interaction analysis, 1512–1515
initiation by nuclear run-on assays, 1508
multiprotein, purification/sequencing, 1516–1517
osteoblast cell culture models, 1504, 1506–1507
promoter analysis
assay selection, 1509–1510
reporter genes, 1511–1512
transfection methods, 1510–1511
Transcytosis, 274–275
Transfection
biolistic, description, 1511
methods, 1510–1511
stable, assay selection, 1509–1510
transient, assay selection, 1509
Transforming growth factor α
effects on osteoclasts, 121
osteoporosis pathophysiology and, 1279
phosphate transport regulation, 323–326
Transforming growth factor β
binding protein, 911–912
in bone
effects of FGF, 837
effects of PTH/PTHrP, 495
characterization, 904–905
connexin regulation by, 293
effects on
bone cells *in vitro,* 905–906
chondrocytes, 47
osteoclast formation, 948–949
VEGF-A expression, 891–892
gene expression in bone
effects of glucocorticoids, 732–733
targeted disruption of, 904
isoforms, 903–904
latency, 909–911
-RANKL, cross-communication between, 121
receptors, 906–907
role in
BMD, 1266–1267
bone cancer metastasis, 911–912
collagen synthesis, 194–196
osteoblast apoptosis, 160
osteoblast development, 71–73, 72
signaling pathways, 908–909
superfamily, 903
Transgenes, tissue-directed, 1494–1496
Transilial bone biopsy
in clinical research, 1633
complications, 1617
histology, 1628–1629
morphometric methods, 1629, 1631
in patient care, 1632
practical considerations, 1633
procedure, 1615–1616
reference data, 1631–1632
regions evaluated, 1629, 1631
specimen processing, 1617
timing, 1633
Transmission disequilibrium test, 1262
Transplantation
heart, associated bone loss, 1561–1562
kidney, associated bone loss, 1562
as osteopetrosis therapy, 1224–1225
TRAP. *see* Tartrate-resistant acid phosphatase
T scores. *see* Desitometry
Tumor necrosis factors
effects on bone, 963–964
expression, 109
α
effects on VEGF-A expression, 891
osteoporosis pathophysiology and, 1278–1279
role in collagen synthesis, 197–198
in osteoblasts, effects of estrogen, 662–663
receptor-associated, 118–119
role in
HHM, 1094–1095
MAH, 1087–1088
osteoclast
apoptosis, 158–159
formation, 693–695
ovariectomy-induced bone loss, 699–702
β, in cancer cells, 1098
Tumors
bone disease, 1376–1377
extrathyroidal neuroendocrine, 630
nonneuroendocrine, 630
suppressor genes, 1022, 1024
Turnover, bone
effects of
GH, 1443–1446
IGF-I, 1447–1448
Mg, 350
raloxifene, 682–683
as fracture risk marker, 1296–1298
postmenopausal rate, animal model, 1637
Tyrosine kinase, 889

U

Ultrasound, BMD, 1292–1293
Urokinase-type plasminogen activator, 257
Uterus, effects of PTH/PTHrP, 523–524

V

Vascular endothelial growth factor
  in bone formation, effects of FGF, 837
  effects on
    bone, 893–896
    cartilage, 893–896
    osteoclast formation, 948–949
  ligand family
    alternative splicing, 884–885
    postranslation processing, 885
    structure, 885–886
    viral, 886
  receptors
    activation, 889–890
    expression, 891–892
    structure, 886
    types, 887–888
  type A
    activities, 889–891
    expression, 891–892
    mechanisms, 889–891
Vasculature
  cells, uniquness, 883
  PTH in, 473–475
  PTHrP in
    effects on
      anatomy, 532–533
      contractile mechanisms, 532–533
      smooth muscles, 534
    expression, 533–534
    mode of action, 535–536
  role in remodeling, 311
Vasoactive intestinal peptide
  characterization, 781–783
  clinical observations, 791–792
Vasodilation, VEGF-A-induced, 891
VEGF. *see* Vascular endothelial growth factor
Versican (PG-100), 226
Viral vectors, description, 1510–1511
Viscoelasticity, 23–24
Vitamin D
  absorption, genetic determinants, 1269
  binding proteins, receptor interactions, 1413–1415
  compounds
    activating enzymes, 1412–1413
    analogs, 1409, 1411, 1416
    binding proteins, 1413
    clinical applications, 1411–1412
    genomic mechanisms, unraveling, 1417–1418
    hepatic clearance, 1416
    modeling, 1418
    molecular mechanisms of action, 1416–1417
    prodrugs, 1408
    target cell catabolic enzymes, 1415–1416
  deficiency, animal models, 1205
  effects of osteocytes, 98–99
  effects on chondrocytes, 47
  endocrine system, 548–549
  enhancer domain, 178
  function, 1195–1196
  hereditary deficiencies (*see* Osteomalacia; Rickets)
  metabolism
    effects of
      glucocorticoids, 725–726
      Mg depletion, 348–349
      PTH, 492–493
      sunlight, 573–574, 590–591
    in type II rickets, 1199
  osteoporosis pathophysiology and, 1280–1281
  production, hereditary deficiency, 1197–1198
  pseudohypoparathyroidism therapy, 1154–1156
  related genes
    response elements, 577–579
    transcriptional regulation, 579–581
  role in
    collagen synthesis, 199
    FBHH, 1036
    MAH, 1087
Vitamin D_3
  analogs, 561–565
  as arthritis treatment, 598
  conformational flexibility, 545–548
  effects on
    bone, 574–575
    calcitriol levels, 1204
    differentiation, 576–577
    hormone secretion, 577
    immune system, 577
    intestine, 575
    kidney, 575–576
    parathyroid glands, 576
    proliferation, 576–577
  genomic mechanism of action, 574
  nuclear receptor
    characterization, 551–552
    dimerization, 552–553
    evidence for, 558–559
    hormone response element, 553–554
    knockout model, 556–558
    ligand binding, 554–555
    ligand shape, 559–561
    mutations, 556
    receptor structure, 555–556
  photosynthesis, 587–590
  posttranscritpional effects *in vitro,* 1202–1203
  role in
    malignancy-associated hypercalcemia, 1081
    PTH gene regulation, 409–412
    PTHrP repression, 1086–1087
  signal transduction pathways, 549–551
  as skin disease treatment, 595–597
  structure, 545
  transcriptional regulation by, 577–579
  transcritpional effects *in vitro,* 1202–1203

Vitamin D receptors
in cancer cells, 591–592
characterization, 551–552
compound interactions, 1413–1415
deficient
DNA binding, 1201
hormone binding, 1201
nuclear uptake, 1201
dimerization, 552–553
evidence for, 558–559
function, 1195
genes, role in BMD, 1264–1265
heterodimerization, 580
homodimerization, 580
hormone response element, 553–554
in immune system, 592–594
interacting proteins complex, 581
knockout model, 556–558
ligand binding, 554–555
ligand shape for, 559–561
mutations, 556
phamacologic actions, 595
phosphorylation, 579–580
physiolgic actions, 595
receptor structure, 555–556
regulation, 579
reproductive system, 594–595
-transcription machinery
interaction, 580–581
Vitamin K, role in osteoclast apoptosis, 159
Vitronectin, 231

W

Water metabolism, 379–381
WHO. *see* World Health Organization
Wolff's law, 17, 99, 1477
World Health Organization
osteopenia definition, 1582
osteoporosis classification, 1573–1574
Wounding healing, 821
Woven bone, 315

X

X-linked hypophosphatemic rickets, 326–329
X-linked recessive hypophosphatemic rickets, 333
X-ray energy, 1612–1613

Y

Yield point, 21–22
Young's modulus, 21

Z

Zone of polar activity, 38, 40
ZPA. *see* Zone of polar activity